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DEVELOPMENT and APPLICATION of a NEW METHODOLOGY for SEPARATION and ANALYSIS of SUBMICROMETER-SIZED FUNGAL PARTICLES in LABORATORY and FIELD STUDY

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ABSTRACT

Mold exposure is associated with adverse health effects, but spore counts have not shown strong correlation with health outcomes. Submicrometer-sized fungal fragments have been suggested as potential contributors to the adverse health effects because of their small size, large quantities, and biological properties. However, the contribution of submicrometer-sized fungal fragments to mold exposure and adverse health effects is poorly characterized due to the lack of suitable sampling and analytical methods. The purposes of this research were to test and optimize a newly developed Fragment Sampling System combined with (1→3)-β-*D*-glucan assay for separation and analysis of submicrometer-sized fungal fragments, to apply the new methodology to characterize size-fractionated particulate (1→3)-β-*D*-glucans aerosolized from fungi grown on MEA, ceiling tile, and gypsum board for one and six months, and to field-test the new methodology in moldy homes.

Penetration and spore bounce tests were performed for optimization using PSL particles, *Aspergillus versicolor*, and *Stachybotrys chartarum*. (1→3)-β-*D*-glucan of collected samples was analyzed by the *Limulus* Amebocyte Lysate assay. This investigation confirmed the successful separation of submicrometer-sized fungal fragments from intact spores and the feasibility of quantification of these samples. The results also revealed that aerosolization of particulate (1→3)-β-*D*-glucan can be influenced by the material type and fungal species. The release of particles and particulate (1→3)-β-*D*-glucan increased with the age of the fungal culture. A poor correlation between numbers of submicrometer-sized particles and (1→3)-β-*D*-glucan mass was observed. The field study showed that the actual contribution of submicrometer-sized fungal fragments to total mold exposure was higher than that estimated by laboratory-generated data.

Moreover, the amount of spore-sized (1→3)-β-*D*-glucan did not correlate with the amount of fragment-sized (1→3)-β-*D*-glucan.

In conclusion, this research indicates that the new methodology is a promising tool for separation and analysis of submicrometer-sized fugal fragments. A considerable amount of submicrometer-sized particulate (1→3)-β-*D*-glucan was observed in the field study. However, numbers of submicrometer-sized particles or the amount of spore-sized (1→3)-β-*D*-glucan cannot be used to estimate the (1→3)-β-*D*-glucan mass of fungal fragments due to a poor correlation between these parameters. Therefore, measurement of fungal fragments should be included when assessing mold exposure in moldy indoor environments.

EXECUTIVE SUMMARY

Epidemiological studies indicate that indoor exposures to molds contribute to occupant respiratory symptoms and diseases. However, attempts to correlate health outcomes with spore counts have not indicated strong associations. Simultaneous release of submicrometer-sized fungal fragments with intact spores from mold-contaminated surfaces in large quantities has been demonstrated in earlier studies, which investigated the aerosolization of spores from moldy surfaces. It was reported that these submicrometer-sized particles contain biologically active agents such as fungal antigens and mycotoxins. These findings indicate that fungal fragments (e.g., < 1.0 μm) may potentially contribute to the adverse health effects related to fungal exposures. However, the quantification of fungal fragments in moldy buildings has previously been hindered by the lack of suitable field-compatible sampling and analytical methods.

Analytical techniques for fungi based on their constituents, such as glucans, extracellular polysaccharides, and antigens, have recently been suggested as alternatives for mold assessment. Their health impact (e.g., toxic or allergenic potential) as well as the ease and low cost of performing the assay rather than cultivation and microscopic counting of mold spores have caused many to use (1→3)-β-*D*-glucan as a measure of total fungal biomass. However, the contribution of (1→3)-β-*D*-glucan in submicrometer-sized fungal fragments to exposure and health outcomes is poorly characterized. Therefore, more information is needed on the release of size-selective concentrations of particulate (1→3)-β-*D*-glucan in order to assess and understand mold exposure and associated health effects.

The objectives of this dissertation are to test and optimize a new methodology for separation and analysis of submicrometer-sized fungal particles using a newly developed

Fragment Sampling System and the (1→3)-β-*D*-glucan assay; to apply the new methodology to characterize airborne (1→3)-β-*D*-glucan levels in submicrometer-sized particles released from fungal species grown on different building materials for various periods of time; and to field-test the new methodology in moldy homes.

In the first objective, penetration and spore bounce tests of a newly developed Fragment Sampling System were performed in the laboratory using monodisperse polystyrene latex (PSL) particles and particulate matter aerosolized from sporulating *Aspergillus versicolor* and *Stachybotrys chartarum* cultures. The Fragment Sampling System consists of two Sharp-Cut cyclone samplers ($PM_{2.5}$ and $PM_{1.0}$) and a 25-mm after-filter (preloaded with a gamma-sterilized polycarbonate filter; pore size of 0.4 μm). Aerosolized fungal particles were separated into three distinct size fractions: (i) >2.5 μm (spores); (ii) 1.0–2.5 μm (mixture of spores and fragments); and (iii) < 1.0 μm (submicrometer-sized fragments). In addition, the amount of (1→3)-β-*D*-glucan in each size fraction was analyzed by the *Limulus* Amebocyte Lysate (LAL) assay in order to test the feasibility of (1→3)-β-*D*-glucan analysis for quantification of submicrometer-sized fungal fragments.

Experiments conducted with PSL particles showed that the 50% cut-off values of the two cyclone samplers under the test conditions were 2.25 μm and 1.05 μm, respectively. These results indicate that the majority of fungal spores (and/or spore-sized particles) would be efficiently collected by the two Sharp-Cut cyclone samplers in the Fragment Sampling System since aerodynamic diameters of most spores are greater than 1.05 μm. No particle bounce onto the after-filter was observed when the total particle number entering the Fragment Sampling System

was kept below 1.6×10^8. Thus, collection of purified submicrometer-sized fungal fragments can be attained if particle load is controlled below a certain upper limit.

The results of the (1→3)-β-*D*-glucan assay showed that the concentration of airborne (1→3)-β-*D*-glucan in the fragment (< 1.0 μm) and spore size range varied from 5.4 to 1,005.2 ng/m^3. The detection limit was 0.015 ng/m^3 for the fragment size range and 0.013 ng/m^3 for the spore size range. This result indicates that the (1→3)-β-*D*-glucan assay is sensitive enough for the analysis of all tested fungal aerosols including submicrometer-sized fungal fragments, as well as intact spores. Therefore, the new methodology may provide a representative method for future exposure assessment of submicrometer-sized fungal fragments.

Mold-contaminated building materials may contain biologically active agents (e.g., mycotoxins, (1→3)-β-*D*-glucan, and allergens), which have been associated with adverse health effects, but the release of these components from material surfaces into the air is not well understood. In the second objective, the release of total (and size-fractionated) particulate (1→3)-β-*D*-glucan from the surface of three artificially mold-contaminated material samples (malt extract agar (MEA), ceiling tile, and gypsum board) incubated for one to six months was investigated. (1→3)-β-*D*-glucan on the surfaces of moldy materials and in the air samples collected from these materials were analyzed by the LAL assay. The aerosolization ratio was defined as the amount of airborne (1→3)-β-*D*-glucan divided by the amount of (1→3)-β-*D*-glucan on the surface of material sample.

Results showed that the aerosolization of particulate (1→3)-β-*D*-glucan was influenced by the material type (P<0.001) and fungal species (P<0.01). Aerosolization ratios varied from < 0.1% to 92.0%. The highest value was measured for *S. chartarum* grown on gypsum board for

one month and the lowest value was found for *S. chartarum* grown on MEA for six months. For *A. versicolor*, aerosolization ratios of particulate (1→3)-β-*D*-glucan released from the three material types were not significantly different. However, corresponding ratios of *S. chartarum* released from ceiling tiles and gypsum board were significantly higher than from MEA (P<0.001). This can be attributed to the exudate (e.g., slime materials) of *S. chartarum*, which covers spores and increases as the fungus is growing. The exudate was very abundant on *S. chartarum* grown on MEA, but not as much when it grew on ceiling tile or gypsum board. This was supported by the finding that the low aerosolization ratio of particulate (1→3)-β-*D*-glucan was due to low concentrations of airborne (1→3)-β-*D*-glucan, but not due to low amount of (1→3)-β-*D*-glucan on the surface.

In addition, the size-fractionated concentrations of particulate (1→3)-β-*D*-glucan, as well as numbers of particles released from the surface of moldy material samples were investigated. Results showed that numbers of released particles ranged from 0.5×10^6 to 1.6×10^9 particles/m^3 in the fragment and spore size range. The highest value was measured for *S. chartarum* grown on gypsum board for six months in the fragment size range and the lowest value was observed for *S. chartarum* grown on MEA grown for six months in the spore size range. The numbers of submicrometer-sized particles released from 6-month old cultures were always significantly higher that those from 1-month old (P<0.001). This can be explained by the changes in the moisture content (decreased up to 18%) and fungal biomass (increased up to 319%).

The size-fractionated concentrations of particulate (1→3)-β-*D*-glucan ranged from 4.3×10^{-1} to 9.8×10^2 ng/m^3 in the fragment size and from 1.0×10^1 to 4.7×10^4 ng/m^3 in the spore size range. The highest value was measured for *S. chartarum* grown on gypsum board for one month

in the spore size range and the lowest value was observed for *A. versicolor* grown on MEA for six months in the fragment size range. Similar increases with incubation time as for particle numbers were observed for airborne concentrations of (1→3)-β-*D*-glucan in the fragment size range. The increases were most pronounced for fungi grown on gypsum board ($P<0.01$ for *A. versicolor* and $P<0.05$ for *S. chartarum*). However, this trend was not as clear as with numbers of released particles. Particle numbers in the fragment size range showed a poor correlation with the mass concentrations of particulate (1→3)-β-*D*-glucan. This may be due to variation in the (1→3)-β-*D*-glucan content per fragment particle attributed to a wide range of size distribution of fungal fragments and the variation in the origin of the fragments within the fungal structure. Moreover, poor correlation was found between the submicrometer-sized fragment and spore results either for particle numbers ($r=-0.04$, $P>0.05$) or for (1→3)-β-*D*-glucan mass ($r=0.14$, $P>0.05$). In addition, the ratio of (1→3)-β-*D*-glucan concentrations in the fragment size range to those in the spore size range (F/S ratio) varied from < 0.01 to 0.67. The average F/S ratios of 6-month old cultures were higher for *S. chartarum* (0.11) than for *A. versicolor* (0.02). Results of F/S ratio indicate that the contribution of fragments to the total fungal exposure was higher for *S. chartarum* than for *A. versicolor*.

Finally, the new methodology was examined and utilized for characterizing exposures to submicrometer-sized fungal fragments in five mold-contaminated homes located in New Orleans, Louisiana (three homes) and Southern Ohio (two homes). Five samples per home (three indoor samples and two outdoor samples) were collected during the summer of 2006 (June–September). The sampling was repeated in two homes in the winter (December, 2006–January, 2007). To evaluate the performance of the Fragment Sampling System, the Button Sampler was utilized in parallel, and the concentrations of total (non size-fractionated) (1→3)-β-*D*-glucan collected by

the Fragment Sampling System and the Button sampler were compared. In addition, the size-fractionated concentrations of (1→3)-β-*D*-glucan were determined and compared with corresponding data from the laboratory.

The concentrations of total (1→3)-β-*D*-glucan varied from 0.2 to 15.9 ng/m^3 in field samples. The corresponding concentrations measured with the Fragment Sampling System strongly correlated with those measured with the Button Sampler (r=0.92; P<0.001). The difference in the concentrations of (1→3)-β-*D*-glucan measured with these two methods was on average 8.1% and was not significantly different (P>0.05). This result proves the capability of the Fragment Sampling System combined with the (1→3)-β-*D*-glucan assay for quantifying fungal particles in the fragment and spore size fraction under field conditions.

The size-fractionated concentrations of (1→3)-β-*D*-glucan varied from 0.02 to 0.72 ng/m^3 for the fragment size range and from 0.09 to 12.9 ng/m^3 for the spore size range. A clear seasonal variation in the size-fractionated concentrations was also observed: in the summer, the concentration of (1→3)-β-*D*-glucan was significantly lower in the fragment size range than in spore size range (P<0.001), but there was no difference in the winter data. The F/S ratios ranged from 0.01 to 2.16. The F/S ratio was higher in the winter (average = 1.02) than in the summer (0.23) coinciding with a lower relative humidity in the winter. Furthermore, F/S ratios under field conditions were higher than those obtained from laboratory experiments (0.11 for *S. chartarum* and 0.02 for *A. versicolor*). No correlation between the (1→3)-β-*D*-glucan concentrations in the fragment size fraction with those in the spore size fraction was observed (r=0.33; P>0.05). These findings indicate that submicrometer-sized fungal particles may comprise a significant portion of

the fungal exposure in moldy indoor environment, but their mass cannot be quantified based on mass concentration of spores.

In conclusion, the new methodology is a promising tool for separation and analysis of submicrometer-sized fungal fragments in laboratory and field environments. The aerosolization of particulate (1→3)-β-*D*-glucan was influenced by the material type and fungal species. In addition, the release of particles and particulate (1→3)-β-*D*-glucan increased with the age of the fungal culture in general. The increase was most consistent for particle numbers in the fragment size range. Results of field sampling indicate that the actual (field) contribution of submicrometer-sized fungal fragments to the overall exposure may be very high, even much greater than that estimated in the laboratory-based studies.

The present Ph.D. research indicates that a large proportion of airborne (1→3)-β-*D*-glucan can exist as submicrometer-sized fungal fragments in moldy buildings and corroborates the role of fragments as a potential contributor of adverse health effects in moldy indoor environments. Long-term mold (or water) damage in buildings may increase the contributions of submicrometer-sized fungal fragments to overall mold exposure. Furthermore, no correlation between submicrometer-sized fragment data (e.g., particle numbers and (1→3)-β-*D*-glucan mass) and corresponding data of spores was observed either in the laboratory or field environments. This indicates that the fragment concentration cannot be estimated based on spore data. Therefore, measurement of submicrometer-sized fungal particles should be included when determining exposure in moldy indoor environments to obtain more comprehensive exposure profiles when investigating health outcomes related to fungal exposure. (1→3)-β-*D*-glucan mass in the fragment particles did not show a good correlation with numbers of fragment particles.

Measurement of submicrometer-sized fungal particles using other analytical methods based on other fungal constituents, besides (1→3)-β-*D*-glucan assay also deserves further investigation.

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LIST OF PEER-REVIEWED PUBLICATIONS

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II. **Sung-Chul Seo**, Tiina Reponen, Linda Levin, Tiffany Borchelt, Sergey A. Grinshpun. Aerosolization of particulate (1→3)-β-*D*-glucan from moldy materials. *Appl. Environ. Microbiol.* (submitted) [Specific aim 2].

III. **Sung-Chul Seo**, Tiina Reponen, Sergey A. Grinshpun. Size-fractionated (1→3)-β-*D*-glucan concentrations aerosolized from different moldy building materials. *Atmos. Environ.* (submitted) [Specific aim 3].

IV. Tiina Reponen, **Sung-Chul Seo**, Faye Grimsley, Taekhee Lee, Carlos Crawford, Sergey A. Grinshpun. Fungal fragments in moldy houses: A field study in homes in New Orleans and southern Ohio. *Atmos. Environ.* 41(37): 8140-8149 [Specific aim 4].

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FIGURE 3-1. Number concentrations of particles (particles/m^3) in the fragment size range aerosolized from each material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present geometric means, and error bars present geometric standard deviation of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid line presents significantly different geometric means between the two incubation periods. Asterisks present the level of statistical difference (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

FIGURE 3-2. Number concentrations of particles (particles/m^3) in the spore size range aerosolized from each material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present geometric means, and error bars present geometric standard deviation of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid line presents significantly different geometric means between the two incubation periods. Asterisks present the level of statistical difference (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

FIGURE 3-3. Airborne concentrations of particulate (1→3)-β-*D*-glucan (ng/m^3) in the fragment size range aerosolized from each material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present geometric means, and

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BACKGROUND

Introduction

The estimated fraction of buildings with mold and moisture problems in Northern Europe and North America is as high as 20–50%, based on data from the United Kingdom (30–45%) (Platt et al., 1989; Hunter et al., 1988), the Netherlands (20–25%) (Verhoeff et al., 1995; Adan 1994; Brunekreef et al., 1992), Finland (20–32%) (Nevalainen et al., 1998; Koskinen et al., 1999), Canada (up to 30%) (Dales et al., 1991), and the United States (36–52%) (Spengler et al., 1993; Salo et al., 2005; Cho et al., 2006). Most moisture and water damage is due to poorly manufactured constructions and inadequate maintenance (Hilling et al., 1998; Miller et al., 2000), which can induce mold growth on building materials. Therefore, concerns about adverse health effects associated with mold growth in the indoor environments have increased over the past several years. Possible health outcomes can generally be grouped into three major categories as shown in Table 1.

Table 1. Health effects associated with fungi (Robbins et al., 2000).

	Allergic	Infectious	Toxic
Health outcome	Sensitization and immune response (e.g., allergic rhinitis, asthma, and hypersensitivity pneumonitis).	Growth of the fungus in or on the body (e.g., aspergillosis and histoplasmosis).	Disruption of cellular function and interaction with DNA (e.g., mycotoxins)

Several epidemiological studies which have relied on either self-reported mold and dampness or surveyor-assessed moisture and visible mold have found an association between

dampness or visible mold and respiratory illness of children and adults as summarized by the Institute of Medicine of the National Academies of Science (IOM, 2004): people living or working in buildings contaminated with mold are more likely to develop respiratory symptoms and diseases than people in non-moldy buildings (Dearborn et al., 1999; Etzel et al., 1998; Meklin et al., 2002; IOM, 2004). The relative risk of allergic illness in water-damaged houses is at least two times greater than in homes without dampness. (Dales et al., 1991; Dekker et al., 1991; Kilpeläinen et al., 2001).

Although many epidemiological studies have reported positive correlation between the visible signs of mold damage and health symptoms, the association between the concentration of airborne fungal spores and adverse respiratory effects has not been well-documented and is still controversial (Rao et al., 1996; Cooley et al., 1998; Robbins et al., 2000). For example, health problems in moldy buildings are associated with exposure to high concentration of airborne fungal spores only in few studies (Platt et al., 1989; Waegemaekers et al., 1989), while several field studies show that the concentration of airborne fungal spores in problem buildings is not significantly higher than in homes without mold problems (Nevalainen et al., 199; Strachan et al., 1990; Garrett et al., 1998; Chew et al., 2003). In a Finnish study, the mean fungal spore level in the samples collected in moldy homes was even lower than that in reference homes (Nevalainen et al., 1991). A British study could not find a significant difference between median counts of colony forming units obtained in moldy and non-moldy homes, respectively (Strachan et al., 1990). Similarly, studies performed in Australia and the United States (Boston area) did not find a significant association between evidence of dampness or visible mold and spore concentrations (Garrett et al., 1998; Chew et al., 2003). Thus, these findings indicate that spore concentration may not be an adequate measure for fungal exposure.

Need for the new sampling methods

The existence of larger hyphal fragments in the ambient air has been recognized for some time (Glikson et al., 1995; Li and Kendrick, 1995), but has so far been overlooked when assessing exposures in moldy buildings. Hyphal fragments have been shown to represent 6–56% of the total fungal particle counts in field samples based on microscopic sample analysis (Li and Kendrick, 1995; Foto et al., 2005; Green et al., 2005). This method is limited typically to particles >1 μm (Green et al., 2006). Recent laboratory-based studies (Górny et al., 2002 and 2003; Cho et al., 2005) have reported that large quantities of submicrometer-sized fungal and actinomycete fragments (ranging from 30 nm to 1 μm) are released together with intact spores from contaminated surfaces. These studies demonstrated that the number of released smaller-sized fragments was always higher, up to 500 times, than the number of intact spores. Furthermore, the number of spores and fragments did not correlate.

Assessing exposure to smaller-sized fungal fragments may be important for several reasons. Smaller-sized fragments remain airborne much longer than larger spores and can penetrate deeply into the alveolar region when inhaled. Fungal fragments have been shown to contain fungal antigens (Górny et al., 2002) and mycotoxins (Brasel et al., 2005a and 2005b). Moreover, recent epidemiological investigations have reported that fine particles (< 2.5μm) are significantly associated with elevated mortality and morbidity risk (Magari et al., 2001; Pope, 2000; Pope et al., 1999; Gold et al., 2000; Pekkanen et al., 2002). The small size, large quantities, and biological properties of fungal fragments suggest that these particles may potentially contribute to the adverse health effects and raises the need for further characterization of fungal fragments in moldy buildings.

However, the quantification of fungal fragments, including those of nanoscale sizes, has previously been hindered by the lack of suitable field-compatible sampling and analytical methods. Cascade impactors (e.g., Electrical Low Pressure Impactor (ELPI) and Andersen multi-stage sampler) would be an alternative that potentially offers a sharp separation of fragments from intact spores. Their application toward fungal particles is, however, problematic since spores are prone to bounce from upper to lower stages, making it difficult to separate fungi-associated submicrometer-sized particles from fungal spores (Trunov et al. 2001; Cho et al. 2005). Therefore, it has been of utmost importance to develop a sampling system for separating submicrometer-sized fungal particles from intact spores to investigate specific health effects of these corresponding sized particles. We have recently developed a new field-compatible Fragment Sampling System for separating and collecting submicrometer-sized fungal particles based on Sharp-Cut cyclone samplers to reduce particle bounce. In the current Ph.D. study, this new system was tested and optimized for better performance in laboratory and field environments.

Rationale for selection of appropriate analytical method

It should be noted that submicrometer-sized fungal particles can not be analyzed by traditional methods such as culture-based analysis (i.e., colony counts) and microscopic analysis (i.e., total spore counts). A major problem with culture-based analysis is that the majority of the fungal fragments may not be culturable due to loss of cellular or the absence of a cell when derived from intra- and extracellular structures of fungal colonies (Górny et al., 2002). The microscopic analysis is limited to particles above 0.2 μm in size. Furthermore, none of these analytical methods take into account the potential toxic nor allergenic effects of submicrometer-

sized fungal fragments. Thus, these methods are unable to detect submicrometer-sized fungal particles.

Analytical techniques for fungal molecules, such as ergosterol, mycotoxins, extracellular polysaccharides (EPS) and (1→3)-β-*D*-glucan, have been developed. Measuring fungal constituents may be more representative of total fungal exposures since these methods do not rely on viable spores and measure components that may have direct health effects (e.g., toxic or allergenic potential) (Institute of Medicine 2004; Douwes 2005).

