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Indoor Emissions as a Primary Source of Airborne Allergenic Fungal Particles in Classrooms

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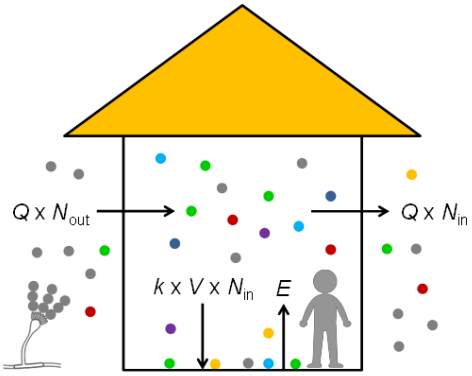
ABSTRACT

This study quantifies the influence of ventilation and indoor emissions on concentrations and particle sizes of airborne indoor allergenic fungal taxa and further examines geographical variability, each of which may affect personal exposures to allergenic fungi. Quantitative PCR and multiplexed DNA sequencing were employed to count and identify allergenic fungal aerosol particles indoors and outdoors in seven school classrooms in four different countries. Quantitative diversity analysis was combined with building characterization and mass balance modeling to apportion source contributions of indoor allergenic airborne fungal particles. Mass balance calculations indicate that 70% of indoor fungal aerosol particles and 80% of airborne allergenic fungal taxa were associated with indoor emissions; on average 81% of allergenic fungi from indoor sources originated from occupant-generated emissions. Principal coordinate analysis revealed geographical variations in fungal communities among sites in China, Europe, and North America ($p < 0.05$, ANOSIM), demonstrating that geography may also affect personal exposures to allergenic fungi. Indoor emissions including those released with occupancy contribute more substantially to allergenic fungal exposures in classrooms sampled than do outdoor contributions from ventilation. The results suggest that design and maintenance of buildings to control indoor emissions may enable reduced indoor inhalation exposures to fungal allergens.

1 **TOC ABSTRACT ART**



$$N_{out}Q + E = N_{in}Q + kVN_{in}$$

Q, ventilation rate
N_{in}, indoor concentration
N_{out}, outdoor concentration
k, deposition-rate coefficient
V, room volume
E, indoor emission rate

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INTRODUCTION

Fungal aerosol particles are ubiquitous in indoor and outdoor environments [1-4], and human exposure to allergenic fungi can cause allergic respiratory diseases, resulting in significant public health and socioeconomic burdens [5]. Inhalation of airborne fungal particles can result in deposition of allergenic material in the human respiratory tract, which can subsequently induce IgE-mediated type I hypersensitivity. Atopy to fungal allergens is related to asthma severity [6]; thus, induced hypersensitivity from fungal inhalation can further exacerbate allergic symptoms in sensitized individuals. To date, approximately 150 allergenic fungal taxa have been identified [7], and at least 3% to 10% of the global population are affected by fungal sensitization [8].

People spend most of their time indoors [9]. Both indoor and outdoor sources contribute to allergenic airborne fungal particles. Consequently, it is important to understand indoor-outdoor relationships as well as to characterize source contributions in occupied indoor environments. Each year, an estimated 28-50 Tg of fungal materials are emitted into the earth's atmosphere [10, 11]. Owing to the magnitude of these fungal emissions and studies that document higher outdoor air fungal concentrations than those indoors [1], most indoor fungi are believed to have originated outdoors in buildings that are not water-damaged [12, 13]. Airborne fungi are also thought to originate from human activities such as resuspension from flooring and surfaces [14-17]. Direct human emissions, such as via skin shedding, may also contribute for obligate skin fungi such as *Malassezia* spp. [18]. Qian et al. [19] revealed up to a 5-fold increase in indoor airborne fungal concentrations in a university classroom in response to human occupancy. Dannemiller et

al. [20] reported that the human skin-associated *Malassezia* was among the most highly abundant fungal taxa in residential house dust.