Ergosterol is a constituent of membranes in mycelia, spores, and vegetative cells of fungi (Newell, 1992). Ergosterol has, thus, been widely used to estimate fungal biomass in various environments because a strong correlation has been found between ergosterol content and fungal dry mass (Newell, 1992; Suberkropp et al., 1993). However, the amount of ergosterol in fungal cultures is not constant, and ergosterol rapidly degrades after the death of fungal hyphae (Mill-Lindblom et al., 2004). Thus, degradation of ergosterol may make it difficult to analyze it in smaller-sized fungal particles, most of which are non-viable piece of fungal structures.

Mycotoxins, by-products of fungal metabolic processes, can be used to detect the presence of certain fungi in the environment. However, detection of airborne mycotoxins is very difficult due to low concentrations, except in environments that have high concentration of airborne spores such as agriculture (Olsen et al., 1988; Sorenson et al., 1990; Xu et al., 2006). Advanced analytical instruments such as gas/liquid chromatography with mass spectroscopy are required to analyze mycotoxins (Portnoy et al, 2004). Therefore, this method has several limitations for the analysis of the airborne submicrometer-sized fungal particles.

EPS have potential usefulness as surrogate fungal biomass measures, as they are produced in mycelial cell walls under almost any growth conditions. EPS levels have been reported to significantly correlate with total culturable fungi, and levels in living room floor dust were positively associated with home dampness (Douwes et al., 1999). Thus, EPS of specific fungal species may be a good marker for fungal exposure if measured in settled house dust (Chew et al., 2001); however, there is no evidence of EPS causing adverse health effects (Wouters et al., 2005).

β-glucans are the major water-insoluble structural cell wall component (up to 60% of the dry weight) of most fungi (Douwes 2005). Physical environmental factors such as temperature and water activity do not influence the (1→3)-β-*D*-glucan content of fungal cell walls (Foto et al. 2004; Fogelmarker and Rylander. 1997). (1→3)-β-*D*-glucan has, thus, been used as a measure of total fungal biomass (Chew, 2001; Rao et al. 2004; Thorn and Rylander, 1998; Foto et al., 2005; Lee et al., 2006). Most of all, (1→3)-β-*D*-glucan exposures have been associated with common respiratory symptoms. Thorn and Rylander (1998) reported the correlation between exposure to (1→3)-β-*D*-glucan and adverse health symptoms (e.g., an increased prevalence of atopy; decrease in Forced Expiratory Volume (FEV_1); and a slightly increased amount of myeloperoxidase in serum). Moreover, a recent study demonstrated an association between infants' exposure to (1→3)-β-*D*-glucan and a decrease in wheezing and allergen sensitization indicating a strong immuno-modulating effect of this fungal component (Iossifova et al., 2007a).

As described above, many of the fungal biomass surrogate markers, except (1→3)-β-*D*-glucan, may still be inappropriate indicators for assessment of fungal exposure since they may not provide information that is sufficiently specific to make correlations with adverse health

effects (Macher, 1999). On the other hand, (1→3)-β-*D*-glucan analysis has a low detection limit, is commercially available, and is a less time consuming analysis (Douwes et al., 2003a), which is an advantage in large population-based studies. Therefore, (1→3)-β-*D*-glucan was selected as a surrogate marker for the analysis of submicrometer-sized fungal particles, and the feasibility of (1→3)-β-*D*-glucan analysis for quantifying these particles was investigated in this Ph.D. research.

Mold growth on building materials

Fungal growth on building materials, such as ceiling tiles and gypsum board, can occur if there is enough moisture available (Foarde et al., 1993; Pasanen et al., 1994; Gravesen et al., 1999). The material components (e.g., cellulose) and accumulated soil (or dust) can serve as nutrients (Streifel, 1988) and promote growth of mold on the building materials (Karunasena et al., 2000).

Mold contamination of building materials was investigated in several studies in an attempt to better understand exposure patterns. These studies have had two primary foci: (i) identifying and evaluating the growth of fungi (or their metabolites) on the surface of building materials under controlled conditions; (ii) characterizing how the release of fungal particles is affected by environmental factors (e.g., air velocity and humidity). It was observed that particle release from moldy materials increased with increase in air velocity and decrease in relative humidity. Particle release can also be affected by vibration, texture of materials, and the morphology of the colonies (Foarde et al., 1999; Górny et al., 2001; Kildesø et al., 2003; Pasanen et al., 1991; Sivasubramani et al., 2004a). The composition and the moisture content of building

materials have been shown to be critical factors for fungal growth (Chang et al., 1995; Foarde et al., 1993; Karunasena et al., 2000). Bioactive agents such as endotoxins, mycotoxins, and (1→3)-β-*D*-glucan have been found on naturally and artificially mold-inoculated building materials (Andersson et al., 1997; Nielsen et al., 1998 and 1999; Gravesen et al., 1999; Murtoniemi et al., 2003). However, very little is known about the aerosolization patterns of these components.

As noted above, (1→3)-β-*D*-glucan has been used as an indicator of mold exposure, and moreover been associated with adverse health effects and immuno-modulating effects. Thus, more information is needed on the release of particulate (1→3)-β-*D*-glucan from moldy materials in order to assess and understand mold exposure and associated health effects, and to reduce knowledge gap between laboratory and field conditions. In addition, the release of size-selective particulate (1→3)-β-*D*-glucan from building materials is still not known. This information is needed for better understanding of exposure to submicrometer-sized fungal fragments in moldy environments, considering characteristics of specific smaller-sized particles (below 1.0 μm) which may cause adverse health effects. The current Ph.D. study included the investigation of the release of size-fractionated particulate (1→3)-β-*D*-glucan from three different material samples that were artificially contaminated with fungi.

In summary, this Ph.D. study comprises the test and optimization of a newly-developed Fragment Sampling System for separating submicrometer-sized fungal particles (i.e., fungal fragments). The feasibility of (1→3)-β-*D*-glucan assay as an appropriate method for the quantification of aerosolized fungal fragments and intact spores was investigated as well. Finally, the methodology was applied to investigate the concentration of size-fractionated particulate

(1→3)-β-*D*-glucan in a laboratory study and on a field study in order to better understand the potential exposure to airborne fungal fragments in moldy environments.

OBJECTIVES, HYPOTHESES, AND SPECIFIC AIMS

OBJECTIVES

The objectives of this dissertation are: (1) to test and optimize the new methodology for separation and analysis of submicrometer-sized fungal particles using a newly developed Fragment Sampling System; (2) to apply the new methodology to characterize airborne (1→3)-β-*D*-glucan levels of submicrometer-sized particles released from fungal species grown on different building materials for various periods of time; and (3) to field-test the new methodology and collect size-fractionated (1→3)-β-*D*-glucan data in moldy homes.

HYPOTHESES AND SPECIFIC AIMS

In order to achieve the objectives of this research, the new methodology was first developed (specific aim 1) and then utilized to test two hypotheses.

Specific aim 1. Test and optimize the new Fragment Sampling System for separation of submicrometer-sized fungal fragments aerosolized from moldy surfaces in laboratory-based experiments, and measure the (1→3)-β-*D*-glucan concentration of particles in the submicrometer size range.

Hypothesis I

The release of particulate (1→3)-β-*D*-glucan in the submicrometer size range from mold-contaminated surfaces is significantly dependent on growth media and age of the fungal culture.

The following specific aims were performed in order to test the first hypothesis:

Specific aim 2. Determine the total aerosolization ratio (the amount of airborne (1→3)-β-*D*-glucan divided by the amount of (1→3)-β-*D*-glucan on the surface) of particulate (1→3)-β-*D*-glucan released from different moldy materials**.**

Specific aim 3. Measure and compare the airborne (1→3)-β-*D*-glucan concentrations of submicrometer-sized fungal fragments and intact spores released from different growth media incubated for different periods under laboratory conditions.

Hypothesis II

The contribution of submicrometer-sized fungal fragments to mold exposure is significantly higher in the field than what is expected based on laboratory experiments.

The following specific aim was performed in order to test the second hypothesis:

Specific aim 4. Evaluate the new methodology in moldy environments, and compare the ratios of airborne (1→3)-β-*D*-glucan concentrations in the fragment size range to those in the spore size range (F/S ratios) measured in moldy homes with corresponding ratios measured in laboratory experiments.

SPECIFIC AIM 1

Test and optimize the new Fragment Sampling System for separation of submicrometer-sized fungal fragments aerosolized from moldy surfaces in laboratory-based experiments, and measure the (1→3)-β-*D*-glucan concentration of particles in the submicrometer size range.

1.1. INTRODUCTION

Evidence exists indicating that indoor exposures to molds contribute to occupant respiratory symptoms and diseases (e.g., allergic rhinitis, asthma, hypersensitivity pneumonitis) (Institute of Medicine, 2004). However, attempts to correlate health outcomes with spore counts have not indicated strong associations. Recently, the simultaneous release of large quantities of submicrometer-sized fungal fragments together with intact spores from mold-contaminated surfaces has been demonstrated (Górny et al., 2002 and 2003: Cho et al., 2005). Moreover, it was reported that these small-sized particles contain biologically active agents such as fungal antigens and mycotoxins (Górny et al., 2002; Brasel et al., 2005). These findings support that fungal fragments may potentially contribute to the adverse health effects. However, the quantification of fungal fragments (e.g., < 1.0 μm) for further characterizations of these airborne particles in moldy buildings has previously been hindered by the lack of suitable field-compatible sampling and analysis methods.

In specific aim 1, the performance of the new field-compatible Fragment Sampling System was tested for separating submicrometer-sized fungal particles using standardized monodisperse polystyrene latex (PSL) particles or fungal particles aerosolized from cultures of two common indoor molds, *Aspergillus versicolor* and *Stachybotrys chartarum* (Hyvärinen et al., 2002). In addition, we measured the amount of particulate (1→3)-β-*D*-glucan in the fragment and spore size fractions for testing the feasibility of the *Limulus* Amebocyte Lysate (LAL) assay.

1.2. MATERIAL AND METHODS

1.2.1. The new Fragment Sampling System

The new Fragment Sampling System consists of two Sharp-Cut cyclone samplers ($PM_{2.5}$ and $PM_{1.0}$; BGI Inc., Waltham, MA) and a 25-mm after-filter cassette preloaded with gamma-sterilized polycarbonate filter (0.4 μm of pore size; SKC Inc., Eighty Four, PA) (Figure 1-1). The two cyclone samplers are connected in sequence so that intact spores (>2.5 μm) are captured in the $PM_{2.5}$ cyclone sampler, a mixture of fragments and spores (1.0–2.5 μm) is collected in the $PM_{1.0}$ cyclone sampler, and submicrometer-sized fragments are collected onto the after-filter.

Two optical particle counters (OPC: Model 1.108; Grimm Technologies, Inc., Douglasville, GA) and a condensation nucleus counter (CNC: P-Trak®; TSI Inc., Shoreview, MN) were used to continuously measure particle numbers. The size-selective OPC covers the size range of 0.3–20.0 μm in 15 channels while the CNC measures the total number of smaller particles between 0.02–1.0 μm. Two OPCs (OPC-1 and OPC-2) were incorporated into the system, one upstream and one downstream of the $PM_{2.5}$ cyclone sampler. The number of fungal particles collected into the $PM_{2.5}$ cyclone sampler was calculated as the difference between the concentrations obtained in the two OPCs. A CNC located downstream of the $PM_{1.0}$ cyclone sampler measured the number of submicrometer-sized fragments that would be retained on the after-filter. In separate tests, measurement differences between the two OPCs in 15 size channels were found to be within 6.0% when tested with NaCl aerosol particles (Seo et al., 2007a). In addition, the collection efficiency of the after-filter was found to be over 99.2% in the size range of 0.01–0.3 μm.

The design flow rate of the Sharp-Cut cyclone samplers is 16.7 l/min. However, the Fragment Sampling System consists of two types of direct reading instruments which take part of the air flow from the system (1.2 l/min for OPC; 0.7 l/min for CNC). Therefore, the actual flow rates in this system differed from the design flow rates and the corrected values were 20.5 l/min at the inlet (Q_{total}), 19.3 l/min ($Q_{PM2.5}$) through the $PM_{2.5}$ cyclone sampler, 17.4 l/min ($Q_{PM1.0}$) through the $PM_{1.0}$ cyclone sampler, and 16.7 l/min (Q_{Filter}) through the after-filter. Thus, we anticipated some changes in the aerodynamic particle cut-off sizes of the cyclone samplers, corresponding to 50% penetration values.

The Fragment Sampling System was tested under controlled laboratory conditions using monodisperse PSL particles and the particulate matter aerosolized from *in vitro* cultures of *A. versicolor* and *S. chartarum*. The entire experimental setup was operated inside a Class II Biosafety cabinet (SterilchemGard: Baker Company Inc., Sanford, ME).

1.2.2. Penetration testing using PSL particles

As the flow rates in this system differed from the design flow rates of the two Sharp-Cut cyclone samplers, we determined the particle penetration characteristics of the $PM_{2.5}$ and $PM_{1.0}$ cyclone sampler under our experimental conditions with PSL particles (Bangs Laboratories Inc., Fishers, IN), with mean diameter values of less than 10% of coefficients of variation. A mixture of PSL particles of three aerodynamic sizes, 0.54 μm, 1.79 μm and 3.94 μm, was used to simulate a broad range of fungal particle size fractions. PSL particles were aerosolized as described by Aizenberg et al. (2000). Briefly, a six-jet nozzle Collision Nebulizer (BGI Inc., Waltham, MA) was used to generate aerosols from PSL suspensions (10% of concentration: 1 ml

of PSL particle suspension + 9 ml of sterile deionized water) at a flow rate of 6 l/min and a pressure of 20 psi. The aerosol flow was adjusted to 30 l/min by dilution with HEPA-filtered clean air and neutralized using a 10-Ci^{85} Kr electric-charge neutralizer (Model 3012; TSI Inc., St. Paul, MA) before entering the test chamber.

Aerosolized PSL particles which passed through the $PM_{2.5}$ or $PM_{1.0}$ cyclone sampler were measured and enumerated by two OPCs up- and downstream of each cyclone sampler. For penetration testing of the $PM_{1.0}$ cyclone sampler, the CNC located downstream of the $PM_{1.0}$ cyclone sampler was replaced by an OPC-1 (see Figure 1-1) and the total flow rate was adjusted to match the inlet flow rate of the $PM_{1.0}$ cyclone sampler ($Q_{PM1.0}$). Penetration values for each cyclone sampler were calculated using the following equation:

$$Penetration\ \ rate\ (\%) = \frac{N_{downstream}}{N_{upstream}} \times 100 \qquad \textbf{[1]}$$

where $N_{downstream}$ and $N_{upstream}$ are the down- and upstream numbers of PSL particle in each size range measured by the two OPCs. The test was repeated three times to ensure the accuracy of the results.

1.2.3. Testing with fungi

1.2.3.1. Preparation of test aerosols

The microbiological testing was conducted with particles aerosolized from cultures of *A. versicolor* and *S. chartarum*. An isolate of *A. versicolor* (RTI 367; Research Triangle Institute International, Research Triangle Park, NC) and an isolate of *S. chartarum* (JS51-05) that was

characterized as non-toxic by Jarvis et al. (1998) were utilized. Both of these species produce asexual spores (conidia).

Fungal cultures for aerosolization tests were prepared as described by Schmechel et al. (2003). Briefly, *A. versicolor* and *S. chartarum* were allowed to grow on 2% malt extract agar (MEA: 20 g/l of Dextrose, 20 g/l of malt extract, 20 g/l of agar, 1 g/l of Peptone; Difco, Becton Dickinson, Sparks, MD) for one week. Fungal spores were harvested from matured sporulating cultures by applying 1 g of dry, autoclaved glass microbeads (0.4–0.6 mm in diameter: B. Braun Biotech International, Melsungen, Germany) per Petri plate. The beads were gently shaken across the sporulating culture, and then were transferred into a 50-ml sterile tube (Fisher Scientific, Pittsburgh, PA) containing sterile deionized water (5-stage Milli-Q Plus System; Millipore Corp., Bedford, MA) with 0.05% Tween 80 (Sigma Chemicals Co., St. Louis, MO). Spore concentrations were adjusted to 10^6 spores/ml using a brightline hemacytometer (Model 3900; Hausser Scientific Company, Horsham, PA) with less than 20% of the coefficient of variation (C.V.) and 0.1 ml were used to inoculate fresh agar plates for particle aerosolization experiment.

Fungal particles were aerosolized from agar surfaces with a Fungal Spore Source Strength Tester (FSSST), which was designed to aerosolize fungal particles from contaminated surfaces using high-speed HEPA-filtered air-jets (Sivasubramani et al., 2004a). The FSSST was operated at a flow rate of 20.5 l/min to match the total flow rate of the Fragment Sampling System. Individual culture plate was kept for four minutes in the FSSST. If longer aerosolization times were needed, spent plates were replaced by new ones without shutting down the Fragment Sampling System. Aerosolized fungal particles were collected into the collection cups of each Sharp-Cut cyclone sampler and onto the after-filter according to their aerodynamic size.

1.2.3.2. Bounce testing

Cyclones operate by the principle of centrifugal impaction. While this method causes less particle bounce than inertial impaction, bounce can occur if the collection cup of the cyclone sampler becomes overloaded. In specific aim 1, spore bounce from the $PM_{1.0}$ cyclone sampler onto the after-filter was investigated to determine the highest total aerosol numbers of particles that could enter the system at which no spores were observed on the after-filter. To test this, particles, originating from culture plates, were collected with the Fragment Sampling System at sequentially increasing loads.

First, one culture plate was used to generate fragments and spores. After collection, particles were removed from the after-filter for microscopic evaluation. For this, particles collected onto the polycarbonate filter were suspended in two ml of sterile deionized water with 0.05% Tween 80 in a 10-ml sterile tube (Fisher Scientific, Pittsburgh, PA) by vortexing for two minutes followed by a 10-min ultrasonic bath agitation (Fisher Ultrasonic Cleaners: Model FS20; 3 qt., 120 V 50/60 Hz, 1 A, 80 W, without heater, Fisher Scientific Inc., Pittsburgh, PA) (Wang et al. 2001). Aliquots of 0.5 ml were filtered though a 13-mm mixed cellulose ester (MCE) filter with a pore size of 1.2 μm (Millipore Corporation, Bedford, MA) using an analytical stainless-steel vacuum filter holder (Fisher Scientific, Pittsburgh, PA). After being dried for 30 minutes, the filter was placed on a glass slide and cleared by a modified instant acetone-vaporizing unit with a continuous flow of acetone vapor. A drop of Lactophenol Cotton Blue Stain (Becton Dickinson, Sparks, MD) was placed in the center of the acetone-cleared slide. This slide was covered with a square 25 × 25 mm^2 cover glass and then the edge of the cover glass was sealed by transparent nail enamel. Spore counts were performed on 40 randomly selected microscopic

fields using a light microscopy (Leitz Laborlux S, Leica Mikroskopie und Systeme GmbH, *Wetzler*, Germany) at a 400 (10 × 40) magnification (spores/cm^2).

This procedure was repeated using two, three, four, and five plates per test until spores were found on the after-filter. Total particle load, measured with the OPC-1 for each test, was used to establish the loading threshold of particles showing bounce.

1.2.4. Collection of airborne fungal particles

After establishing the loading limit of particle bounce, the collection experiments were performed using particles released from *A. versicolor* and *S. chartarum* cultures. Airborne fungal particles were separated and collected into three distinct size fractions as already described above. Four plates were used to keep particle numbers entering the Fragment Sampling System below the threshold value while collecting sufficiently large samples for (1→3)-β-*D*-glucan analyses. During the aerosolization experiment, the concentrations of aerosolized particles (particles/l) were measured by the two OPCs and the CNC and this aerosolization experiment was repeated three times as well.

1.2.5. (1→3)-β-*D*-glucan assay

Collected fungal particles were extracted from the after-filter and the collection cups of each cyclone sampler, and analyzed for (1→3)-β-*D*-glucan by the kinetic chromogenic *Limulus* Amebocyte Lysate (LAL) method (Glucatell®: Associates of Cape Cod, East Falmouth, MA).

After-filters containing submicrometer-sized fungal particles were unloaded from sealed cassettes inside a Biosafety cabinet and aseptically transferred to a pyrogen-free tube

(Pyrotubes®, 13 × 100 mm borosilicate glass tube; Associates of Cape Cod, East Falmouth, MA). Two ml of pyrogen-free water (LAL Reagent Water®; Associates of Cape Cod, East Falmouth, MA) containing 0.05% Tween 80 were added and filters were allowed to soak at room temperature (20–24°C) for one hour. Particles were extracted by vortexing and ultrasonic agitation as described above. The resulting particle suspensions were used for both microscopic enumeration and (1→3)-β-*D*-glucan analysis. Only samples that did not have intact spores on the after-filter, as determined microscopically, were analyzed for (1→3)-β-*D*-glucan. Fungal particles collected into the collection cups of each $PM_{1.0}$ and $PM_{2.5}$ cyclone sampler were extracted using two and nine ml of pyrogen-free water containing 0.05% Tween 80, respectively, according to each collection cup size (3 and 10 ml, respectively). Extraction was performed as described for the after-filter. Aliquots of each extract (0.5 ml) were used for the analysis.

For the analysis, 0.5 ml of 0.6 M NaOH (Fisher Scientific, Pittsburgh, PA) was added to each 0.5-ml extract. This suspension was shaken using a mechanical shaker (Wrist-action Shaker®: Model 75; Burrell Scientific, Pittsburgh, PA) for one hour in order to extract (1→3)-β-*D*-glucan from the suspended fungal particles by unwinding its triple-helix structure and making it water-soluble (Foto et al., 2004; Iossifova et al., 2007b).

For quality control purposes, intra- and interplate coefficients variation (C.V.) were calculated. Fungal extract from a single plate or from three plates were analyzed for intraplate and interplate C.V.s, respectively. The C.V. of (1→3)-β-*D*-glucan content for each analysis was estimated from three aliquots per plate and each analysis was repeated three times.

To destroy and/or remove residual (1→3)-β-*D*-glucan between each run, the experimental setup including metal parts and rubber O-rings were washed with soap, and sequentially rinsed with tap water and a 2-propanol (Fisher Scientific Company, GA) wash using ultrasonic agitation in a bath for 10 minutes. Finally, all metal parts were heated for one hour at 240 °C. For quality control purposes, the cleaning efficiency was verified by repeating the extraction protocol (Appendix C) for cleaned, blank cyclone collection cups and analyzing the extracts for (1→3)-β-*D*-glucan. Blank filters and neat extraction solution were also analyzed monthly. (1→3)-β-*D*-glucan results were expressed as concentrations (ng/m^3) and as content per particle (particle-specific (1→3)-β-*D*-glucan content; ng/particle). The OPC and CNC data were utilized to calculate the content per particle.

1.2.6. Data analysis

Log-transformed particle numbers and concentrations of airborne (1→3)-β-*D*-glucan in the fragment (< 1.0 μm) and spore size range were shown to have normal distributions as determined by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Thus, data were statistically analyzed by ***t***-test using Statistical Analysis System (SAS) software (SAS for Window version 9.1; SAS Institute Inc., Cary, NC) at a significance level of 5%.

1.3. RESULTS

1.3.1. Penetration and bounce test

PSL particle penetration curves for the $PM_{2.5}$ and $PM_{1.0}$ cyclone sampler are shown in Figure 1-2. The penetration rate (%) of PSL particles obtained for different particle sizes is

plotted as a function of the optical particle diameter (D_{op}). Figure 1-2 shows the experimental 50% penetration value for the $PM_{2.5}$ and for the $PM_{1.0}$ cyclone sampler to be 2.25 μm and 1.05 μm, respectively.

Spore numbers found on the after-filter are plotted in Figure 1-3 as a function of total particles entering the Fragment Sampling System as measured with the OPC-1. For both fungal species, spore bounce was observed when more than 1.6×10^8 particles were measured with the OPC-1. In addition, the lower detection limit of spore numbers observed by the microscope was calculated as 55 spores.

1.3.2. (1→3)-β-*D*-glucan content of aerosolized submicrometer-sized fragments and intact spores

All the quality control samples of cleaned collection cups, blank filters, and extraction solutions were below the detection limit of the LAL assay (2.54 pg/ml). The median coefficient of variation (C.V.) was 10.3% (range: 6.6–14.9%) for the intraplate variability and 26.0% (6.0–30.7%) for the interplate variability. Spores on the after-filter were found only in one instance when the total number of particles measured with the OPC-1 was 1.35×10^8. This value was close to the threshold value that we established for particle bounce.

Figure 1-4(A) presents the (1→3)-β-*D*-glucan concentration (ng/m^3) in the fragment and spore size fractions collected from *A. versicolor* and *S. chartarum* cultures. The (1→3)-β-*D*-glucan concentrations (the single measurement values) ranged from 5.4×10^0 to 1.0×10^3 ng/m^3 in the fragment and the spore fractions. For *A. versicolor*, the average (1→3)-β-*D*-glucan concentrations in the fragment and spore size fractions were 2.3×10^1 and 8.1×10^2 ng/m^3,

respectively. For *S. chartarum*, the corresponding values were 8.6×10^{0} and 2.4×10^{2} ng/m^3. The (1→3)-β-*D*-glucan concentration in the fragment-spore mixture size fraction was 4.6×10^{1} ng/m^3 for *A. versicolor* and 1.4×10^{1} ng/m^3 for *S. chartarum* (these results are not shown in Figure 1-4(A)). For comparison, the detection limit for the (1→3)-β-*D*-glucan assay was 1.3×10^{-2} ng/m^3 for the spore size fraction and 1.5×10^{-2} ng/m^3 for the fragment size fraction. Statistical analysis (***t***-test) confirmed that the (1→3)-β-*D*-glucan concentration was higher in the spore than in the fragment fraction for both fungal species ($P<0.05$).

Figure 1-4(B) shows numbers of fungal particle (particles/m^3) released from *A. versicolor* and S. *chartarum* cultures. Total particle numbers were calculated as a function of the flow rate in each cyclone sampler and the sampling time. The results for both species were very similar for the fragment size fractions. However, the number of released particles in the spore fraction was 176-fold higher for *A. versicolor* than for *S. chartarum*

Figure 1-4(C) shows the particle-specific (1→3)-β-*D*-glucan contents (ng/particle) for the fragment and spore size fractions. The particle-specific content ranged from 0.3×10^{-7} to 1.9×10^{-4} ng/particle in all experiments. The lowest content was detected in the fragment fraction and the highest was found in the spore fraction. For *A. versicolor*, the average particle-specific (1→3)-β-*D*-glucan content was 5.2×10^{-7} ng/particle in the fragment fraction and 3.0×10^{-6} ng/particle in the spore fraction. The corresponding values for *S. chartarum* were 1.4×10^{-7} and 1.5×10^{-4} ng/particle, respectively.

1.4. DISCUSSION

For penetration test with PSL particles, some deviation from the nominal design penetration values (D_{50}) was observed. This can be attributed to the increased flow rate in the Fragment Sampling System. This result indicates that the majority of fungal spores (and/or spore-sized particles) would be efficiently collected by the two cyclone samplers in the new Fragment Sampling System since aerodynamic diameters of most spores are greater than 1.05 μm (Cho et al., 2005; Reponen, 1995; Baron and Willeke, 2001).

Spore bounce test demonstrated that spore bounce may still occur in the cyclone sampler when the collection cup becomes overloaded. The different size and morphology of particles may result in the irregular surface on the inside wall of collection tubes when the particles are collected. Furthermore, fungal particles have more elastic and/or spiny surface, which does not provide good contact between the particle and the collection medium (Trunov et al., 2001). Thus, this irregular surface and an elastic layer of aerosol particles may lead to particle bounce as soon as the inside wall of the collection cup becomes saturated with an elastic layer of aerosol particles. The loading threshold of 1.6×10^8 particles was set for further testing including field sampling to avoid particle bounce.

The concentrations of (1→3)-β-*D*-glucan in the fragment and spore size fractions of *A. versicolor* were 2.7 and 3.3 times higher than the corresponding fractions of *S. chartarum.* This could be explained by a higher particle aerosolization rate for *A. versicolor* compared to *S. chartarum*. This will result in the release of higher absolute particle numbers and consequently to higher (1→3)-β-*D*-glucan concentration. In addition, (1→3)-β-*D*-glucan concentrations of *A. versicolor* and *S. chartarum* in the spore size fraction comprised 92.1% and 91.5% of total

(1→3)-β-*D*-glucan concentration, respectively. These results indicate that intact spores were the main contributor to the total amount of airborne (1→3)-β-*D*-glucan.

The Gucatell® LAL assay is not specific for only fungal glucan, but will also account for (1→3)-β-*D*-glucan originating from pollen and some bacteria. However, as many epidemiological studies have reported that (1→3)-β-*D*-glucan itself has strong immuno-modulating effects (Rylander, 1996; Thorn et al., 2001; Beijer et al., 2003; Iossifova et al., 2007a), it appears to be an important parameter for exposure assessment by itself and not only as a surrogate for fungi. Other fungal components, such as ergosterol, extracellular polysaccharides (EPS), and fungal antigens could also be used as surrogate markers of fungi in the future.

1.5. CONCLUSIONS FOR SPECIFIC AIM 1

Laboratory tests showed that submicrometer-sized fragments can be successfully separated from intact spores if the total particle load of the Fragment Sampling System is kept below a certain upper limit. The (1→3)-β-*D*-glucan assay also proved to be sensitive enough for the analysis of all tested fungal aerosols including submicrometer-sized fungal fragments, as well as intact spores. In conclusion, the new Fragment Sampling System has the potential to collect a wide range of submicrometer-sized fungal fragments as well as intact spores, and thus this new methodology for the separation and analysis of submicrometer-sized fungal particles may provide a more representative method for future exposure assessment of fungal aerosols.

SPECIFIC AIM 2

Determine the total aerosolization ratio (the amount of airborne (1→3)-β-*D*-glucan divided by the amount of (1→3)-β-*D*-glucan on the surface) of particulate (1→3)-β-*D*-glucan released from different moldy materials.

2.1. INTRODUCTION

Mold-contaminated building materials may contain biologically active agents such as (1→3)-β-*D*-glucan, allergens, and mycotoxins, which have been associated with adverse health effects. However, most previous studies have focused either on identifying and evaluating the growth of fungi (or their metabolites) on building materials under controlled conditions or on characterizing how the release of fungal particles (e.g., fungal spores) is affected by environmental factors (e.g., air velocity and humidity). The release of biological components, in particular (1→3)-β-*D*-glucan, from material surfaces into the air is not well understood. Therefore, more information is needed on the release of particulate (1→3)-β-*D*-glucan from moldy materials in order to assess and understand mold exposure and associated health effects.

In specific aim 2, the new methodology (tested and optimized in specific aim 1) was utilized to investigate the release of particulate (1→3)-β-*D*-glucan from three different material samples that were artificially inoculated with mold. The aerosolization ratio of particulate (1→3)-β-*D*-glucan from moldy material samples was determined as the amount of airborne (1→3)-β-*D*-glucan divided by the amount of (1→3)-β-*D*-glucan on the surface.

2.2. MATERIAL AND METHODS

2.2.1. Test microorganisms

A. versicolor and *S. chartarum* were used as test microorganisms and were prepared for the experiments as described in specific aim 1 (section 1.2.3.1).

2.2.2. Test materials

Three types of growth materials were utilized to measure the (1→3)-β-*D*-glucan on the surface and in air samples: malt extract agar (MEA), white ceiling tiles (Armstrong World Industries, Lancaster, PA), and a wall-papered gypsum board (National Gypsum Company, Buffalo, NY). MEA is a common growth medium for fungi (Hung et al., 2005). White ceiling tile, a building material commonly used in the United States (Reynolds et al., 1990), consists of man-made mineral fibers; its porous texture is expected to support fungal growth. The wall-papered gypsum board represents a material with a high potential for the release of fungal particles due to its smooth and flat surface.

Five milliliters of 2% MEA per Petri plate was used for fungal cultivation as this condition was observed to generate more airborne fungal particles than high volume of agar per Petri plate (Seo et al., 2007a). The ceiling tile and gypsum board pieces were cut to the same round shape and dimensions as a Petri plate (diameter: 8.7 cm, area: 59.4 cm^2) with a thickness of 0.7 cm. Pre-cut pieces of building materials were autoclaved and placed into sterile Petri plates inside a Class II Biosafety cabinet (Sterilchem Gard; Baker Company, Inc., Sanford, ME). Altogether, 204 samples of each type of material were prepared [144 for aerosolization experiments (2 species × 6 incubation times × 3 replicate experiments × 4 material samples/aerosolization experiment) + 24 for blanks (2 species × 6 incubation times × 2 material samples/aerosolization experiment) + and 36 for testing the amount of (1→3)-β-*D*-glucan and counting spores on the surface of the material samples (2 species × 6 incubation times × 3 replicates)].

2.2.3. Fungal inoculation and incubation

Each fungal suspension was prepared as described in specific aim 1 (section 1.2.3.1). An aliquot of 0.1-ml fungal suspension was inoculated onto 2% MEA plates and extended using a cell spreader (Fisher Scientific Company, Pittsburgh, PA) for even growth on the surface of MEA plates. The ceiling tile and gypsum board pieces were first allowed to absorb 10 ml of autoclaved and deionized water each to establish a high water activity (a_w). This pretreatment produced a thin water film on the surface of these materials and made it easier to evenly spread the inoculum. After being soaked for one hour, each building material sample was supplemented with one ml of malt extract broth (20g/l) for simulating an external nutrient source such as settled dust on the real material surfaces. Finally, each ceiling tile and gypsum board piece was inoculated with 0.1 ml of fungal suspension.

After inoculation, the material samples (Petri plates containing MEA as well as the ceiling tile and gypsum board materials) were placed in six different chambers (2 species × 3 different materials) in order to avoid contamination between species. The 5.3-liter incubation chambers (Figure 2-1) were aerated with filter-sterilized air (0.2 μm of pore size; GE Osmonics Inc., MN) once a day for 10 minutes at a flow rate of 0.53 l/min (Murtoniemi et al., 2003). Inoculated material samples were incubated at room temperature (21–24°C) and a relative humidity of 97–99% for 1, 2, 3, 4, 5 and 6 months, respectively. This humidity was achieved by placing a saturated K_2SO_4 solution (150 g/l) at the bottom of the incubation chamber (Korpi et al., 1998). Temperature and humidity in each chamber were monitored once a day by a traceable humidity-temperature pen (Fisher Scientific Company, Pittsburgh, PA). Different materials at the same ambient relative humidity differ in their moisture content due to difference in their chemical

composition and structure (Flannigan, 1992). Moisture content on the surface of materials samples, as well as relative humidity, can affect the release of fungal particles, and thus it was measured by a moisture meter (Protimeter®: Model BLD5800; General Electric, MA) immediately before using the materials for the experiment. Moisture content was expressed as a percentage of the mass of water in a given volume of a material [(wet mass − dry mass) × 100/(dry mass)]. For a specific material, this percentage is calculated as a wood-equivalent value.

2.2.4. Characterization of test materials with respect to spore numbers and amount of (1→3)-β-*D*-glucan on their surfaces

Following a pre-determined incubation time period, the spore numbers and the amount of (1→3)-β-*D*-glucan were determined on the moldy surfaces. Three pieces of approximately one cm^2 (1 cm × 1 cm) were cut from a material sample using a sterile scalpel (Fisher Scientific Company, Pittsburgh, PA). Altogether, nine pieces of each type of material (3 pieces/material sample × 3 material samples) were prepared to count spore numbers and measure (1→3)-β-*D*-glucan. Each piece was suspended in 10 ml of pyrogen-free water (LAL Reagent Water®; Associates of Cape Cod, East Falmouth, MA) with 0.05% Tween 80 in a 50-ml pyrogen-free tube.

Fungal particles were then extracted from each 1-cm^2 material piece using a vortex touch mixer (Model 231; Fisher Scientific, Pittsburgh, PA) for two minutes followed by a 10-min agitation in ultrasonic bath (Fisher Ultrasonic Cleaners: Model FS20; 3 qt., 120 V 50/60 Hz, 1 A, 80 W, without heater, Fisher Scientific Inc., Pittsburgh, PA) (Wang et al., 2001). Spore counts in the extracts were performed using a filter-based method as described in specific aim 1 (section

1.2.3.2). The total number of spores per material sample was finally calculated by multiplying the spore number obtained from the material piece of 1-cm^2 by the area of one material sample (59.4 cm^2).

In addition, a 0.5-ml aliquot was taken from one out of three extracts per each material type for the analysis of (1→3)-β-*D*-glucan on the surfaces of the material samples. Three material samples in each type were utilized for calculating geometric means of spore numbers and (1→3)-β-*D*-glucan concentrations.

2.2.5. Collection of fungal particles aerosolized from test materials

Aerosolization and collection of fungal particles were described in specific aim 1 (section 1.2.1 and 1.2.3.1). In specific aim 2, the results for the three size fractions were combined to characterize the aerosolization of total particulate (1→3)-β-*D*-glucan released from the moldy materials.

To assure sufficient amount of particulate (1→3)-β-*D*-glucan in air samples, 3–4 material samples were used for the collection of one air sample. Fungal particles from each material sample were aerosolized for three minutes. During the aerosolization experiment, the number concentrations of aerosolized particles were measured by an optical particle counter (OPC: Model 1.108; Grimm Technologies, Inc., Douglasville, GA) located upstream of the $PM_{2.5}$ cyclone. These results were used to determine case by case the maximum number of material samples that can be used for the experiment without overloading the Fragment Sampling System (Seo et al., 2007a). In addition, the aerosol concentration was used in the data analysis after it was normalized as numbers of released particles per material sample (particles/material sample). The

aerosolization experiment was repeated three times for each combination of fungal species and material type.

2.2.6. (1→3)-β-*D*-glucan assay

In specific aim 2, (1→3)-β-*D*-glucan in samples collected from the surface of each material type and from the air were analyzed similarly as describe in specific aim 1 (section 1.2.5).

2.2.7. Determination of the aerosolization ratio of particulate (1→3)-β-*D*-glucan

For characterizing airborne (1→3)-β-*D*-glucan released from material samples, the aerosolization ratio of particulate (1→3)-β-*D*-glucan was determined by measuring the (1→3)-β-*D*-glucan in samples collected from the surface immediately before the aerosolization and from the air during aerosolization.

As described above, an aliquot taken from the extract of a surface piece of a material sample was used for estimating (1→3)-β-*D*-glucan in the extraction suspension ($BG_{surface\text{-}extract}$, ng/ml). The total amount of (1→3)-β-*D*-glucan on the surface per material sample ($BG_{surface}$, ng) was calculated using the following equation:

$$BG_{surface}\ (ng) = \frac{BG_{surface-extract} \times V_{extract}}{A_{one\ piece}} \times A_{material\ sample} \quad \textbf{[2]}$$

where $V_{extract}$ is the volume of extraction solution (10 ml), $A_{one\ piece}$ is the area of a surface piece of a material sample used for the (1→3)-β-*D*-glucan analysis (1 cm^2), and $A_{material\ sample}$ is the total area of one material sample (59.4 cm^2).

Airborne particulate (1→3)-β-*D*-glucan released from material samples was calculated by adding the particulate (1→3)-β-*D*-glucan in the three size fractions collected by the Fragment Sampling System ($BG_{air\text{-}extract}$, ng/ml). As several material samples were utilized to collect one air sample for (1→3)-β-*D*-glucan assay, the amount of airborne (1→3)-β-*D*-glucan was also normalized as airborne mass released per material sample ($BG_{airborne}$, ng) and calculated using the following equation:

$$BG_{airborne}\ (ng) = \frac{BG_{air-extract} \times V_{extract}}{N_{air}} \quad \textbf{[3]}$$

where N_{air} is the number of material samples spent for the collection of the air sample (3 or 4). Finally, the aerosolization ratio of particulate (1→3)-β-*D*-glucan was calculated using the following equation:

$$Aerosolization\ ratio\ (\%) = \frac{BG_{airborne}}{BG_{surface}} \times 100 \quad \textbf{[4]}$$

2.2.8. Data analysis

Spore numbers, $BG_{surface}$, $BG_{airborne}$, aerosolization ratios and numbers of released particles were shown to have log-normal distributions as determined by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Therefore, geometric means (GM) and geometric standard deviations (GSD) were calculated to describe the center and spread of the data. Different species, incubation

times, and material samples were examined in order to investigate their effects on log-transformed outcome variables (spore numbers, $BG_{surface}$, $BG_{airborne}$, and aerosolization ratios) using a general linear model (GLM).

Comparisons of outcome variables among material samples incubated for different time intervals were tested using a method of analysis of variance (ANOVA). Tukey's adjustment for multiple comparisons was applied in order to maintain overall 5% significance levels for hypothesis testing. A Pearson correlation coefficient was obtained to estimate the correlation between the outcome variables. The ***t***-test was performed to compare moisture content on the surface of material samples between incubation times. SAS software (SAS for Window version 9.1; SAS Institute Inc., Cary, NC) was used; and a significance level of 0.05 was applied unless indicated otherwise.

2.3. RESULTS

2.3.1. Numbers of aerosolized particles from material samples

Figure 2-2 shows numbers of aerosolized particles (particles/l) per material sample as measured by the OPC. Numbers of aerosolized particles (including fungal spores and fragments) showed varying trends with incubation time, depending on the fungal species and test materials. Results of ANOVA revealed significant decreasing trends for *A. versicolor* grown on MEA (P<0.001) and for *S. chartarum* grown on gypsum board (P<0.001). In contrast, significant increasing trends were found for *A. versicolor* grown on ceiling tile (P<0.001) and gypsum board (P<0.05), and for *S. chartarum* grown on ceiling tile (P<0.001). Paired comparisons of means between incubation periods (Tukey's range test) also showed that the means of one and six

months were significantly different. Thus, in most experiments only samples that were collected from material samples incubated for one and six months were further used for counting spore numbers and analyzing (1→3)-β-*D*-glucan.

2.3.2. Spore numbers on the surface of material samples

With increased incubation time, powdery colonies on the cultures of *A. versicolor* and the exudate (e.g., slime material) on the cultures of *S. chartarum* were observed. The exudate was particularly abundant on *S. chartarum* grown on MEA, but less profuse when it grew on ceiling tile or gypsum board. The results on spore numbers on the surface per material sample are shown in Figure 2-3. The average coefficient of variation (C.V.) for three replicate spore enumerations performed for one material samples was observed less than 10% (9.8%) indicating that fungi generally grew on the surface uniformly. The single measurement values ranged from 4.6×10^6 to 8.0×10^8 spores/one material sample. The highest value was measured for *A. versicolor* grown on MEA and the lowest value was found for *A. versicolor* grown on ceiling tile. The general linear model (GLM) demonstrated that spore numbers were influenced by the material type and species ($P<0.001$).

Comparisons among the material samples (ANOVA) followed by Tukey's range test showed that spore numbers on the three materials were significantly different for all experimental groups (*A. versicolor*: $P<0.05$ for 1-month and $P<0.001$ for 6-month; *S. chartarum*: $P<0.001$ for 1- and 6-month): spore numbers of *A. versicolor* grown on MEA and gypsum board were significantly higher than those on ceiling tile; for *S. chartarum*, spore numbers were significantly higher on gypsum board than on MEA and ceiling tile.

With respect to the comparison between species, almost all data sets showed significant differences. For fungi grown on MEA, spore numbers of *A. versicolor* were significantly higher that those of *S. chartarum* for both incubation times ($P<0.01$ for 1-month and $P<0.001$ for 6-month). For ceiling tile, however, the surface population of *A. versicolor* significantly exceeded the *S. chartarum* population only for the 6-month data set ($P<0.05$). For gypsum board, corresponding values of *A. versicolor* were significantly lower than those of *S. chartarum* at 1-month ($P<0.001$), but the 6-month data showed reverse results ($P<0.001$).

For *A. versicolor*, spore numbers on the material samples significantly increased with incubation time for MEA ($P<0.05$) and for gypsum board ($P<0.001$). In contrast, spore numbers of *S. chartarum* either did not change (ceiling tile and gypsum board) or significantly decreased ($P<0.05$ for MEA). In addition, correlation analysis showed the correlation coefficient between spore numbers and airborne particle numbers measured by the OPC was significant and strong ($P<0.001$, r=0.89).

2.3.3. Surface (1→3)-β-*D*-glucan

The (1→3)-β-*D*-glucan (ng) on the surface per material sample ($BG_{surface}$) according to incubation times are shown in Figure 2-4. The single measurement values ranged from 9.0×10^2 to 7.6×10^4 ng/one material sample. The highest and lowest values were measured in *S. chartarum* grown on gypsum board for six months and *A. versicolor* grown on gypsum board for one month, respectively. GLM showed that (1→3)-β-*D*-glucan on the surface of material samples was influenced by the incubation time and the material type ($P<0.01$).

For *A. versicolor*, a significant increase in the amount of (1→3)-β-*D*-glucan following the

6-month incubation period was observed for gypsum board (P<0.001) but not for the other two materials. For *S. chartarum*, significant differences between 1- and 6-month data sets were found for all material samples; these differences were represented by a decrease for MEA (P<0.01) and increase for ceiling tile (P<0.01) and gypsum board (P<0.05).