The goal of this study was to determine how indoor emissions and building ventilation influence the composition and concentrations of airborne allergenic fungal particles in common, densely occupied indoor environments. The research test sites were classrooms in elementary schools located in the United States, the European Union, and China. Multiplexed DNA sequencing and quantitative PCR (qPCR) were applied to characterize fungal diversity in size-resolved aerosol samples and to quantify specific fungal allergens in samples obtained from a regime that included vacant and occupied conditions indoors and also included indoor and outdoor sampling. Concentration and diversity estimates of allergenic fungi were incorporated into a mass-balance model of the classrooms to separately quantify the indoor source contributions and the contributions from outdoor air introduced via ventilation. Integrating building science methods with molecular-based measurements of allergenic fungal taxa allows for new insights into how building design, operation, and occupancy influence human exposure to fungal allergens.

EXPERIMENTAL

Sampling sites. Sampling was performed at seven primary schools from August 2010 to October 2011. Sites included elementary schools in the suburbs of Aarhus and Copenhagen, Denmark (AHS, CPH); in Berlin, Germany (BER); in New Haven, Connecticut, USA (NHV); in Salinas, California, USA (SAL); and in two preschools in Lanzhou, Gansu Province, China (LZU1 and LZU2). In Lanzhou, sampling campaigns

were conducted in summer and winter at each location, as denoted by LZU1s, LZU1w, LZU2s, and LZU2w, respectively. Additional details about sampling sites and sampling times are reported elsewhere [21].

Aerosol and floor dust sampling. Non-viable Andersen samplers (New Star Environmental, Roswell, GA, USA) were simultaneously deployed indoors (in) and outdoors (out) to collect airborne particles. The cut-point aerodynamic diameter ranges of the six impactor stages were 0.4-1.1, 1.1-2.1, 2.1-3.3, 3.3-4.7, 4.7-9.0, and >9.0 μm. Sterile polycarbonate nucleopore filter substrates were used. Sampling was performed under occupied (occ) and vacant (vac) conditions. Air sampling was conducted on 3-4 consecutive days using the same filters. Sampling under occupied conditions was performed for the duration of human occupancy in the classrooms, typically from 9 am to 3 pm, whereas the unoccupied sampling was typically conducted during the weekend. Dust collection occurred by sweeping the entire floor and collecting the accumulated dust during one day of human activity and during the entire time of vacancy, respectively. Both non-sieved (total PM) and sieved (PM37) dust samples were analyzed. A CO_2 monitor (LI-COR, Lincoln, NE, USA) tracked indoor concentrations as a basis for computing air exchange rates in each location.

DNA extraction and DNA sequencing. DNA on aerosol filters and in floor dust was extracted using previously reported methods [19, 22]. Multiplexed sequencing on the 454 GS-FLX platform was then performed on the indoor and outdoor air samples, and on total and PM37 floor dust for all sites. The internal transcribed spacer (ITS) region of fungal rDNA was amplified with universal fungal primers ITS1F and ITS4 [23]. Purified amplicons were sequenced at the Duke Institute for Genome Sciences and Policy. Raw

sequence data are deposited in the European Nucleotide Archive under accession number PRJEB4575.

Trimmed, high quality sequences were prepared and taxonomically assigned using BLASTn [4, 24-26]. BLAST results were phylogenetically binned based on the least common ancestor method [27]. Allergenic fungi were identified against an archived list of known fungal allergens [7]. Human skin-associated yeasts of *Candida*, *Cryptococcus*, *Malassezia*, *Pichia*, *Rhodotorula*, and *Trichosporon* selected from literature sources [18, 28, 29] were also analyzed.

Prior to richness analyses, sequences were denoised using QIIME [30]. Sequences were then clustered into operational taxonomic units (OTUs) based on 97% sequence similarity [4, 26]. For richness analysis, the numbers of observed OTUs were obtained based on random subsamples of 300 sequences from each library. For β-diversity analysis, non-phylogenetic Morisita Horn distances between samples were computed and collapsed into their main principal coordinates for analysis [31]. The analysis of similarity (ANOSIM) program in QIIME was used to test for differences in fungal communities across sample types and geographical locations.