Significant differences among three material types (ANOVA) followed by Tukey's range test were found for all 1-month data sets (P<0.001 for *A. versicolor* and for *S. chartarum*, respectively): the surface (1→3)-β-*D*-glucan was higher for MEA inoculated either with *A. versicolor* or *S. chartarum* than for ceiling tile and gypsum board. With respect to surface (1→3)-β-*D*-glucan between both species, *S. chartarum* grown on gypsum board incubated for one month had significantly higher (1→3)-β-*D*-glucan than *A. versicolor* grown similarly (P<0.01).

Moisture contents on the surface of material samples ranged from 4.4% to 19.5% (Table 2-1). Average moisture content of materials incubated for one month was 16.2 ± 1.8%, and corresponding value for six months was 7.6 ± 2.2%, which shows an average decrease by a factor of 2.1. This decrease was significantly different (P<0.001).

2.3.4. Airborne (1→3)-β-*D*-glucan

The mass of aerosolized particulate (1→3)-β-*D*-glucan (ng) released from one material sample ($BG_{airborne}$) is shown in Figure 2-5. The single measurement values ranged from 2.0×10^0 to 1.6×10^4 ng/one material sample. The highest and lowest values were measured in *S. chartarum* grown on gypsum board for one month and on MEA for six months, respectively. GLM showed that airborne (1→3)-β-*D*-glucan released from material samples is influenced by

incubation time and the material type ($P<0.01$).

For *A. versicolor*, airborne (1→3)-β-*D*-glucan released from ceiling tile ($P<0.01$) and gypsum board ($P<0.001$) significantly increased with incubation time. For *S. chartarum*, the effect of incubation time on airborne (1→3)-β-*D*-glucan was observed for cultures grown on MEA ($P<0.001$) and ceiling tile ($P<0.05$). Overall, airborne (1→3)-β-*D*-glucan increased with incubation time, except for *S. chartarum* grown on MEA, where significant decrease was observed.

Significant differences in the airborne (1→3)-β-*D*-glucan among three material types (ANOVA) were found for all data sets, except *A. versicolor* incubated for six months (*A. versicolor*: $P<0.05$ for 1-month incubation; *S. chartarum*: $P<0.05$ for 1-month and $P<0.001$ for 6-month incubation]. Tukey's range test also showed that airborne (1→3)-β-*D*-glucan aerosolized from *A. versicolor*, which was grown on MEA for one month, appeared to be higher than those on ceiling tile and gypsum board. Similarly, for *S. chartarum*, corresponding values for 1- and 6-month data sets were lower on MEA than on ceiling tile and on gypsum board.

Additionally, differences between both species were found when fungi grown on MEA: airborne (1→3)-β-*D*-glucan was significantly higher for *A. versicolor* than for *S. chartarum* both for one ($P<0.001$) and six months of incubation ($P<0.001$). Pearson Correlation Analysis did not reveal a correlation between surface and airborne (1→3)-β-*D*-glucan ($P>0.05$). However, a significant correlation was found between airborne particle numbers (measured by the OPC) and airborne (1→3)-β-*D*-glucan ($P<0.01$, $r=0.71$).

According to the results discussed above, for *S. chartarum* grown on MEA behaved

differently from what was expected: both surface and airborne (1→3)-β-*D*-glucan decreased with incubation time. Therefore, additional (1→3)-β-*D*-glucan analyses were performed to investigate the monthly tendencies of surface and airborne (1→3)-β-*D*-glucan in more detail. The monthly data of surface and airborne (1→3)-β-*D*-glucan are shown in Figure 2-6. Results show that both parameters had decreasing trends with time. However, results of ANOVA revealed that this trend was significant only for airborne (1→3)-β-*D*-glucan ($P<0.001$). Post hoc tests showed no significant difference among mean concentrations of airborne (1→3)-β-*D*-glucan at months two through five, but corresponding values were significantly different from the data at one and six months

2.3.5. Aerosolization ratio of particulate (1→3)-β-*D*-glucan

Figure 2-7 presents the aerosolization ratios of particulate (1→3)-β-*D*-glucan (%) released from the surface of material samples. The ratios of single measurements of all material types varied from <0.1% to 92.3%. The highest value was measured for *S. chartarum* grown on gypsum board for one month and the lowest values was found on MEA for six months. GLM showed that the aerosolization ratio of particulate (1→3)-β-*D*-glucan is influenced by the material type ($P<0.001$).

Comparisons among the material types (ANOVA) showed that the aerosolization ratios of particulate (1→3)-β-*D*-glucan from *A. versicolor* grown on all three material types were not significantly different either for 1- or 6-month incubation period. In contrast, corresponding values of *S. chartarum* grown on all material samples were significantly different: the aerosolization ratio of *S. chartarum* grown on MEA was significantly lower than the respective

results for ceiling tile and gypsum board (Tukey's range test).

Incubation time did not affect the aerosolization ratios of either species. With respect to species difference, the aerosolization ratios were significantly higher for *A. versicolor* than in *S. chartarum* only for MEA.

2.4. DISCUSSION

The lowest aerosolization ratio of particulate (1→3)-β-*D*-glucan was found for *S. chartarum* grown on MEA. This can be attributed to the exudate of *S. chartarum*, which covers spores and hyphae and increases as the fungus is growing (Karunasena et al., 2004). This was supported by the finding that the low aerosolization ratio of particulate (1→3)-β-*D*-glucan was due to low concentrations of airborne (1→3)-β-*D*-glucan, but not due to low amount of (1→3)-β-*D*-glucan on the surface. There was no difference in the amount of surface (1→3)-β-*D*-glucan for *S. chartarum* grown on the three different materials for six months, and respective values of MEA incubated for one month were even higher than those of ceiling tile and gypsum board. Furthermore, numbers of particles (measured by the OPC) aerosolized from MEA were also lower than those of ceiling tile and gypsum board. Considering that (1→3)-β-*D*-glucan could be carried by airborne particulate matter (Seo et al., 2007a), it is feasible that low concentrations of airborne (1→3)-β-*D*-glucan can be attributed to low airborne particle numbers. This was supported by strong correlations between numbers of released particles and airborne (1→3)-β-*D*-glucan.

Kuhn et al. (2005) collected 200 surface samples in water-damaged homes using either swab, tape-lift or bulk sampling and detected *Stachybotrys* spp. in 58.5% of the houses. However,

airborne *Stachybotrys* spores collected by Air-O-Cell™ sampling cassettes were found in only 13.0% of the houses (26/200) and 9.6% of air samples (79/821). The above example and the MEA results in the current study indicate that *S. chartarum* spores do not readily become airborne, which may be due to the exudates produced on certain growth conditions. Furthermore, large spore size also enhances rapid gravitational settling of the aerosolized spores compared to that of many common indoor air spores, such as *A. versicolor*. For example, gravitational settling from a height of one meter would take 96 minutes for *A. versicolor* spores (aerodynamic diameter, d_a = 2.4 μm) (Reponen et al, 1996) but only 26 minutes for *S. chartarum* spores (d_a = 4.6 μm) (Sorenson et al., 1997) assuming standard density (1,000 kg/m^3) spheres at 293 K and 101 kPa (1 atm) (Baron and Willeke, 2001).

Therefore, it was surprising that the aerosolization of particles and particulate (1→3)-β-*D*-glucan from *S. chartarum* grown on building materials (e.g., ceiling tile and gypsum board) was comparable to that of *A. versicolor*, which has much smaller spores and produces powdery colonies. It appears that the release of *S. chartarum* particles from building materials can be abundant under favorable conditions. Sivasubramani et al. (2004b) studied the spore release from contaminated surfaces in four moldy homes using the FSSST. They reported a relative efficiency of 0.45 ± 0.31% for *S. chartarum* spore release from painted drywall. In the current study, the average aerosolization ratios varied from 3.19% to 12.2% for *S. chartarum* grown on building materials and from 0.01% to 0.03% for corresponding values from MEA. The absolute values are not directly comparable due to different sampling time and different air flow rate in the FSSST, but the results suggest that the use of MEA for the aerosolization test is likely to underestimate the release of *S. chartarum* particles from building materials. MEA was used for both fungal species in the present study, but other types of media (e.g., cellulose agar) may be considered for

future studies.

For *A. versicolor*, the aerosolization ratios of particulate (1→3)-β-*D*-glucan from the three material types were similar. The calculated number of particles aerosolized from *A. versicolor* grown on ceiling tile incubated for six months was around 6.4×10^4 particles/cm^2, which is very close to that (around 4.1×10^4 particles/cm^2) measured during the first three minutes by Górny et al. (2001) who also used *A. versicolor* grown on ceiling tiles, but incubated the cultures for 12 months. Sivasubramani et al. (2004a) measured the aerosolization during 10 minutes, but reported very similar values for *A. versicolor* grown on building materials for 12 months: 9.4×10^4 particles/cm^2 for ceiling tile and 5.6×10^4 particles/cm^2 for gypsum board. However, numbers of particles aerosolized from MEA incubated for one and six months in this study were much higher than those reported by Sivasubramani et al. (2004a) and Górny et al. (2001), who incubated *A. versicolor* on MEA for one week. This could be explained by different flow rate of the FSSST (Sivasubramani et al. used 12.5 l/min while 20.5 l/min was used in the present study) and different culture incubation times on MEA (1-week in the Sivasubramani et al. and Górny et al. studies versus 1- and 6-month in the present study). Longer incubation time in the present study increases fungal biomass on the surface and decreases the relatively low moisture content (5.8%) on the surface of MEA, both of which may enhance the release of fungal particles (Pasanen et al., 1991).

Other studies have also investigated the release of fungal spores (Zoberi, 1961; Pasanen et al., 1991; Foarde et al., 1999; Kildesø et al., 2003) and reported that the release rate of particles increased with increased air velocity and decreased relative humidity. However, the results of these studies are not directly comparable with the current study because of differences in the

objectives and methodologies. The objective of the previous studies was to investigate the effect of air velocity and air humidity on the release of fungal spores. These studies did not investigate the effect of incubation time, material type or the difference between *S. chartarum* and *A. versicolor*, which were the objectives of the current study. Furthermore, the different methodologies used to measure the released particles prohibit the direct comparison of the numerical values. In the current study, particle counts were obtained by the OPC and the concentration of (1→3)-β-D-glucan was measured, whereas the other studies measured either the culturable or total count of spores.

Generally, the spore numbers and (1→3)-β-*D*-glucan level, which can be attributed to the increase in fungal biomass (e.g., spores and mycelia), on the surface of material samples significantly increased with incubation time. Karunasena et al. (2000) reported that the number of colony forming units of *S. chartarum* grown on cellulose ceiling tile significantly increased up to 311 fold with incubation time: from the initial loading concentration of 1.40×10^4 to 4.35×10^6 CFU during the first seven days of incubation. Chang et al. (1995) also observed statistically significant growth of *P. glabrum, P. chrysogenum,* and *A. niger* (an increase of CFUs by more than 2 orders of magnitude during the first 28 days of incubation) at relative humidity of 94% or greater. In the present study, spore numbers on the surface increased with time up to around 320 times for *A. versicolor*, and 26 times for *S. chartarum* as the incubation period increased from one to six months. It indicates that the incubation time (7 days) Karunasena et al. used was at the initial stage of logarithmic fungal growth, and therefore, they reported a drastic CFU increase, whereas the incubation period (1-6 months) in the current study may be close to the stationary phase.

However, for *S. chartarum* grown on MEA, the spore numbers and the surface (1→3)-β-*D*-glucan level significantly decreased with time. Overall, a decrease of 65.6% was observed in the spore numbers and 62.3% in surface (1→3)-β-*D*-glucan between 1- and 6-month incubation. This may be explained by the changes occurring during spore germination. Minamikawa et al. (1984) also reported a decrease of around 23% in dry weight of germinating spores and a decrease of about 50% in insoluble glucan of germinating spores of *Adiantum capillus-veneris* during the 8-day experimental period. The investigators concluded that insoluble glucans were used as energy sources. In addition, Stone and Clark (1992) reported that microorganism produce intra- and extracellular enzymes that can degrade polymeric substances containing (1→3)-β-glucosidic linkages, which are utilized as a carbon source. Decreasing trend was only observed for *S. chartarum* grown on MEA, which also had the lowest moisture content. It is well known that different materials at the same ambient relative humidity differ in their moisture content due to difference in their chemical composition and structure (Flannigan, 1992). This study did not reveal similar decreasing trend for *A. versicolor*. The minimum water activity (a_w) needed for growth of *A. versicolor* is 0.65-0.70, whereas *S. chartarum* requires higher water activity (a_w>0.9) (Górny et al., 2001). This suggests that *A. versicolor* might have exhibited more efficient colony growth than *S. chartarum* under the same environmental conditions.

As noted above, the significant correlation between the numbers of airborne particles and the airborne concentration of particulate (1→3)-β-*D*-glucan suggests that both of these parameters are related to the aerosolized fungal biomass. Nonetheless, this correlation was not consistently observed. For example, airborne particle numbers of *S. chartarum* grown on MEA did not change during 6-month incubation time, but airborne (1→3)-β-*D*-glucan significantly decreased with time. On the other hand, released particle numbers of *A. versicolor* grown on

MEA and *S. chartarum* grown on gypsum board significantly decreased with time, but there was no change in the airborne concentration of particulate (1→3)-β-*D*-glucan. The OPC has a limited particle size range and does not measure particles smaller than 0.3 μm. A recent study reported that fungal fragments may contain considerable amount of (1→3)-β-*D*-glucan (Seo et al., 2007a) and could contribute to the total amount of (1→3)-β-*D*-glucan in the air. It may thus be expected that fungal fragments un-accounted for by the OPC may influence the amount of airborne (1→3)-β-*D*-glucan. The size distribution of airborne particles containing (1→3)-β-*D*-glucan is addressed in specific aim 3.

2.5. CONCLUSIONS FOR SPECIFIC AIM 2

In conclusion, the results indicate that the aerosolization ratio of particulate (1→3)-β-*D*-glucan is mainly influenced by the type of water-damaged material. The lowest aerosolization ratio was found for *S. chartarum* grown on MEA. However, comparable amounts of (1→3)-β-*D*-glucan were aerosolized from *S. chartarum* and *A. versicolor* grown on building materials. Use of MEA in aerosolization experiments is likely to underestimate the release of *S. chartarum* particles from building materials. These results strongly suggest that future laboratory or animal experiments should include building materials as growth substrate for fungi. The results also provide important background information for the interpretation of field measurement data.

SPECIFIC AIM 3

Measure and compare the airborne (1→3)-β-*D*-glucan concentrations of submicrometer-sized fungal fragments and intact spores released from different growth media incubated for different periods under laboratory conditions.

3.1. INTRODUCTION

Release of submicrometer-sized fungal fragments together with intact spores from mold-contaminated surfaces in large quantities has been demonstrated in earlier studies. However, the contributions of fungal fragments to exposure and health outcomes are poorly characterized. Several investigations have reported that adverse health outcomes (e.g., respiratory and cardiac responses) are strongly associated with the number concentrations of ultrafine particles (<0.1 μm), rather than with the mass or number concentrations of larger particles (Peters et al., 1997; Penttinen et al., 2001; von Klot et al., 2002). Therefore, it is necessary to measure the biological properties (e.g., (1→3)-β-*D*-glucan) of submicrometer-sized particles for better understanding of exposure to fungal aerosols in moldy environments.

In specific aim 2, aerosolization of particulate (1→3)-β-*D*-glucan from moldy building materials was characterized non-size-selectively. In specific aim 3, the data collected in specific aim 2 was analyzed size-selectively in order to characterize the (1→3)-β-*D*-glucan in submicrometer-sized fungal fragments released from moldy materials.

3.2. MATERIAL AND METHODS

3.2.1. Preparation of material samples

Three material types (MEA, ceiling tile, and gypsum board) were utilized as test materials and were prepared as described in specific aim 1 (section 1.2.3.1) and aim 2 (section 2.2.2).

3.2.2. Fungal inoculation and incubation

Two fungal species (*Aspergillus versicolor* strain RTI 367 (Research Triangle Institute International, Research Triangle Park, NC) and a non-toxic *Stachybotrys chartarum* strain JS51-05 (1998)) were used for inoculation and the procedure of inoculation and incubation was described in specific aim 2 (section 2.2.3).

3.2.3. Collection of size-selective fungal particles aerosolized from material samples

Size-fractionated fungal particles aerosolized from each material sample were collected by the Fragment Sampling System, and detailed procedures including the aerosolization of particles from fungal cultures were described in specific aim 1 (section 1.2.1 and 1.2.3.1) and specific aim 2 (section 2.2.5).

3.2.4. (1→3)-β-*D*-glucan assay

(1→3)-β-*D*-glucan of particles in the submicrometer and spore size range was analyzed by the LAL assay as described in specific aim 1 (section 1.2.5).

3.2.5. Data analysis

The concentrations of particulate (1→3)-β-*D*-glucan as well as numbers of airborne particles were shown to have log-normal distributions as determined by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Therefore, geometric means (GM) and geometric standard deviations (GSD) were calculated to describe the center and spread of the data. Different species, incubation times, and material samples were examined in order to investigate their effects on log-

transformed concentrations of size-fractionated (1→3)-β-*D*-glucan and airborne particles using a general linear model (GLM). The variability of three experimental repeats was estimated by modeling replicate measurements as a random effect. A Pearson correlation coefficient was obtained to estimate the correlation between concentrations of particulate (1→3)-β-*D*-glucan and numbers of airborne particles.

The analysis of variance (ANOVA) tested the differences of airborne particle numbers (or the concentrations of particulate (1→3)-β-*D*-glucan) released from three material types. These tests were followed by investigation of differences between species and incubation times for each material type. Tukey's adjustment for multiple comparisons was applied in order to maintain overall 5% significance levels for hypothesis testing. Statistical Analysis System (SAS) software (SAS for Window version 9.1; SAS Institute Inc., Cary, NC) was used and a significance level of 0.05 was applied unless indicated otherwise.

3.3. RESULTS

3.3.1. Particle numbers aerosolized from material samples

Figure 3-1 shows numbers of particles in the fragment size range aerosolized from the three material types incubated for one and six months. The single measurement values ranged from 1.0×10^6 to 1.6×10^9 particles/m^3. The highest and lowest values were measured for *S. chartarum* grown on gypsum boards for six months and on MEA for one month, respectively. The generalized liner models (GLM) demonstrated that numbers of airborne fragment-sized particles were statistically significantly influenced by incubation period, species, and material type (P<0.001).

Mean numbers of aerosolized particles in the fragment size range from all 6-month old cultures were significantly higher than the respective values from 1-month old cultures (P<0.001). This difference was more pronounced for fungi grown on building materials (ceiling tiles and gypsum board) than on MEA. Differences between the two species were observed for all data sets, except ceiling tile incubated for one month. For MEA, numbers of aerosolized particles from *A. versicolor* grown for one and six months were significantly higher than those from *S. chartarum* (P<0.05 for one month and P<0.01 for six months incubation). In contrast, corresponding values aerosolized from building materials were significantly higher for *S. chartarum* than for *A. versicolor* (P<0.01 for 6-month ceiling tile, and 1- and 6-month gypsum board).

Comparisons among three material types (ANOVA) showed that there were differences between the material types for all data sets. For *A. versicolor*, numbers of particles released from MEA and gypsum boards incubated for one and six months were significantly higher than those released from ceiling tile (P<0.01). For *S. chartarum*, corresponding values for gypsum board incubated for one month were higher than those for 1-month old MEA and ceiling tile, but respective data for fungi grown on MEA for six months were lower than those of ceiling tile and gypsum board with the same incubation period (P<0.01).

Numbers of aerosolized particles in the spore size range from the three material types are shown in Figure 3-2. The single measurement values ranged from 0.5×10^6 to 8.6×10^8 particles/m^3. The highest value was observed for *A. versicolor* grown on MEA for one month and the lowest value was found for *S. chartarum* grown on MEA for six months. The GLM

demonstrated that similar to fragment-sized particles, numbers of spore-sized particles were significantly influenced by incubation periods, species, and material type ($P<0.001$).

For *S. chartarum*, the differences in particle numbers between the two incubation times were found for all material types ($P<0.001$ for MEA; $P<0.01$ for ceiling tile and gypsum board). Numbers of aerosolized particles from ceiling tile increased with time, but corresponding values for MEA and gypsum board decreased with time. For *A. versicolor*, the only significant increase with time was observed for gypsum board. Differences between the two fungal species occurred for almost all material types. For MEA, numbers of aerosolized particles from *A. versicolor* grown for one and six months were significantly higher than those from *S. chartarum* ($P<0.001$). For building materials, corresponding values from *S. chartarum* grown on ceiling tiles for one and six months were higher than those from *A. versicolor* ($P<0.05$), while opposite results were obtained for gypsum board incubated for six months.

Significant differences among the three material types (ANOVA) were observed. Released particle numbers from *A. versicolor* grown on MEA and gypsum board were significantly higher than those from ceiling tile ($P<0.001$). For *S. chartarum*, the particle numbers in the spore size range showed similar trends as numbers of particles in the fragment size range. Corresponding values for gypsum board incubated for one month were higher than those for MEA and ceiling tile ($P<0.01$), and after six months of incubation, numbers of aerosolized particles from ceiling tile and gypsum board were higher than those aerosolized from MEA ($P<0.001$).

The ratio of particle numbers in the fragment size range to those in the spore size range (particle F/S-ratio) is presented in Table 3-1 and varied from 0.011 to 104.2. The highest value was measured for *S. chartarum* grown on MEA for six months and the lowest for *A. versicolor* grown on MEA for one month. Average particle F/S-ratios were calculated as 1.01 ± 0.82 (average ± standard deviation of three repeats) for *A. versicolor* and 23.29 ± 22.33 for *S. chartarum*. A significant difference between the two incubation times was observed for *S. chartarum* grown on all three material types ($P<0.001$). Furthermore, for all material types incubated for six months, particle F/S-ratios for *S. chartarum* were significantly higher than those for *A. versicolor* ($P<0.001$).

3.3.2. Size-fractionated concentrations of particulate (1→3)-β-*D*-glucan aerosolized from material samples

Figure 3-3 presents the concentrations of particulate (1→3)-β-*D*-glucan in the fragment size range aerosolized from the three material types. The single measurement values ranged from 4.3×10^{-1} to 9.8×10^{2} ng/m^3. The highest and lowest values were measured for *A. versicolor* grown on MEA for six months and on gypsum board for one month, respectively. The GLM showed that the (1→3)-β-*D*-glucan concentrations of fragment-sized particles were influenced by the species and the material type ($P<0.001$).

Species differences were found for MEA incubated for one and six months and ceiling tile for one month. The concentrations of (1→3)-β-*D*-glucan aerosolized from *A. versicolor* were significantly higher than those from *S. chartarum* ($P<0.05$ for 1-month and $P<0.01$ for 6-month MEA; $P<0.01$ for 1-month ceiling tiles). Differences among the three material types were found

only for *A. versicolor* grown for one month. The concentrations of (1→3)-β-*D*-glucan released from MEA and ceiling tile were higher than the corresponding concentrations from gypsum board.

The concentrations of particulate (1→3)-β-*D*-glucan in the spore size range are shown in Figure 3-4. The single measurement values ranged from 1.0×10^{1} to 4.7×10^{4} ng/m^3. The highest and the lowest values were measured for *S. chartarum* grown on gypsum board for one month and on MEA for six months, respectively. The GLM demonstrated that the (1→3)-β-*D*-glucan concentrations of spore-sized particles were influenced by the incubation period and material type (P<0.001).

Differences in the (1→3)-β-*D*-glucan concentration between the two incubation times were mainly observed for *A. versicolor*: the concentrations of (1→3)-β-*D*-glucan increased with time for all three material types (P<0.05). In contrast, the corresponding values for *S. chartarum* grown on MEA decreased with time. Differences among the three material types (ANOVA) followed by Tukey's range test were observed for *S. chartarum* grown for six months (P<0.001): furthermore, the concentrations of (1→3)-β-*D*-glucan released from ceiling tile and gypsum board were statistically significantly higher than the respective concentrations from MEA

The ratio of (1→3)-β-*D*-glucan concentrations in the fragment size range to those in the spore size range (β_G F/S-ratio) is shown in Table 3-2 and varied from 0.2×10^{-3} to 6.7×10^{-1}. The highest value was measured for *S. chartarum* grown on MEA for six months and the lowest for *S. chartarum* grown on gypsum board for one month. The average β_G F/S-ratios were 0.02 ± 0.02

for *A. versicolor* and 0.06 ± 0.16 for *S. chartarum*. A significant difference between the two incubation times was observed for *A. versicolor* grown on ceiling tiles only.

3.3.3. Correlation analyses

According to the results presented above, the concentrations of particulate (1→3)-β-*D*-glucan did not always follow the same trends as numbers of particles aerosolized from material samples. Furthermore, the results in the fragment size range had different patterns from those in the spore size range. The scatter plots of the particle numbers versus the concentrations of particulate (1→3)-β-*D*-glucan are shown in Figure 3-5. The correlation analysis including data in the fragment and spore size range (n=72) failed to show correlation between measurements (Figure 3-5(A): r=-0.05, $P>0.05$). Additionally, analysis including data in the fragment size range only (n=36) revealed low association between these variables (r=0.31, $P>0.05$). In contrast, the data in the spore size range (n=36) showed relatively good correlation between the particle numbers and the concentration of particulate (1→3)-β-*D*-glucan (Figure 3-5(B): r=0.68, $P<0.0001$). Poor correlations were found between the fragment and spore results with respect to either particle numbers (r=-0.04, $P>0.05$) or particulate (1→3)-β-*D*-glucan mass (r=0.14, $P>0.05$).

3.4. DISCUSSION

Overall, the particle numbers released from mold-contaminated surfaces increased with incubation time. This was most clearly seen with numbers of fragment-sized particles, which were always significantly higher for 6-month old cultures than for 1-month old cultures. In the

spore size range, this trend was also observed in many cases. The increased release of particles can be explained by the changes in fungal biomass and moisture content. Spore numbers on the surface increased about up to 320 times for *A. versicolor* and up to 26 times for *S. chartarum* when the incubation time increased from one to six months (Seo et al., 2007b). The average moisture content on the surface of material samples was 17.9% for 1-month old cultures and decreased to 5.3% for 6-month old cultures (Seo et al., 2007b). Dryness on the surface can increase the release of fungal particles by reducing adhesion forces among fungal structures and enhancing these structures to become brittle. However, the numbers of particles in the spore size range released from *S. chartarum* grown on MEA and gypsum board decreased with time. This finding was consistent with the trend in total particle numbers and total mass of particulate (1→3)-β-*D*-glucan aerosolized from material samples reported by Seo et al. (2007b) and may be associated with the slimy materials produced by *S. chartarum* when it grows. Furthermore, the microscopically observed extent of aggregation of slimy materials surrounding the spores was highest on MEA and lowest on ceiling tile (Seo et al., 2007b).

Generally, size-fractionated concentrations of (1→3)-β-*D*-glucan released from material samples had increasing trend with increase in the incubation time, but not as clearly as with numbers of airborne particles. In contrast to particle numbers, the increase in the aerosolized (1→3)-β-*D*-glucan was more notable in the spore size range than in the fragment size range. The concentrations of particulate (1→3)-β-*D*-glucan in the spore size range released from *A. versicolor* grown on all material types for six months were significantly higher than the respective values for one month; however, the opposite trend was found for *S. chartarum* grown on MEA. This is consistent with the decreased numbers of particles in the spore size range

aerosolized from *S. chartarum* grown on MEA. This is also supported by a good correlation between the airborne concentrations of particulate (1→3)-β-*D*-glucan and numbers of aerosolized particles in the spore size range.

For the data in the fragment and spore size ranges combined, the lack of correlation between airborne (1→3)-β-*D*-glucan mass and numbers of aerosolized particles occurred due to poor correlation between these two parameters in the fragment size range. This helps explaining why in the concurrent study (specific aim 2; Seo et al., 2007b), the total mass of aerosolized (1→3)-β-*D*-glucan did not consistently follow the same trends as the total numbers of aerosolized particles. The difference in the correlation may be due to the different characteristics of particles in the spore and fragment size ranges. Fungal spores released from material samples have relatively monodisperse size distribution (Reponen et al., 1998; Kanaani et al., 2007), and thus, spore numbers are expected to be associated with the concentration of (1→3)-β-*D*-glucan originating from pure fungal cultures. On the other hand, fungal particles in the fragment size range are derived from intra- and extracellular structures of fungal colonies when aerosolized (Górny et al., 2002). Fungal fragments originated from intracellular components are expected to contain less (1→3)-β-*D*-glucan than those originating from the cell wall. Fungal fragments vary in size more than fungal spores (Cho et al., 2005) and causes additional variation in the (1→3)-β-*D*-glucan mass per particle. Thus, (1→3)-β-*D*-glucan content per particle can vary more in the fragment than in the spore size range, and results in poor correlation between numbers of fragment-sized particles and the mass concentrations of (1→3)-β-*D*-glucan. In addition, biodegradation of building materials, which is caused by fungal growth, may contribute to this difference. When fungi utilize cellulose-containing building materials as a nutrient source,

biodegradation of materials occurs (Gutarowska et al., 2007) and may result in the generation of small particles, which may not contain (1→3)-β-*D*-glucan. Thus, these particles cannot contribute to (1→3)-β-*D*-glucan concentrations in air samples and further reduce the correlation between the two parameters.

Significant differences between the two species were observed for the particle numbers and (1→3)-β-*D*-glucan mass concentrations. However, the extent of these differences varied depending on the incubation time and material type. The most pronounced difference was found for fungal particles in the spore size range released from MEA: both the particle numbers and concentrations of particulate (1→3)-β-*D*-glucan in the spore size range were lower for *S. chartarum* than for *A. versicolor*. For *S. chartarum*, the differences between building materials and MEA were more pronounced than for *A. versicolor*. This finding is consistent with the aerosolization ratios of non-size-fractionated particulate (1→3)-β-*D*-glucan previously reported by Seo et al. (2007b). The results suggest that the use of laboratory media such as MEA is likely to underestimate the release of *S. chartarum* spores from real building materials.

The average particle F/S-ratios were slightly higher for *S. chartarum* (0.36) than for *A. versicolor* (0.16) for 1-month old cultures. This difference was more pronounced for 6-month old cultures: the particle F/S-ratios of *S. chartarum* grown for six months were up to 36-fold higher than those of *A. versicolor*. Generally, the β_G F/S-ratios were also higher for *S. chartarum* (0.06) than for *A. versicolor* (0.02). These results indicate that spores were the main contributor to the total (1→3)-β-*D*-glucan mass aerosolized from mold-contaminated materials under laboratory conditions. The contribution of fragments to the total (1→3)-β-*D*-glucan mass was higher for *S. chartarum* than for *A. versicolor*. The spores are also the main contributor to the total particle

numbers released from young cultures (F/S-ratio <1). For older cultures, however, the contribution of fragments became more pronounced than that of spores (F/S-ratio >> 1).

3.5. CONCLUSIONS FOR SPECIFIC AIM 3

The release of particles and particulate (1→3)-β-*D*-glucan generally increased with the age of the fungal culture. This increase was most consistent for particle numbers in the submicrometer size range. Particle numbers correlated with the mass concentrations of particulate (1→3)-β-*D*-glucan only in the spore size range. This is possibly due to variation in the (1→3)-β-*D*-glucan content per fragment particle attributed to a wide size range and test-specific size distribution of fungal fragments as well as the variation in the origin of the fragments within the fungal structure. Results of β_G F/S-ratios indicate that the contribution of fragments to the total mold exposure was higher for *S. chartarum* than for *A. versicolor*. The present study indicates that long-term mold damage in buildings may increase the contribution of submicrometer-sized fungal fragments to the overall mold exposure. The health impact of these particles may be even greater than that of spores, considering the strong association between numbers of fine particles and adverse health symptoms reported in other studies (Magari et al., 2001 and 2002; Gold et al., 2000; Pekkanen et al., 2002). As the fragment results did not correlate with the corresponding spore results, fragment concentrations cannot be estimated based on spore data. Therefore, assessment of submicrometer-sized fungal particles should be conducted along with other mold measurements and analyses when determining exposure in moldy indoor environments.

SPECIFIC AIM 4

Evaluate the new methodology in moldy environments, and compare the ratios of airborne (1→3)-β-*D*-glucan concentrations in the fragment size range to those in the spore size range (F/S ratios) measured in moldy homes with corresponding ratios measured in laboratory experiments.

4.1. INTRODUCTION

Smaller-sized fungal fragments (<1.0 μm) may contribute to mold-related health effects. Previous laboratory-based studies have shown that numbers of fungal fragments can be up to 500 times higher than that of fungal spores, and furthermore they contain biologically active agents such as allergens, mycotoxins, and (1→3)-β-*D*-glucan. However, this has not yet been confirmed in a field study due to lack of suitable methodology. Traditional microbiological methods, such as cultivation and microscopic counting cannot be used for the analysis of fungal fragments as described in the Background ("Rationale for selection of appropriate analytical method" section). In laboratory-based studies, direct-reading particle counters can be deployed for the enumeration of fragments since non-fungal particles are eliminated in the laboratory set-up. In field situations, fungal fragments are masked by other particles, and a specific technique is needed to analyze fungal-derived components.

In specific aim 4, the new methodology was utilized for characterizing submicrometer-sized fungal fragment exposures in mold-contaminated homes located in New Orleans, Louisiana and Southern Ohio.

4.2. MATERIAL AND METHODS

4.2.1. Selection of homes

The field investigation was conducted in five mold-contaminated single-family houses (Table 4-1). Three houses, located in New Orleans, Louisiana, were flooded during hurricane Katrina, and were severely mold-contaminated. Two other houses were located in Southern Ohio:

both had suffered water-damage in the basement and had either water-damage (house 4) or visible mold (house 5). Sampling was performed in all five homes during the summer of 2006 (June–September) and repeated in two homes (one is for New Orleans and this other is for Southern Ohio) in the winter (December, 2006–January, 2007).

4.2.2. Safety measures

Prior to field sampling in New Orleans, respirator fit-tests were performed for all personnel participating in field sampling. Half mask respirators (North Safety Products, Cranston, RI), safety goggles, gloves and tyvek suits with hoods (DuPont Tyvek, Wilmington, DE) were worn during sampling.

4.2.3. Air sampling

Size-selective sampling of fungal particles was performed using the Fragment Sampling System. The flow-rate of 16.7 l/min was achieved using a high-volume air sampling pump (Model SP-280; Air Diagnostics and Engineering Inc., Harrison, ME). It was powered by a car battery (DieHard®: Model 30052; Size 51R, Sears, New Orleans, LA) when testing in New Orleans as no electric power was available in the Katrina-affected areas. The sampling flow rate was calibrated with Drycal® DC-Lite Calibrator (Bios International Corp., Butler, NJ) before and after each measurement.

Sampling time needs to be long enough to collect a sufficient amount of particulate matter in the submicrometer size range for the subsequent analysis. On the other hand, longer sampling times may lead to overloading and spore bounce. During performance tests of the Fragment

Sampling System, it was observed that spore bounce from the collection cup of the second cyclone onto the after-filter can occur if the particle number entering this system exceeds a threshold of 10^8 particles (Seo et al., 2007a). Spores on the after-filter would confound the fragment results as the aim is to collect purified fragments in the submicrometer size fraction. Therefore, sampling times were adjusted to overall concentrations of airborne particles as measured by an optical particle counter (OPC: Model 1.108; Grimm Technologies, Inc., Douglasville, GA) operating in parallel with the Fragment Sampling System. The sampling time varied from 120 to 180 minutes.

Traditional sampling was also performed in parallel using the Button Sampler (SKC, Inc., Eighty Four, PA) loaded with a 25-mm polycarbonate filter (pore size 0.4 μm: GE osmosis Inc., Minnetonka, MN). (1→3)-β-*D*-glucan concentrations of samples collected by the Button Sampler were compared to those collected by the Fragment Sampling system to evaluate the performance of the new system. To reduce pressure drop of the filter in the Button Sampler, a metal screen with a sparse mesh was used as support pad. The Button samplers were attached to battery operated pumps (Model 4004; BGI Inc., Waltham, MA), which produced a flow rate of 4 l/min. The sampling time was the same as for the Fragment Sampling System.

Indoor samples were collected in mold-contaminated rooms during two days. Altogether, three indoor samples were taken in each home during each season. One outdoor sample per day was collected, except in New Orleans during the summer season, because outdoor sampling of fragments could not be performed there at that time due to limitations of the battery power source. In addition, temperature and relative humidity per sampling were measured using a traceable humidity/temperature pen (Fisher Scientific Company, Pittsburgh, PA).

4.2.4. (1→3)-β-*D*-glucan analysis

4.2.4.1 Size-fractionated samples

Size-fractionated (1→3)-β-*D*-glucan of particles was analyzed by the LAL assay as described in specific aim 1 (section 1.2.5).

4.2.4.2 Button samples

The samples on polycarbonate filters collected by the Button Sampler were extracted as described for the after-filters of the Fragment Sampling System (section 1.2.5). An aliquot was analyzed for (1→3)-β-*D*-glucans by the LAL assay to compare (1→3)-β-*D*-glucans concentrations obtained by the Button Sampler to those obtained by the Fragment Sampling System.

Quality control samples included trip blanks, field blanks, and extraction fluid blanks. The Fragment Sampling System and the Button Samplers were cleaned before individual runs by first washing with soap, rinsed with water, and finally rinsed with ethanol. All metal parts of the cyclones including collection cups and the Button Samplers were treated by heating at 240°C for one hour to remove residual (1→3)-β-*D*-glucan. The cleaning efficiency was verified by performing a LAL-assay for blank samples extracted from a set of cleaned, non-used cyclone collection cups.

4.2.5. Data analysis

Data distribution was analyzed by Shapiro Wilk-test. All data were found to be log-normally distributed, except the total (1→3)-β-*D*-glucan concentration. Thus, geometric means (GM) and geometric standard deviations (GSD) were used for the descriptive statistics. Pearson Correlation Analysis and ***t***-test (or paired ***t***-test) were utilized for data analysis and calculated from log-transformed data. The statistical analyses were performed using SAS/Stat 9.1 (SAS Institute Inc., Cary, NC), and a significance level of 0.05 was used for all statistical tests.

4.3. RESULTS

4.3.1. Meteorological observations

Results on relative humidity and temperature during the environmental sampling are presented in Table 4-2. The highest values for indoor relative humidity (69.3–90.4%) and temperature (28.9–38.8°C) were measured in New Orleans in the summer. The values were lowest during the winter measurement cycle.

4.3.2. (1→3)-β-*D*-glucan concentration in different seasons

The total (1→3)-β-*D*-glucan varied from 0.2 to 15.9 ng/m^3 and was significantly higher in New Orleans than in Southern Ohio (P<0.001). The total (1→3)-β-*D*-glucan measured with the Fragment Sampling System correlated well with those measured with the Button Sampler (r=0.92; P<0.001) (Figure 4-1). The difference in (1→3)-β-*D*-glucan measured with these two methods was on average 8.1% and was not statistically significant (P=0.07). However,

differences dependent on the areas and seasons were observed. In the summer, the measurements of total (1→3)-β-*D*-glucan with the Fragment Sampling System in New Orleans showed a strong agreement with those with the Button Sampler (r=0.94; $P<0.001$), but a poor correlation was observed in corresponding data of Southern Ohio (r=0.49, $P>0.05$). In the winter, low areal differences of the total concentrations of (1→3)-β-*D*-glucan were relatively observed (r=0.70, $P<0.01$).

Similar to the total (1→3)-β-*D*-glucan, the size-fractioned concentrations in both the fragment and spore size fractions were higher in homes in New Orleans than in homes in Southern Ohio (Table 4-3). This difference, however, was significant only in the summer ($P<0.01$ for fragments and $P<0.001$ for spores).

There was a clear seasonal variation in the size-fractionated concentrations: the (1→3)-β-*D*-glucan in spore size fraction ($P<0.01$) was higher in the summer than in the winter, whereas the situation was opposite for the (1→3)-β-*D*-glucan in fragment size fraction ($P<0.05$) (Figure 4-2). In the summer, the (1→3)-β-*D*-glucan concentration was significantly lower in the fragment size fraction than in the spore size fraction ($P<0.001$), but there was no difference in the winter data.

4.3.3. The (1→3)-β-*D*-glucan correlation and ratio of fragment to spore size fraction

There was only a borderline correlation between the (1→3)-β-*D*-glucan concentration in the fragment size fraction with that in the spore size fraction when all the data were combined (r=0.33; $P>0.05$) (Figure 4-3). This was driven by the data obtained in the indoor air in the summer as there was a significant correlation only for this data set (r=0.72; $P<0.01$).

The ratio of the (1→3)-β-*D*-glucan concentrations in the fragment size fraction to that in the spore size fraction (F/S ratio) varied from 0.01 to 2.16 (Table 4-3). In the summer, the F/S-ratio was significantly lower in New Orleans than in Southern Ohio ($P<0.05$) but there was no difference in F/S-ratio between these two locations in the winter. The average F/S ratio for the indoor samples collected in the summer (0.28) was significantly lower than for those collected in the winter (1.02) ($P<0.05$) (Figure 4-4).

4.4. DISCUSSION

The F/S-ratio of (1→3)-β-*D*-glucan ranged from 0.01 to 2.16, and the highest average (F/S =1.02) was found for the indoor samples collected in the winter. The corresponding ratio measured in the laboratory study was 0.018 for fungal particles released from *A. versicolor* grown for six months and 0.105 for *S. chartarum* (Seo et al., 2007c). These findings, thus, indicate that the (1→3)-β-*D*-glucan concentration in the fragment size fraction was often equal and in some cases even higher than that in the spore size fraction.

For the numbers, we have previously shown that numbers of fungal fragments was as much as 500 fold higher than that of spores (Cho et al., 2005). In field situations, numbers of fragments can be estimated based on the mass of (1→3)-β-*D*-glucan. Assuming that the mass concentration of fragments and spores is equal (F/S =1) and the spore size is 3.0 μm, the number ratio (fragment number/spore number) would be 10^3 and 10^6, if fragment size is 0.3 and 0.03 μm, respectively. These results indicate that the actual (field) contribution of fungal fragments to the overall fungal exposure may be very high, even much higher than suggested by our estimates based on the laboratory studies. This can be attributed to the difference in the sampling protocols.

In the laboratory tests, fungi were released from the surface directly into the experimental system by high air velocity jets. In the field, the release occurred naturally through a variety of mechanisms, including air currents and vibrations, and the release of fragments may have been enhanced by the changes in relative humidity. Furthermore, the larger particles (spores) had time to settle down before sampled from the air.

The clinical significance of fungal fragments is currently not known. However, exposure to fine particles in ambient air has been associated with several adverse health outcomes, including respiratory and cardiac responses (Peters et al., 1997; von Klot et al., 2002; Penttinen et al., 2001). These health effects have shown stronger associations with numbers of ultrafine particles (<0.1 μm) than with mass or numbers of larger particles. Smaller-sized fungal fragments have longer lifetimes in the air compared to spores; they can be easily transported by air currents and more efficiently deposited in the alveolar region than intact spores. Based on laboratory studies on the number and size distribution of fungal particles, it has been estimated that the respiratory deposition of *Stachybotrys chartarum* fragments in adults is 230 times higher than that of spores (Cho et al., 2005). When the respiratory deposition model was applied to infants, it was demonstrated that the deposition ratios (fragments versus spores) were 4–5 times higher than those for adults (Cho et al., 2005). High surface area of fragments may further facilitate the bioavailability of fungal components after the particles are inhaled into the human lung. Due to their small size, fragments may be able to evade phagocytosis by macrophages, and can be translocated through systemic circulation (Ibald-Mulli et al., 2002).

(1→3)-β-*D*-glucan in the spore size fraction appeared to follow similar pattern as the total fungal aerosol with respect to the seasonal difference and the difference between homes located

in New Orleans versus Southern Ohio. However, these patterns were drastically different for (1→3)-β-*D*-glucan in the fragment size fraction, which did not correlate with the (1→3)-β-*D*-glucan in the spore size fraction. Highest fragment concentrations were found in the winter, when the fungal spore concentrations were at their lowest levels. This may be attributed to the low relative humidity in the winter, which in turn may increase the release of fungal particles by reducing forces among fungal structures and enhancing these structures to become brittle.

There are no previous reports on (1→3)-β-*D*-glucan analyzed by the LAL-method in New Orleans, but several investigators have used this method for the analysis of air samples in other geographic areas (Thorn and Rylander, 1998; Foto et al., 2005; Lee et al., 2006). Thorn and Rylander (1998) studied 75 homes in Swedish row houses suffering from moisture problems and found the (1→3)-β-*D*-glucan concentrations up to 19.0 ng/m^3. Foto et al. (2005) measured (1→3)-β-*D*-glucan in 110 Canadian homes, most of which had mold problems. They reported concentrations ranging from 0.04 to 20.55 ng/m^3. Lee et al. (2006) reported a range of 0.31–9.35 ng/m^3 for five non-moldy homes in Cincinnati. While the indoor (1→3)-β-*D*-glucan levels measured in New Orleans in this study are much higher than those reported by Lee et al. (2006), they fall in the ranges reported by Thorn and Rylander (1998) and Foto et al. (2005). The levels measured in Southern Ohio in this study are within the range reported by Lee et al. (2006). These comparisons show that the data obtained in New Orleans homes in our study represent fungal exposures in non-renovated homes with significant water-damaged such as flooding in the aftermath of hurricanes Katrina and Rita. On the other hand, the homes in Southern Ohio represent an exposure situation when the concentrations are only slightly or not at all elevated

compared to non-moldy homes. This study showed that fungal fragments may represent a significant portion of the fungal exposures in both types of exposure scenarios.