Taxon-specific fungal concentration and aerodynamic diameter calculations. Taxon-specific fungal concentrations were calculated by multiplying the universal fungal qPCR-derived airborne concentration of each sample [21] by the DNA sequencing-based relative abundance of each taxon [32]. Reported fungal quantities are based on spore equivalent (SE) qPCR values, which were calibrated against pure-cultured *Aspergillus fumigatus* spores [33]. Taxon-specific fungal concentrations for species j (N_j) were calculated by summing over all particle size intervals:

$$N_j = \sum_i n_i \times ra_{i,j} \quad (1)$$

where n_i is the qPCR derived total fungal concentration in the i^{th} particle size interval and $ra_{i,j}$ is the DNA sequence-based relative abundance of the species j in the same particle size interval. Taxon-specific fungal aerodynamic diameters were characterized in terms of their geometric means (d_g) and geometric standard deviations (σ_g) [34]. To compute representative taxon-specific d_g values, particle size distributions were averaged for all sampling sites.

Indoor-outdoor (I/O) ratio and source contribution calculations. The I/O ratios of airborne fungal concentrations were calculated by:

$$\text{I/O} = N_{in} / N_{out} \quad (2)$$

where N_{in} and N_{out} are the indoor and outdoor concentrations, respectively. If N_{in} was below our quantification limit but N_{out} was not, then I/O = 0.01 was assumed. In case N_{out} was below our quantification limit but N_{in} was not, I/O = 100 was assumed. The values 0.01 and 100 were selected as lower and upper limits of the I/O ratios because more than 97% of the sample pairs that had $N_{in} > 0$ and $N_{out} > 0$ showed the I/O ratios within this range, and the smallest and largest quantified I/O ratios were 0.008 and 128, respectively. The I/O ratio was not calculated if both N_{in} and N_{out} were below quantification limits. To compute representative taxon-specific I/O ratios, the geometric means of all sampling sites were used.

Source contributions to indoor airborne fungal particles were estimated using a mass-balance model. We divided the source contributions into two broad categories: (i) indoor emissions and (ii) contributions from outdoor air by ventilation. The former category includes particle resuspension from building surfaces by human activities such

as walking [14-17, 35]; release of previously deposited fungal particles, e.g. from clothing [36]; direct human emissions such as skin shedding; and non-occupant-associated fungal emissions such as natural dispersal from indoor materials [37]. The latter category includes penetration of outdoor air through infiltration and natural or mechanical ventilation. On a time-averaged basis, and for the conditions that existed in the six of the seven sampling sites (excluding NHV because of its complex ventilation regime), indoor fungal aerosol concentrations can be balanced for the sources and the rates of removal according to the following equation [21]:

$$N_{out}Q + E = N_{in}Q + kVN_{in} \quad (3)$$

where Q is the volumetric ventilation rate ($m^3\ h^{-1}$), E is the indoor emission rates of fungal particles (SE/h), k is the size-specific deposition-rate coefficient for airborne particles (h^{-1}), and V is the room volume (m^3). Size-specific deposition rate coefficients were derived from literature [35]. Indoor emissions (E) were determined by means of applying eq (3), utilizing the simultaneously measured indoor (N_{in}) and outdoor (N_{out}) concentrations during occupancy, the air-exchange rate (Q/V) assessed from a material balance on CO_2, and literature-based estimates of the deposition rate coefficient, k [21]. As indicated by the left-hand side of eq (3), source contributions to indoor concentrations comprised two parts: supply from outdoors with ventilation and direct indoor emissions. The proportional contribution of indoor emissions (F_{in}) was estimated as this ratio:

$$F_{in} = \frac{E}{N_{out}Q + E} \quad (4)$$

To obtain representative taxon-specific F_{in} values, the median values of all sampling sites were used.

We further separated the indoor emissions into two components: one associated with human occupancy and a second from building-associated processes that occur independent of occupants. The non-occupant-associated fungal emissions ($E_{\text{non-occ}}$) were estimated based on vacant-condition indoor and outdoor fungal concentrations. Since no ventilation-rate measurements were made for vacant conditions, we assumed a background air-exchange rate of 0.5 per hour for vacant conditions for each classroom [38]. The contribution of human occupancy to overall indoor fungal emission (f_{occ}) was then estimated by:

$$f_{\text{occ}} = \frac{E_{\text{occ}}}{E} = 1 - \frac{E_{\text{non-occ}}}{E} \quad (5)$$

where $E_{\text{occ}} = E - E_{\text{non-occ}}$ represents the occupancy-associated fungal emission rate.