4.5. CONCLUSIONS FOR SPECIFIC AIM 4

The very close agreement between (1→3)-β-*D*-glucan concentrations measured by the Button Sampler and the Fragment Sampling System shows a good capability of the this system combined with (1→3)-β-*D*-glucan assay for quantifying fungal particles in the fragment and spore size fraction under field conditions.

In addition, these findings indicate that the actual (field) contribution of fungal fragments to the overall exposure is very high, even much higher than was earlier estimated resulting from previous laboratory studies. As the exposure to airborne fungal fragments cannot be quantified based on spore concentrations, submicrometer-sized fragment measurements should be included when assessing exposures in moldy buildings.

OVERALL CONCLUSIONS

Successful separation of submicrometer-sized fungal fragments from intact spores and quantification of these samples using the (1→3)-β-*D*-glucan assay in laboratory experiments suggest that the new Fragment Sampling System combined with the (1→3)-β-*D*-glucan assay may provide a more representative method for future assessment of mold exposure (specific aim 1). The release of particulate (1→3)-β-*D*-glucan is influenced by the type of water-damaged material. Furthermore, statistical differences of aerosolization ratios of particulate (1→3)-β-*D*-glucan were observed between a laboratory medium (e.g., MEA) and building materials. These results suggest that future laboratory or animal experiments should always include real building materials as growth substrate for fungi (specific aim 2). The release of particles and particulate (1→3)-β-*D*-glucan generally increased with the age of the fungal culture. The increase was most consistent for particle numbers in the submicrometer size range. As previous investigations have shown strong associations between the numbers of fine particles and adverse health symptoms may increase health impact of these particles, assessment of submicrometer-sized fungal particles should be included when assessing exposure in moldy indoor environments (specific aim 3). Comparison of the total (1→3)-β-*D*-glucan concentrations measured by the Button Sampler and the Fragment Sampling System in moldy homes provided a proof-of-principle for the Fragment Sampling System to collect samples with minimal sample losses. A considerable amount of particulate (1→3)-β-*D*-glucan in the fragment size range was observed in field study. Thus, this finding suggests the need to measure submicrometer-sized fragments when determining exposures in moldy buildings (specific aim 4).

In summary, submicrometer-sized fungal fragments were characterized using the (1→3)-β-*D*-glucan assay and their presence under field conditions was confirmed in this Ph.D. study. The high content of these fragments supports their potential contribution to the adverse health effects in moldy indoor environments. Therefore, measurement of submicrometer-sized fungal fragments can provide more comprehensive exposure profiles in support of investigating health outcomes.

FUTURE DIRECTIONS

- Modification of the Fragment Sampling System for a large-population based study.

  For a large-population based study, it is essential to modify the Fragment Sampling System into a more user-friendly system. To save time for assembling the sampler set-up (e.g., Sharp-Cut cyclone samplers, collection cup, and direct reading instrument) as well as for the easier use by an un-trained person, a more compact design of the collection system is needed. Furthermore, the development of disposable collection cups will be needed in parallel to reduce labor and time for sample extraction. Also, it would be good to develop a a battery-operated system in order to enable to collect samples in places without electricity.

- Animal studies to investigate dose-response relationships of inhaled submicrometer-sized fungal particles and their adverse health effects.

- A large-population based study

  In the current study, the field sampling was performed only in a few moldy homes and no health data were collected. Data are needed to establish a clear cause-and-effect relationship between inhalation of fungal fragments and adverse health effects on occupants. Therefore, further epidemiological studies including the assessment of submicrometer-sized fungal fragments are recommended.

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FIGURES

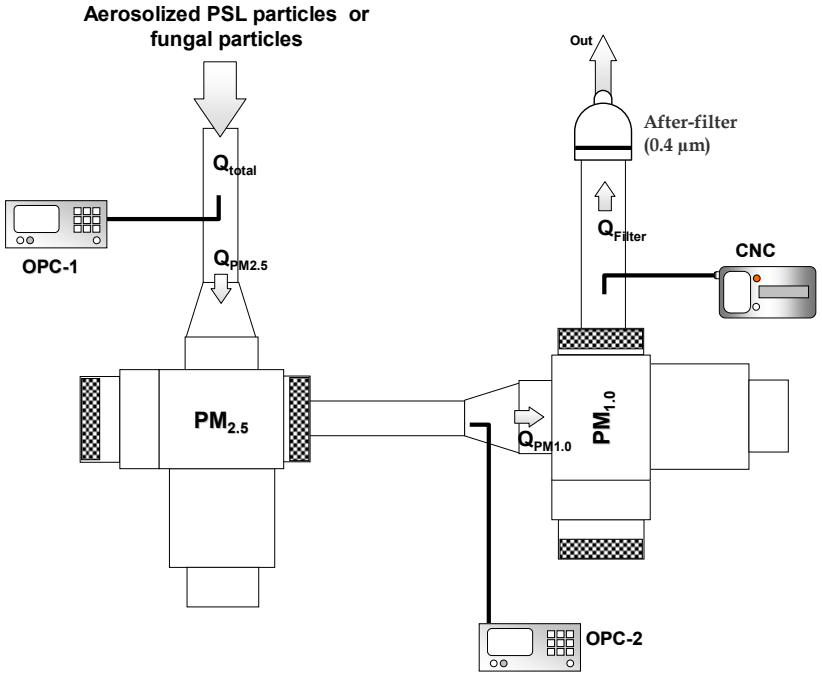

FIGURE 1-1. Experimental set-up: OPC=Optical Particle Counter; CNC=Condensation Nucleus Counter. Q=the air flow rate (l/min).

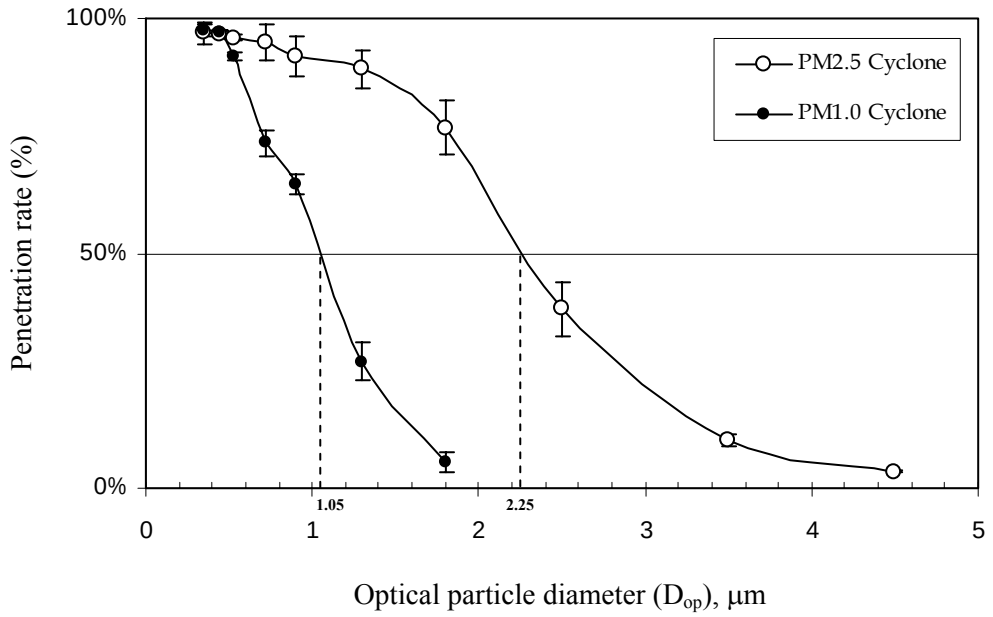

FIGURE 1-2. PSL particle penetration curves and their corresponding 50% penetration values for the Sharp-Cut cyclone samplers at a flow rate of 19.3 l/min for $PM_{2.5}$ and 17.4 l/min for $PM_{1.0}$. Each data point presents the average of three repeated experiments. The error bars represent standard deviations calculated for each group.

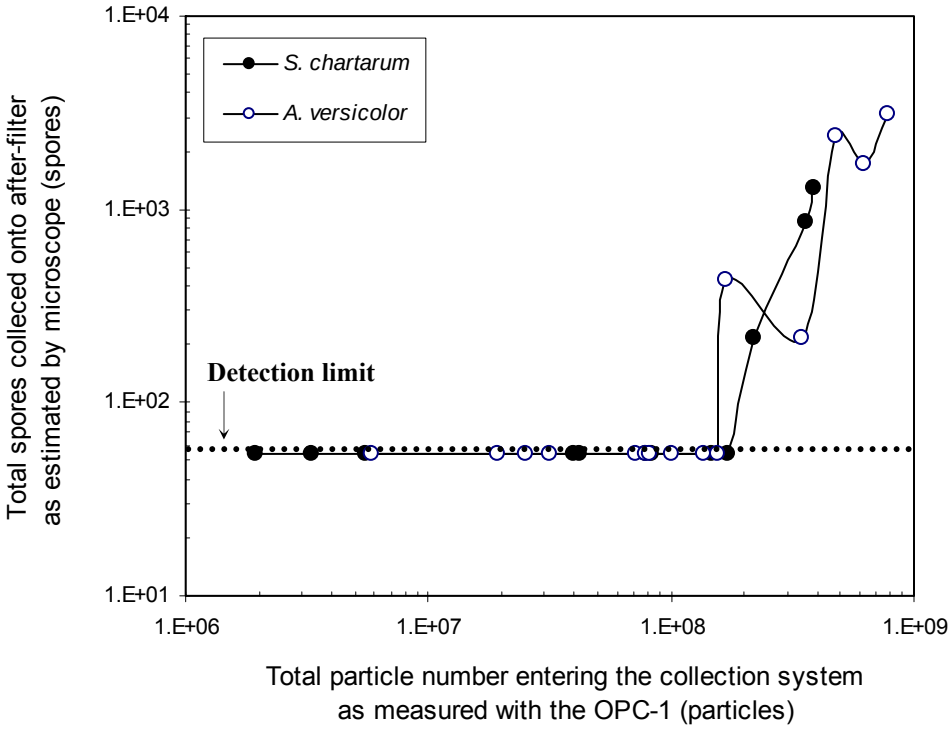

FIGURE 1-3. Spore numbers collected on the after-filter as a function of the total particle number entering the collection system as measured with the OPC-1. The dotted line represents the lower detection limit of spore numbers (55 spores).

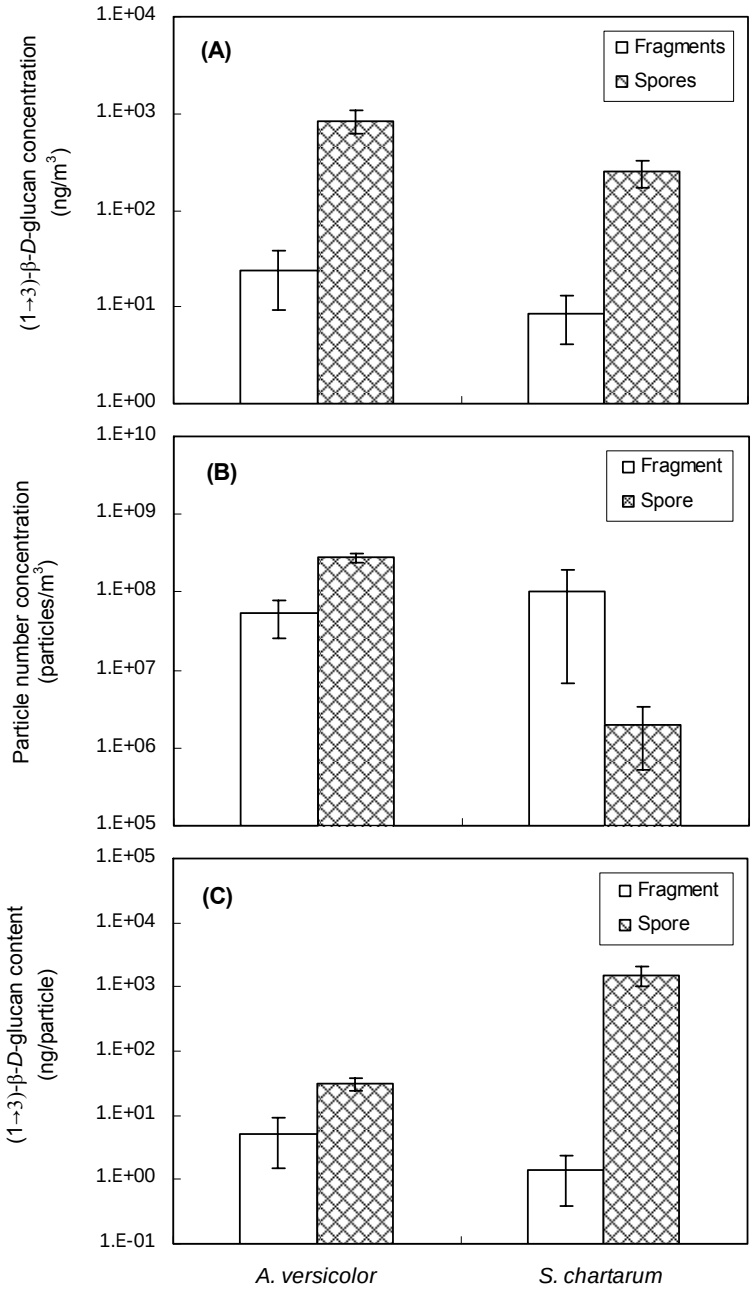

FIGURE 1-4. (1→3)-β-*D*-glucan assay results of samples collected from *A. versicolor* and *S. chartarum* into fragment and spore size fractions: (A) (1→3)-β-*D*-glucan concentration in the air (ng/m^3); (B) particle number concentration (particles/m^3) in the air; (C) particle-specific (1→3)-β-*D*-glucan content (ng/particle). The histograms present the averages of three repeated experiments. The error bars represent the standard deviations calculated for each group.

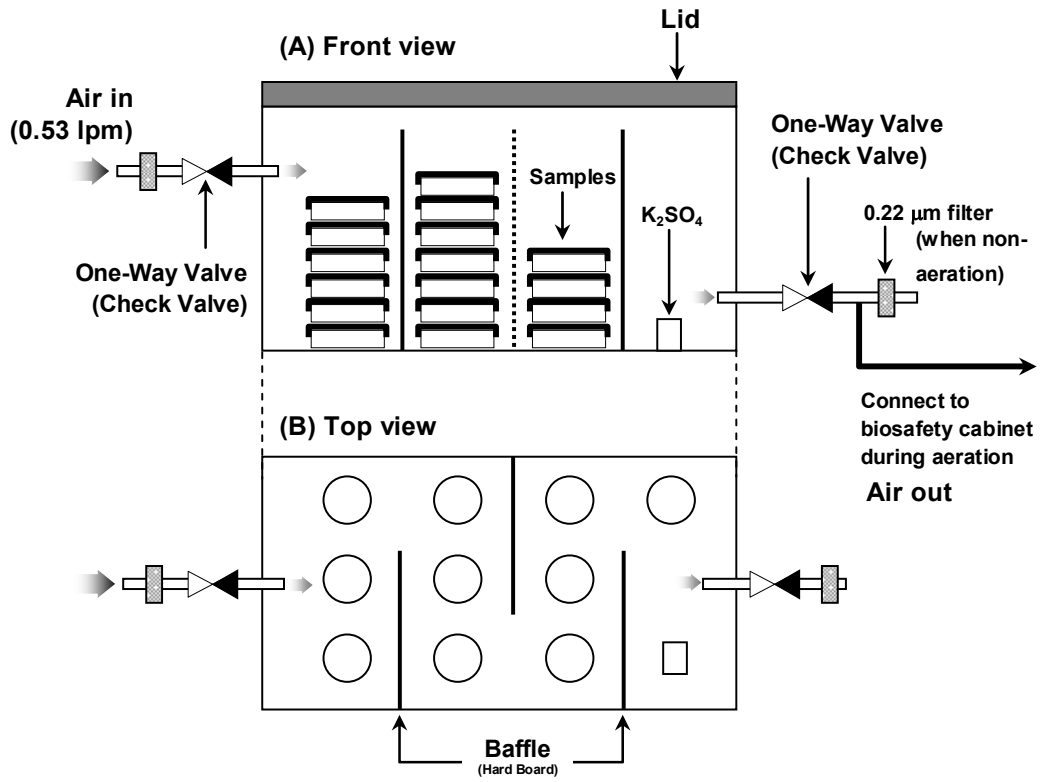

FIGURE 2-1. Experimental set-up of incubation chamber in view from: (A) front; (B) top.

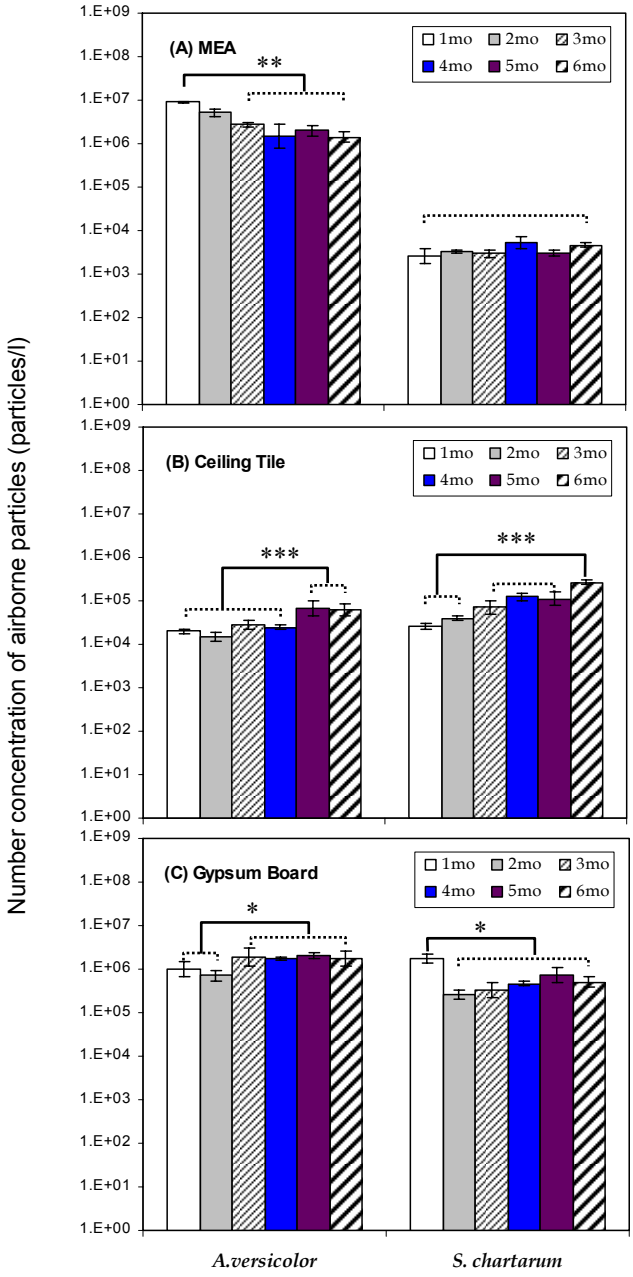

FIGURE 2-2. Numbers of released particles (particles/l) per material sample of each type as measured by an optical particle counter. Histograms present the geometric means, and error bars present geometric standard deviations of three repeats. Dotted lines indicate no significant difference between geometric means; solid lines indicate significantly different geometric means. Asterisks present the significance level of statistical difference (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

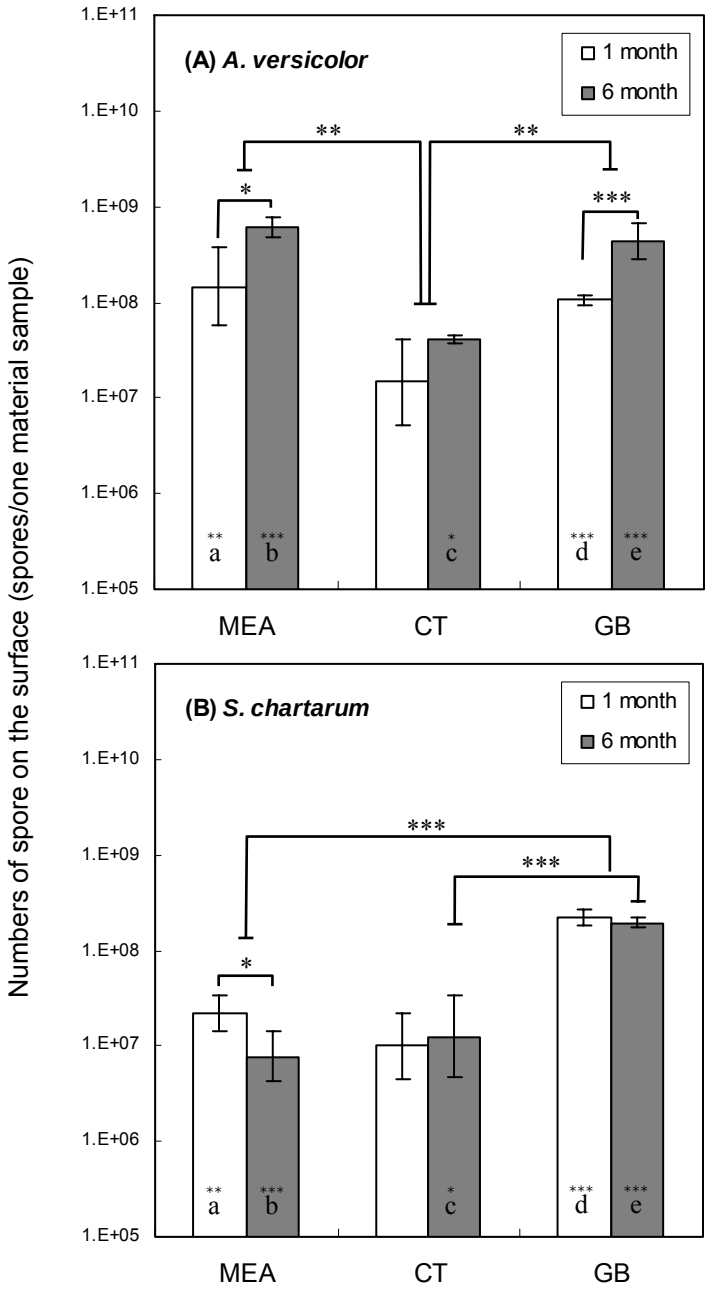

FIGURE 2-3. Spore numbers on the surface per material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present the geometric means, and error bars present geometric standard deviations of nine repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid lines indicate significantly different geometric means of incubation periods and material samples. Asterisks present the significance level of statistical difference (*: P<0.05; **: P<0.01; ***: P<0.001).

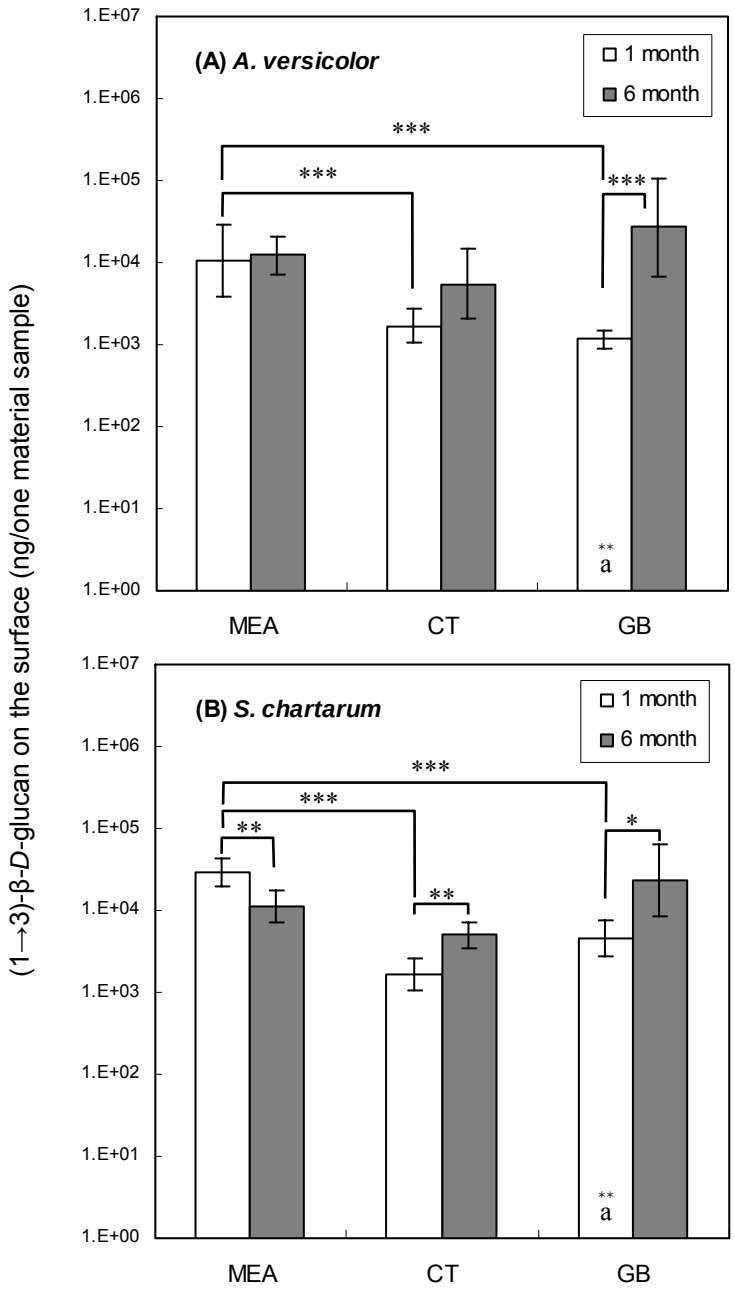

FIGURE 2-4. (1→3)-β-*D*-glucan (ng) on the surface per material sample ($BG_{surface}$) (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present the geometric means, and error bars present geometric standard deviations of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid lines indicate significantly different geometric means of incubation periods and material samples. Asterisks present the significance level of statistical difference (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

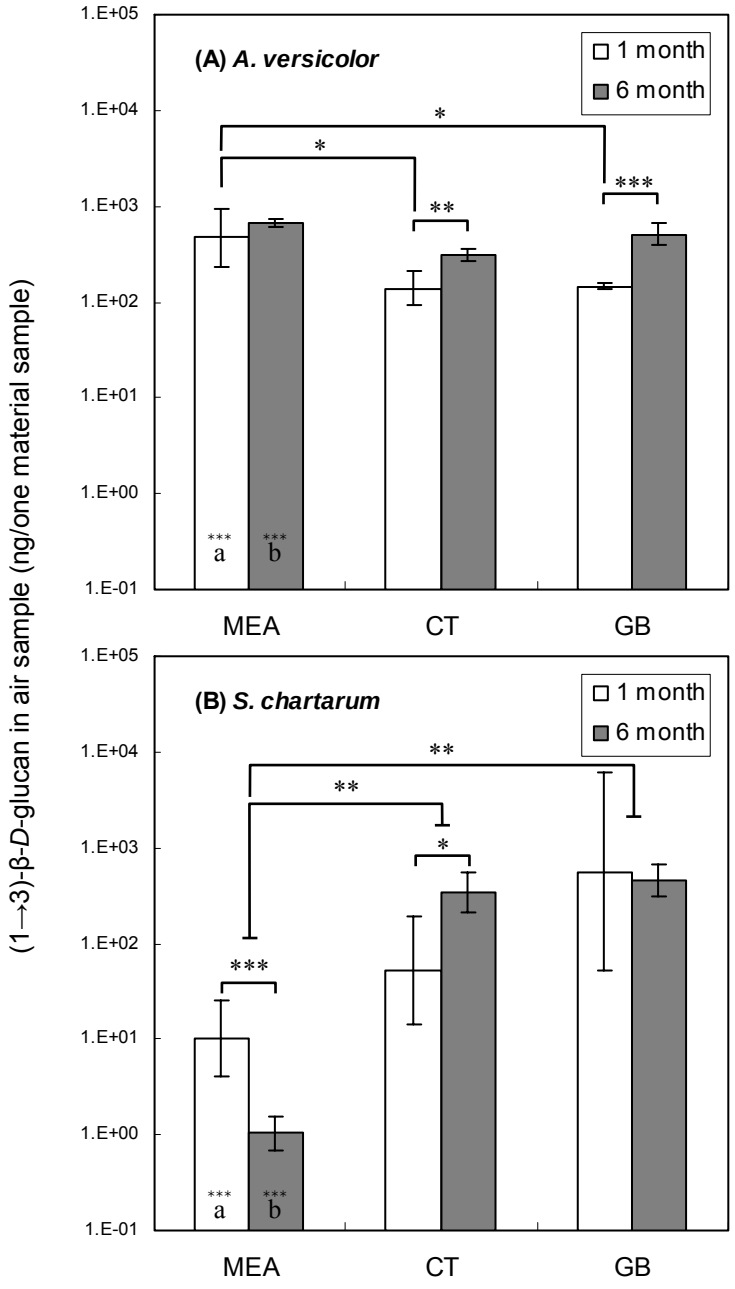

FIGURE 2-5. The amount (ng) of airborne (1→3)-β-*D*-glucan per material sample ($BG_{airborne}$) during three minutes (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present the geometric means, and error bars present geometric standard deviations of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid lines indicate significantly different geometric means of incubation periods and material samples. Asterisks present the significance level of statistical difference (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$).

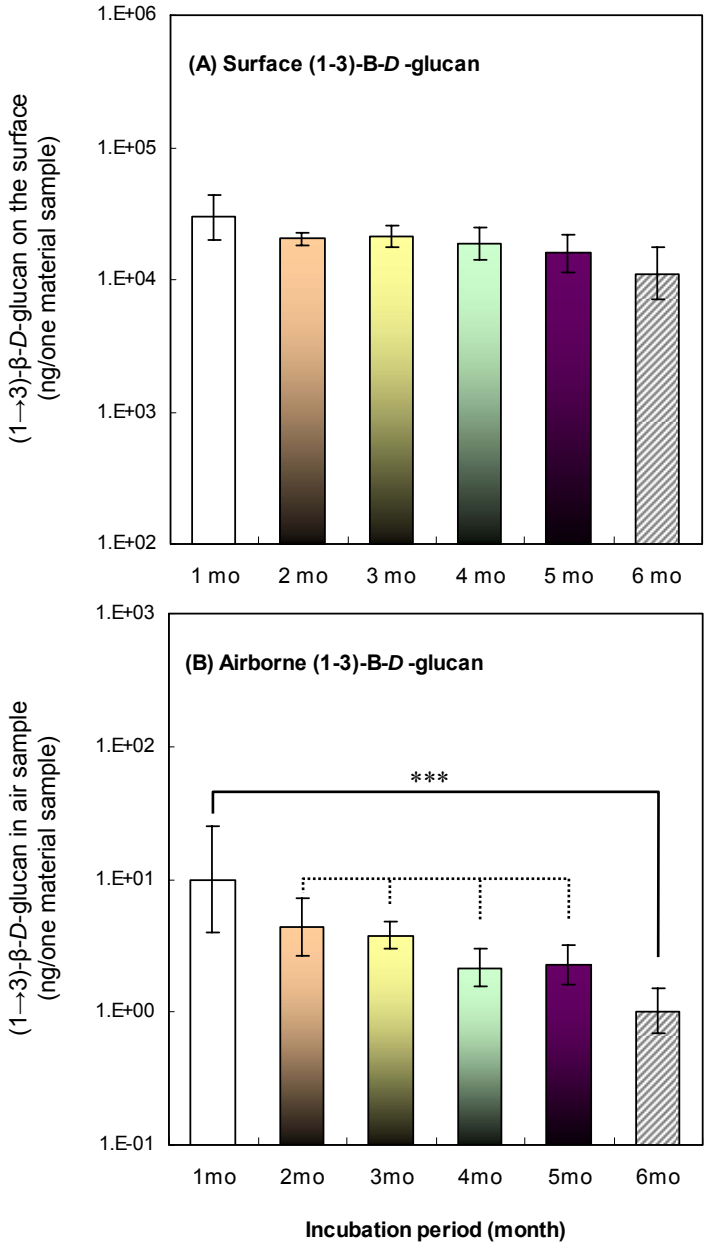

FIGURE 2-6. Monthly data of (A) surface and (B) airborne (1→3)-β-*D*-glucan of *S. chartarum* grown on MEA. Histograms present the geometric means, and error bars present geometric standard deviations of three repeats. Solid line indicates significantly different geometric means of incubation periods and material samples. Asterisk presents the significance level of statistical difference (***: $P<0.001$).

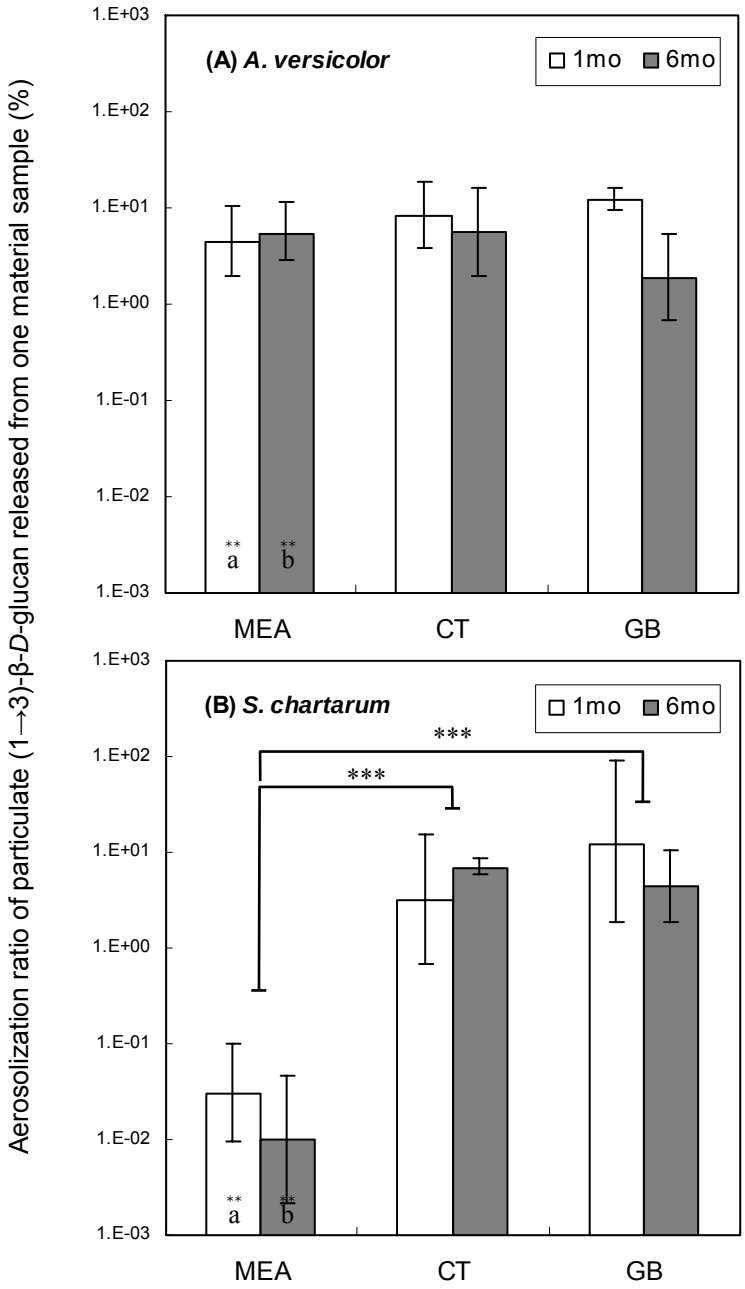

FIGURE 2-7. Aerosolization ratio of particulate (1→3)-β-*D*-glucan (=[$BG_{airborne} \div BG_{surface}$]×100) released from the surface of material samples during three minutes (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present the geometric means, and error bars present geometric standard deviations of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid lines indicate significantly different geometric means of incubation periods and material samples. Asterisks present the significance level of statistical difference (*: P<0.05; **: P<0.01; ***: P<0.001).

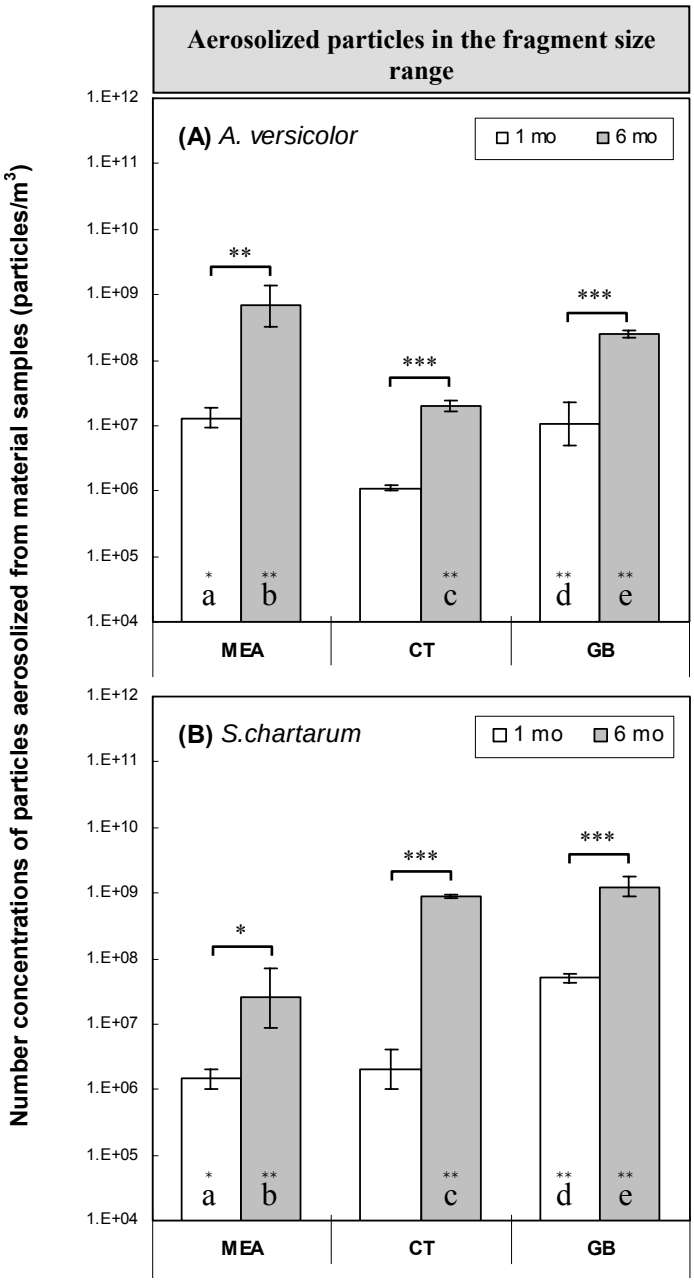

FIGURE 3-1. Number concentrations of particles (particles/m^3) in the fragment size range aerosolized from each material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present geometric means, and error bars present geometric standard deviation of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid line presents significantly different geometric means between the two incubation periods. Asterisks present the level of statistical difference (*: P<0.05; **: P<0.01; ***: P<0.001).

Note: The following significant differences were found between material types; (A) For *A. versicolor*, ceiling tile incubated for one and six months was lower than MEA and gypsum board; (B) for *S. chartarum*, gypsum board incubated for one month was higher than MEA and ceiling tile and MEA for six month was lower than ceiling tile and gypsum board.

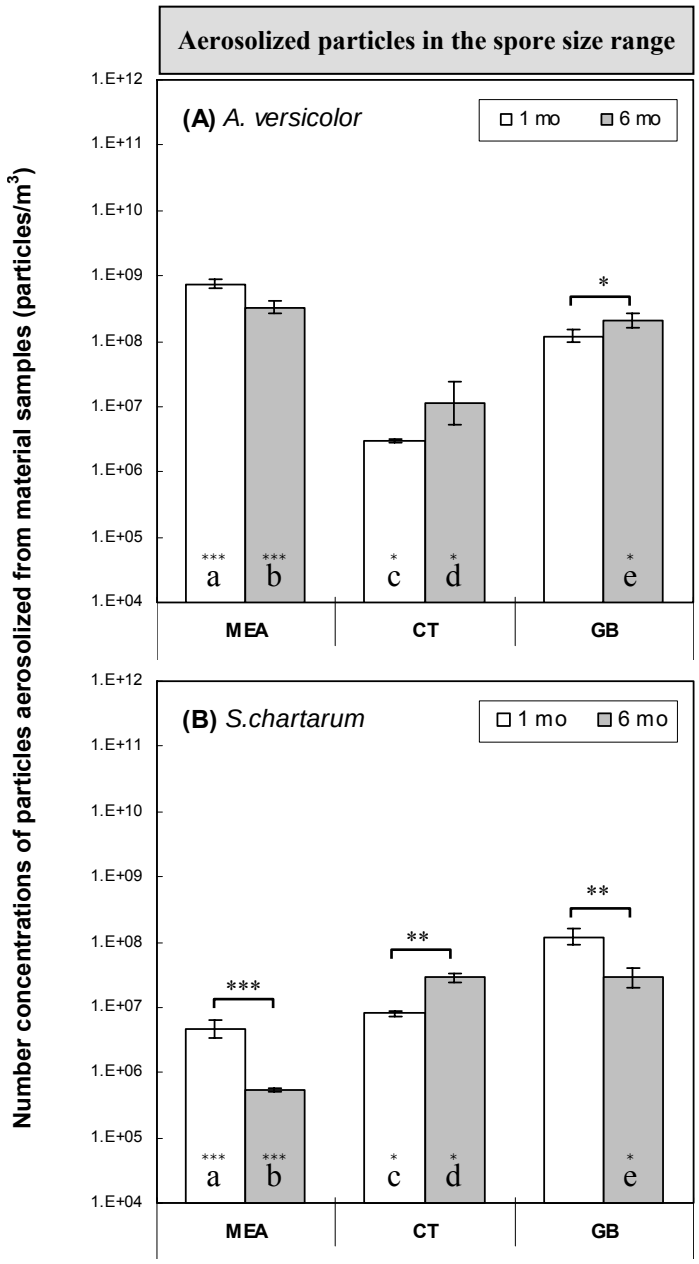

FIGURE 3-2. Number concentrations of particles (particles/m^3) in the spore size range aerosolized from each material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present geometric means, and error bars present geometric standard deviation of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid line presents significantly different geometric means between the two incubation periods. Asterisks present the level of statistical difference (*: P<0.05; **: P<0.01; ***: P<0.001).

Note: The following significant differences were found between material types; (A) For *A. versicolor*, ceiling tile incubated for one and six months was lower than MEA and gypsum board; (B) for *S. chartarum*, gypsum board incubated for one month was higher than MEA and ceiling tile and MEA for six month was lower than ceiling tile and gypsum board.

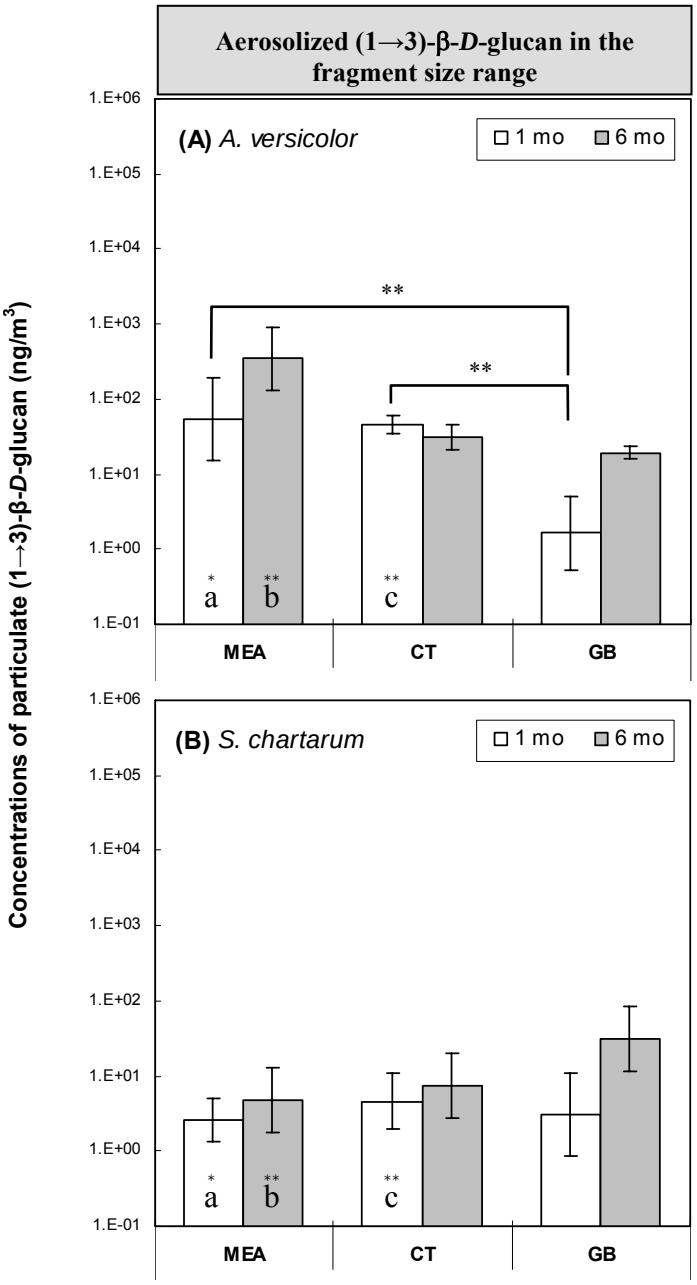

FIGURE 3-3. Airborne concentrations of particulate (1→3)-β-*D*-glucan (ng/m^3) in the fragment size range aerosolized from each material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present geometric means, and error bars present geometric standard deviation of three repeats. Significant difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Solid line presents significantly different geometric means between material types. Asterisks present the level of statistical difference (*: P<0.05; **: P<0.01; ***: P<0.001).