RESULTS

Taxonomic composition. After quality trimming, 127,390 ITS sequences were produced from 177 size resolved aerosol and floor dust samples. Overall, 1,859 unique species and 823 genera were detected. Across the range of particle sizes sampled, the size-resolved fractions of airborne *Ascomycota* increased with increasing particle size, whereas airborne *Basidiomycota* decreased with greater particle size (Figure S1). In addition to size, microbial community structure also varied with geographic location. Relative abundances of fungal classes in *Ascomycota* and orders in *Basidiomycota* are shown in Figures S2 and S3, respectively. Notably, fungal phylum and class compositions for each sampling site are similar across sample types (e.g., indoor *vs.* outdoor), but variations among fungal communities were greater across different geographical regions than across sample type. Principal coordinate analysis results of

fungal communities consistently indicate geographic differences for each of the three sample types (floor dust, indoor air, and outdoor air) among the three sampling-site regions, i.e., China (LZU1 and LZU2), Europe (AHS, BER, and CPH), and North America (NHV and SAL) ($p<0.05$ for floor dust, indoor air and outdoor air) (Figure 1). Within each site, ANOSIM testing showed significant differences in fungal community composition between occupied indoor air and floor dust at 4 out of 6 sampling sites from which dust sampling was undertaken ($p<0.05$ for BER, LZU1s, NHV, and SAL) (Figure S4, Table S1).

Richness and compositions of allergenic fungi. Richness estimates normalized to 300 sequences based on numbers of unique OTUs ranged from 15 to 93 in indoor air and floor dust samples (Tables S2 and S3). On average, fungal richness of indoor air was 1.5 times greater than that of outdoor air ($p<0.05$, paired *t*-tests). No statistically significant difference was observed for average richness values between occupied and vacant indoor fungal aerosol particles ($p>0.05$, paired *t*-tests). Floor dust richness (both PM37 and total PM combined) was 1.4 times the occupied indoor air diversity ($p<0.05$, *t*-test).

Figure 2 illustrates relative abundances of the 40 most abundant genera detected by DNA sequence analysis. On average, the five most abundant genera in air and dust samples were *Cryptococcus* (12.0%), *Alternaria* (5.8%), *Wallemia* (3.7%), *Cladosporium* (2.7%), and *Epicoccum* (2.5%). In total, 20 fungal genera that contain allergenic species were identified, representing 16% of the total sequences (Figure 3). To the species rank, 21 allergenic taxa were identified in indoor air (Figure S5). Figures 2 and 3 also provide insights about abundance differences among locations and the aerodynamic diameter size

bins in which these taxa are typically enriched. These figures also illustrate how the indoor/outdoor relationship and human occupancy interact with taxonomic abundance.

I/O ratios, source contributions, and aerodynamic diameters. Absolute concentrations of allergenic genera, total allergens, skin-associated fungi, and total fungi were estimated by eq (1) based on total fungal concentrations measured by qPCR and relative abundances of each fungal taxon summarized in Table 1. With these specific fungal concentrations, I/O ratios were calculated for both occupied and vacant conditions (Figure 4A). The I/O ratios were higher under occupied conditions for all taxa and groups considered, suggesting that their higher concentrations were associated with human occupancy-generated emissions. Notably, the I/O ratios of allergenic taxa, ranging from 1.0 to 35 under occupied conditions, and the I/O ratio for total allergens (I/O=1.3) were higher than that for total fungi (I/O=0.89), suggesting that human occupancy preferentially enriched these allergen concentrations in indoor air.

Source apportionment of indoor fungal aerosol particles was determined according to eq (4). The contributions to total airborne fungi from indoor emissions were always substantial and varied by sampling site (Table 1). Median values of the indoor emission and ventilation contributions across all sites were 70% and 30%, respectively (Figures 4B and 5), indicating that the dominant source of indoor air fungal aerosols during occupancy was from indoor emissions. The indoor emission contributions of allergenic taxa were each greater than 50% and were also higher than corresponding values for total fungi (Figures 4B and 5), except for *Cladosporium* (60%), which is known to originate outdoors [39]. Furthermore, evidence suggests that the majority of indoor fungal emissions originated from occupant-generation processes, with the

calculated f_{occ} values being 99%, 98%, 98%, 60%, 54%, and 93% in AHS, BER, CPH, LZU1s, LZU2s, and SAL, respectively (median = 95%). For total allergenic taxa, the corresponding values were 98%, 61%, 97%, 71%, 72%, and 91% (median = 81%).

As a positive control for known indoor sources, we assessed the source apportionment for fungal organisms that were associated with human skin. The evaluated F_{in} fractions of skin-associated yeasts of *Candida*, *Cryptococcus*, *Rhodotorula*, and *Trichosporon* were 92%-100% (Figure 4B). An obligate human skin genus *Malassezia* [18] also showed 100% contribution from indoor emissions, although only two sites had the valid datasets to allow for F_{in} calculations for this fungus. To capture variability in allergen ratios among sites, cumulative profiles of F_{in} are shown in Figure 5.

Particle size distributions of indoor fungal particles were shifted by human occupancy. Geometric means of aerodynamic diameters (d_g) of total fungi were greater under occupied conditions than in vacant conditions for all sampling sites ($p<0.05$, paired *t*-test) (Table 1), suggesting that emissions of larger indoor fungal aerosol particles were preferentially associated with human occupancy. This trend was also observed for taxon-specific d_g values. Here, 4 out of 6 allergenic taxa, for which both vacant and occupied data were available, showed greater aerodynamic diameters during occupancy (Figure 4C).

DISCUSSION

Although several prior studies have examined indoor-outdoor relationships of airborne fungal particles [1, 13, 19], this work extends the science of indoor allergenic fungal exposures in two important ways. First, DNA-based sequencing techniques were used to

thoroughly identify and quantify individual taxa and fungal community features of health relevance. This moves fungal exposure science beyond biomarker and culture-based analyses, which cannot discriminate among the broad spectrum of fungal allergens. Second, these microbial data were integrated into a size-resolved aerosol sampling and building-science modeling approach for evaluating the contributions of indoor emissions and ventilation to specific fungal allergen exposure. Our approach reveals that — for the several classroom sites studied — fungal emissions attributable to human occupancy are a significant, and in many cases, a primary source for indoor allergenic fungal particles.

Fungal communities. Fungi are diverse, with an estimated 1.5 million species [40]. Traditionally, researchers have used culture-based or biomarker methods to measure environmental fungal exposure [41, 42]. However, these methods have limited capabilities for identifying the broad range of fungal taxa and cannot provide accurate α and β microbial diversity measures, which might be crucial for connecting health effects and environmental factors with microbial community composition [20, 43, 44].

The data presented here reveal new insights into fungal community composition in the indoor environment. Specifically, comparisons of fungal richness between indoor and outdoor air reveal that the indoor environment (occupied indoor air and floor dust) had a greater fungal richness than outdoor air, which might be informative for health and exposure evaluations. Elevated fungal richness has been shown recently to be associated with lower rates of asthma development [20, 45].

The largest difference in fungal community composition was among samples from different sampling locations rather than between different sample types from the same location. Principal coordinate analyses reveal distinct geographical patterns in

fungal communities of floor dust, indoor air and outdoor air (p<0.05, ANOSIM) (Figure 1). While floor dust fungal compositions are known to be geographically patterned [2], this study confirmed that fungal communities of indoor and outdoor air are also geographically distinct.

Sources of fungi in indoor air. On average, indoor emissions contributed more to the allergenic fungal populations in indoor air than outdoor fungi entering through ventilation. These observations were consistent over air-exchange rates that ranged from 1.0 to 7.4 h^{-1} for the different sites, across variation in total fungal emission rates that spanned more than one order of magnitude, and across three orders of magnitude variation in outdoor air fungal concentration (Table 1). Prior studies have demonstrated that, in buildings with no moisture damage, fungal concentrations in outdoor air are commonly greater than concentrations in indoor air [1]. This finding has contributed to the prevalent paradigm that outdoor air is the most significant source of indoor fungal bioaerosols [12]. By considering I/O ratios for both the occupied and vacant cases, and through the quantitative source comparisons produced here, the relative strengths of indoor versus outdoor sources have been revealed. This work documents that in cases of high occupant density, indoor emission sources of fungi and allergens can dominate over ventilation-based supply from outdoor air, and that occupancy contributes substantially to total indoor emissions.

A significant finding from this work is that indoor fungal allergens are preferentially enhanced in classroom indoor air compared to total fungal particles. As one potential explanation, both laboratory- and field-based studies have shown a strong increase in resuspension rate of floor dust with increasing particle size [14-17, 46]. A prior

study in outdoor air has demonstrated that the proportion of fungal allergens in an aerosol sample increases with increasing aerodynamic diameter, with allergenic fungi comprising 2.0% of total fungi in particles smaller than 9.0 μm, and 15.3% in particles larger than 9.0 μm aerodynamic diameter [4]. Fungi deposited in indoor floor dust may also be entrained or attached to other particles, and this may also account for the larger aerodynamic diameters of indoor fungal aerosols in occupied versus vacant conditions [21]. In addition to size, characteristics that are known to increase the resuspension rate of materials from flooring include the concentration of floor dust and the intensity of human activity [17, 46, 47].

Origins of fungi in house dust. While insights are now emerging about indoor surface-borne fungal communities [48], quantitative knowledge regarding the origin of fungal material in floor dust remains an open research challenge. We found that floor dusts were enriched with human skin-associated yeasts and with taxa producing multicellular dictyospores, which are gravitationally dominant. *Rhodotorula*, *Candida,* *Cryptococcus*, *Malessezia,* and *Trichosporon* are associated with the human microbiome [18] and can be viewed as originating mostly indoors. In this study, these taxa were commonly detected in indoor air and in floor dust (Figures 2 and 3) and large fractions were estimated to come from indoor emissions, i.e., 94% for *Candida*, 100% for *Malassezia*, 100% for *Rhodotorula*, and 100% for *Trichosporon* (Figure 4B). The value was slightly lower for *Cryptococcus* (92%) for which a large variety of environmental reservoirs is also known [49]. Since yeasts are commonly found on human skin, direct human emissions from processes such as desquamation followed by resuspension might play significant roles for increasing their concentrations indoors [50]. Floor dust from this study was enriched in human associated fungi, with the cumulative relative abundance of

Candida, *Cryptococcus*, *Malassezia*, *Pichia*, *Rhodotorula*, and *Tricosporon* equaling 29.6%, much larger than their 3.4% cumulative abundance in outdoor air.

The importance of size on source apportionment can be demonstrated by the fungal allergens *Alternaria* and *Epicoccum*. Distinct increases in the I/O ratios were observed for these taxa under occupied conditions (Figure 4A). These organisms are plant pathogens, typically originating outdoors [3, 4]. These fungi produce large multicellular dictyospores, i.e., 15–25 μm for *Epicoccum nigrum*, and 18–83×7–18 μm for *Alternaria alternata* [51]. Due to their large settling velocities [52], the spores settle rapidly under undisturbed vacant conditions, but can easily be resuspended from the floor by human activities. Indeed, relative abundances of *Alternaria* and *Epicoccum* in floor dust were high, i.e., contributing 7.3% and 3.2% on average of total dust and PM37 floor dusts, respectively (Figures 2 and 3). It was estimated that 74% and 84% of the indoor *Alternaria* and *Epicoccum* concentrations, respectively, were a consequence of indoor emissions (Figure 5).

Human health implications. We used DNA-based methods coupled with building characterization and modeling to better understand dynamics of indoor allergenic fungal aerosol particles. The study has demonstrated the importance of indoor emissions as contributors to microbial allergen exposures indoors and has revealed roles for human occupancy influencing the particle sizes, richness, and diversity of indoor allergenic fungi. Guidelines for promoting healthy schools for asthmatic children typically recommend reducing asthma triggers such as total fungi or fungal allergens. The results reported here point to the importance of reducing indoor emissions associated with occupancy, potentially through more regular and effective floor cleaning and through the

choice of flooring materials that limit particle resuspension. Continued improvements in understanding this system hold the promise of eventually enabling better design of buildings to mediate both beneficial and detrimental microbial exposures.

ASSOCIATED CONTENTS

Supporting Information

Additional figures and tables are available in Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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