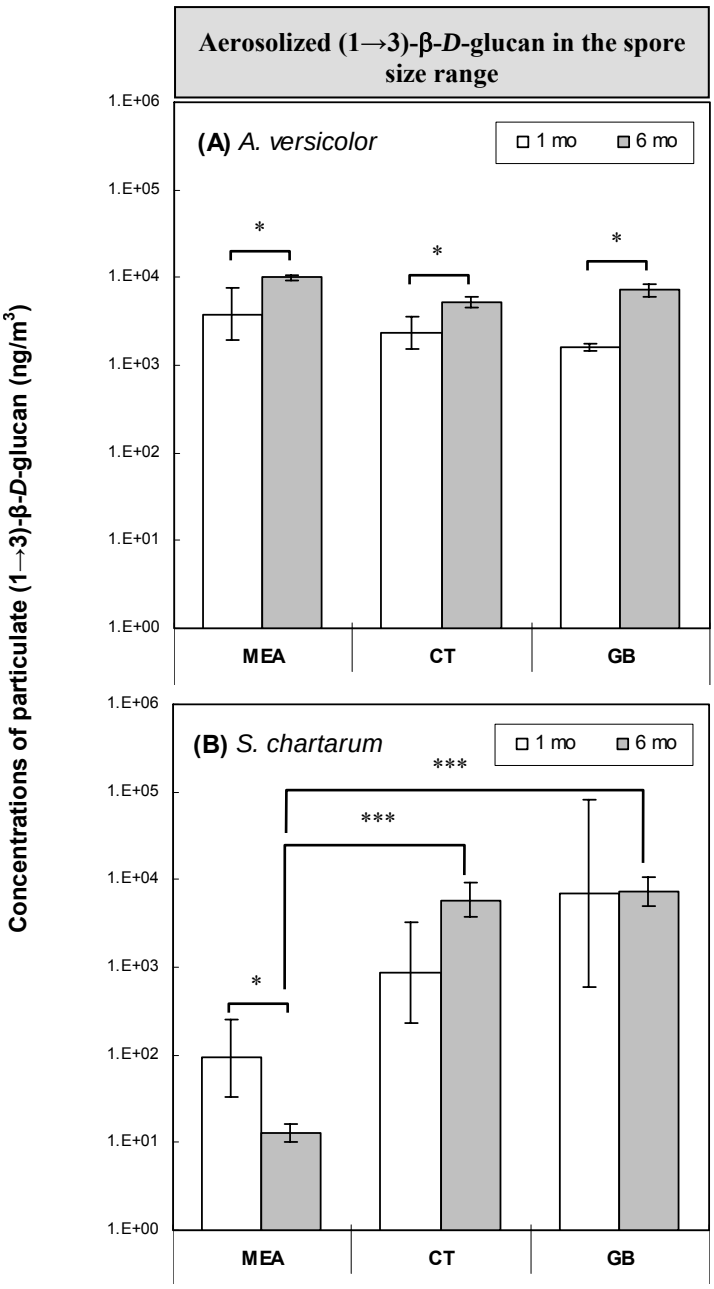

FIGURE 3-4. Airborne concentrations of particulate (1→3)-β-*D*-glucan (ng/m^3) in the spore size range aerosolized from each material sample (MEA=malt extract agar; CT=ceiling tile; GB=gypsum board). Histograms present geometric means, and error bars present geometric standard deviation of three repeats. Solid line presents significantly different geometric means between the two incubation periods and between the material types. Asterisks present the level of statistical difference (*: P<0.05; **: P<0.01; ***: P<0.001).

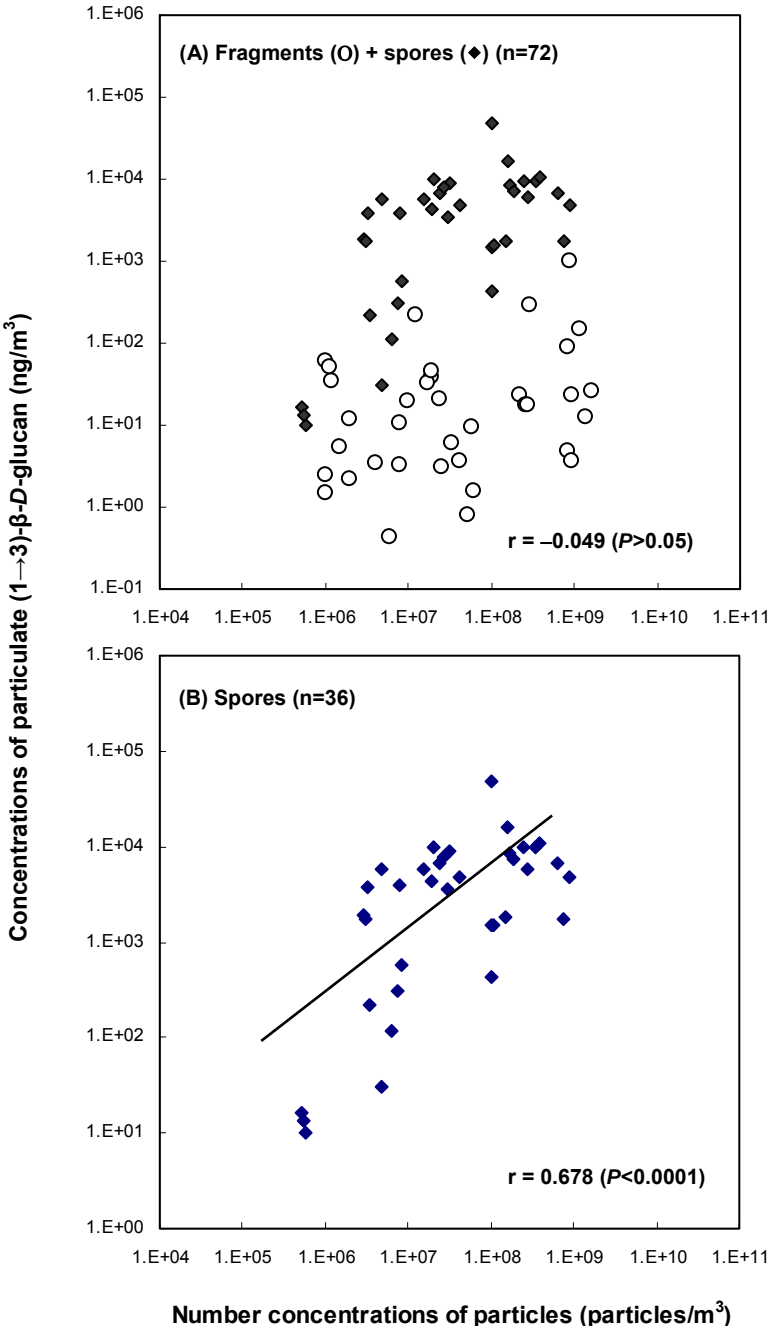

FIGURE 3-5. Scatter plot of number concentrations of particles (particles/m^3) versus airborne concentrations of particulate (1→3)-β-*D*-glucan (ng/m^3): (A) fragments + spores; (B) spores. Solid line presents a linear regression line, and n is the number of samples.

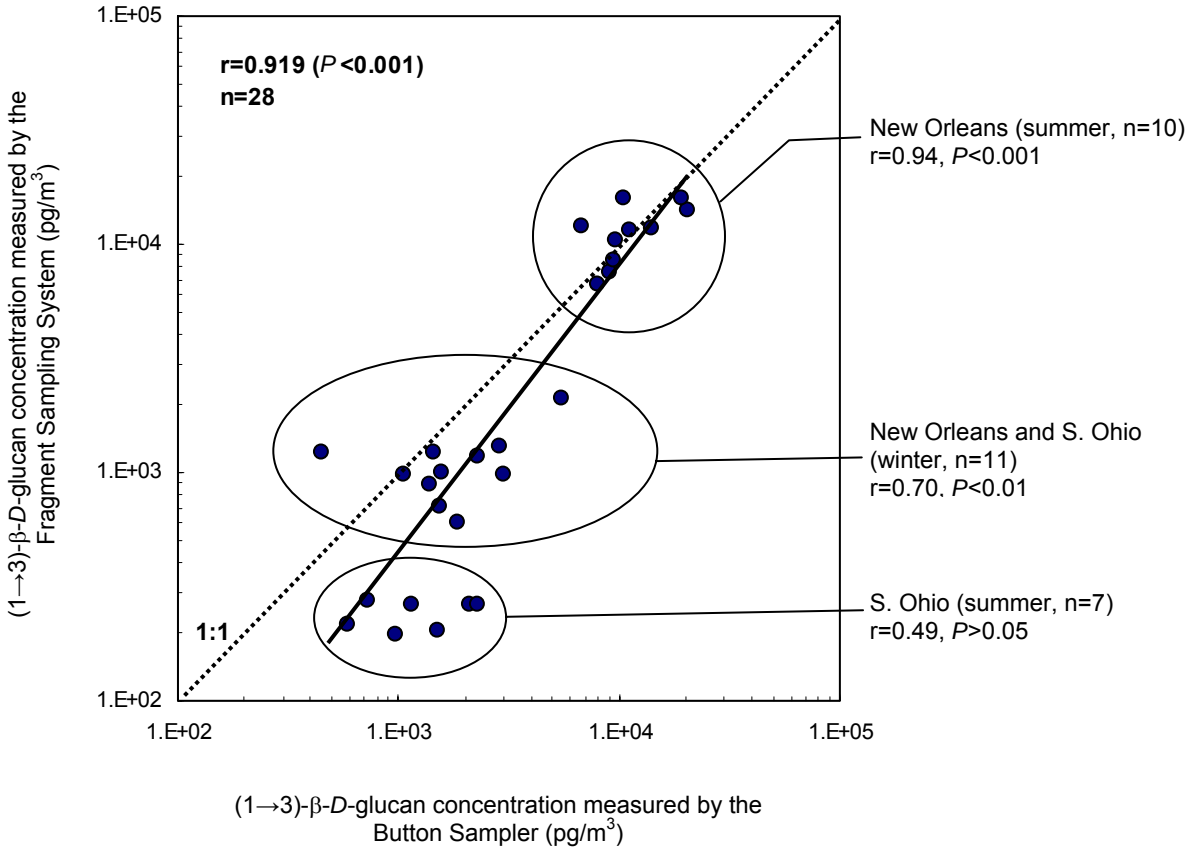

FIGURE 4-1. The correlation between the total (1→3)-β-*D*-glucan concentrations measured by the new Fragment Sampling System and the Button Sampler. Dotted line presents 1:1 ratio; the solid line presents the regression line; and n is the total number of samples.

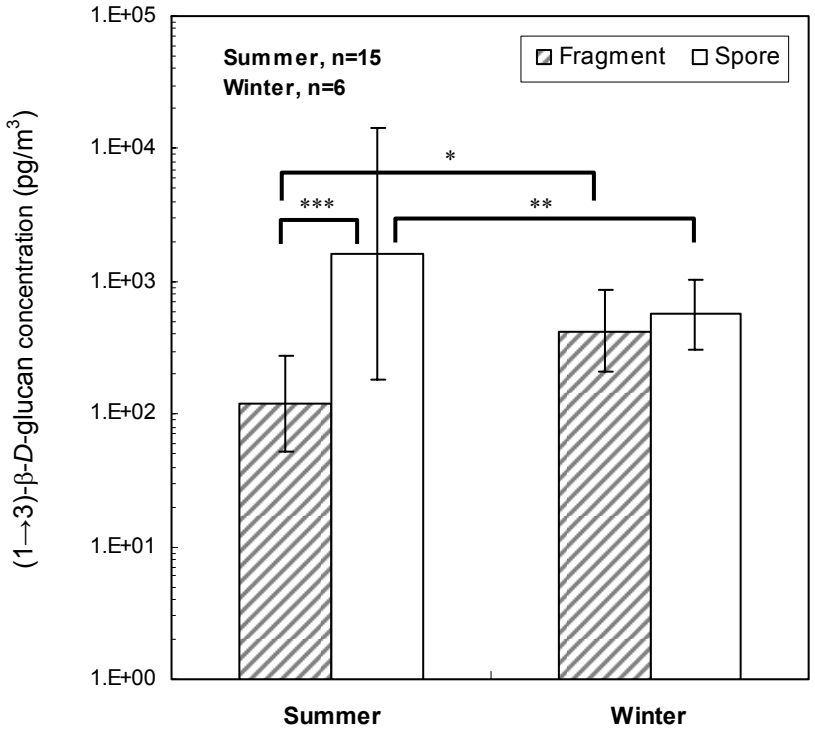

FIGURE 4-2. Geometric means of indoor airborne (1→3)-β-*D*-glucan concentrations (pg/m^3) measured by the new Fragment Sampling System. Error bars represent geometric standard deviations and n is the total number of samples. Solid lines indicate significant difference and asterisks present the significance level of statistical difference (*: P<0.05; **: P<0.01; ***: P<0.001).

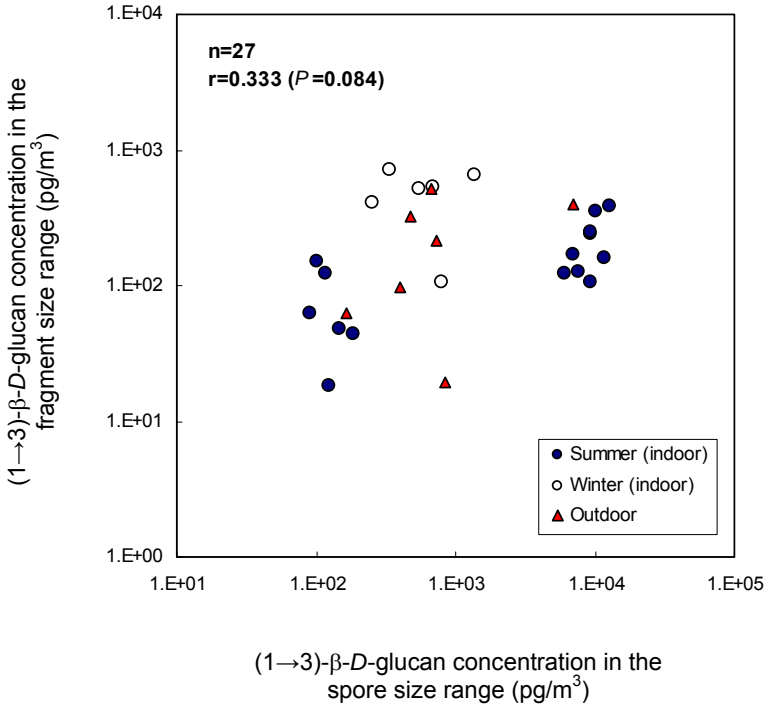

FIGURE 4-3. Correlation between (1→3)-β-*D*-glucan concentrations (pg/m^3) in the fragment and spore size fraction (data obtained by the Fragment Sampling System).

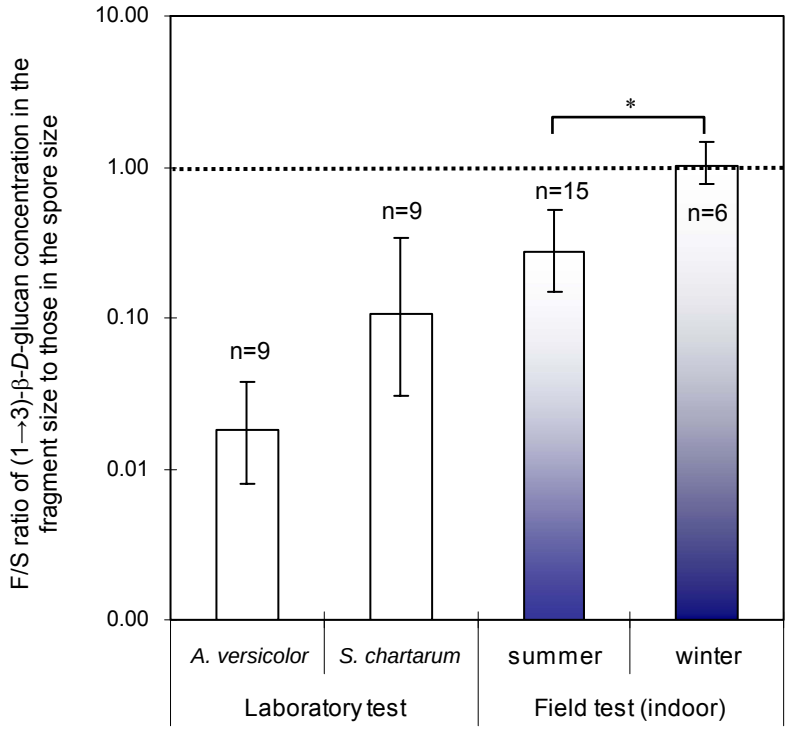

FIGURE 4-4. Ratios of (1→3)-β-*D*-glucan concentrations in the fragment size range to those in the spore size range (F/S ratio) measured in the laboratory (Seo et al., 2007c) and in the field (indoor). Histograms represent the arithmetic average and error bars present standard deviations of samples (n). The dotted line presents F/S-ratio=1, n is total sample number. Solid lines indicate significant difference and asterisk presents the significance level of statistical difference (*: $P<0.05$).

TABLES

Table 2-1. Moisture content on the surface of material samples.

		Moisture content (%)	
		1 month	6 month
A. versicolor	MEA	15.4 ± 3.2	5.8 ± 1.4
	Ceiling tile	16.9 ± 1.8	7.9 ± 1.3
	Gypsum board	13.4 ± 2.9	6.4 ± 2.5
S. chartarum	MEA	17.2 ± 1.2	5.3 ± 0.6
	Ceiling tile	16.2 ± 2.2	9.6 ± 1.6
	Gypsum board	17.9 ± 0.7	10.7 ± 3.1
Total average		16.2 ± 1.8***	7.6 ± 2.2***

Average ± standard deviation of three repeats; asterisks present the significance level of statistical difference (P<0.001).

Table 3-1. Ratios of particle number concentrations in the fragment to those of the spore size fractions (particle F/S-ratio) for the two fungal species.

		MEA	CT	GB	Average
A. versicolor	1 mo	0.019	0.365	0.099	0.161 ± 0.04
	6 mo	2.274[a]	2.038[b]	1.287[c]	1.867 ± 0.97[d]
	average	1.146 ± 1.426	1.202 ± 1.260	0.693 ± 0.705	1.014 ± 0.82[e]
S. chartarum	1 mo	0.352	0.289	0.446	0.363 ± 0.19
	6 mo	60.65[a] ***	32.00[b] ***	46.02[c] ***	46.21 ± 22.17[d] ***
	average	30.50 ± 43.34	16.13 ± 17.49	23.23 ± 27.61	23.29 ± 22.33[e]

Average ± standard deviation of three repeats.

Note: statistical difference between the two species is indicated by a letter symbol (the same letters present a significant difference). Asterisks present the significance level of statistical difference (P<0.001).

Table 3-2. Ratios of (1→3)-β-*D*-glucan concentrations in the fragment to those of the spore size fractions (β_G F/S-ratio) for the two fungal species.

		MEA	CT	GB	Average
A. versicolor	1 mo	0.020	0.021 *	0.001	0.014 ± 0.01
	6 mo	0.045	0.006 *	0.003	0.018 ± 0.03
	average	0.032 ± 0.03	0.013 ± 0.01	0.002 ± 0.01	0.016 ± 0.02
S. chartarum	1 mo	0.066	0.010	0.001	0.026 ± 0.05
	6 mo	0.410	0.002	0.006	0.105 ± 0.23
	average	0.204 ± 0.27	0.006 ± 0.01	0.003 ± 0.004	0.063 ± 0.16

Average ± standard deviation of three repeats; asterisks present the significance level of statistical difference (*: $P<0.05$).

Table 4-1. Summary of the characteristics of the houses.

	House 1	House 2	House 3	House 4	House 5
Location	New Orleans	New Orleans	New Orleans	Southern Ohio	Southern Ohio
Total number of rooms	7	9	8	3	3
Room for sample collection	Living room	Living room	Living room	Basement	Basement
Floor material	Wood	Carpet	Wood	Concrete	Concrete
Room size (m^2)	26.1	29.7	46.2	133.1	35.6
Cause of water damage	Flooding (1.83 m)	Flooding (3.05 m)	Flooding (2.74 m)	Water leakage through wall	Water leakage through wall and window sills
Damaged materials	Visible mold on all surfaces	Visible mold on all surfaces	Visible mold on all surfaces	Several spots of water-damage on dry wall (<0.2 m^2)	Visible mold on dry wall (~3 m^2)
Occupied	No	No	No	No	Yes

Table 4-2. Relative humidity and temperature during the environmental sampling.

		Relative humidity (%)		Temperature (°C)	
		Indoor air	Outdoor air	Indoor air	Outdoor air
Summer	House 1	79.1-89.4	32.9-67.2	30.0-38.8	27.0-33.0
	House 2	69.3-90.4	40.0-53.5	28.9-37.3	32.1-35.8
	House 3	72.8-84.3	52.7-71.6	30.8-38.1	27.8-34.6
	House 4	52.4-74.1	47.9-67.0	15.3-22.0	10.0-26.4
	House 5	57.0-82.7	42.3-59.4	12.9-26.2	14.8-20.3
Winter	House 3	48.4-50.8	36.0-53.4	10.3-17.7	12.8-17.3
	House 5	40.8-48.0	40.6-50.0	11.6-15.1	11.9-14.6

Table 4-3. Indoor airborne (1→3)-β-*D*-glucan concentrations in each size fraction in New Orleans and Southern Ohio, and.

		(1→3)-β-*D*-glucan concentrations (pg/m^3) (Data obtained with the Fragment Sampling System)			
		Total (1→3)-β-*D*-glucan	Fragment fraction	Spore fraction	F/S ratio
Summer	New Orleans	14,607.0 (6,661–15,854) N=9 ***	192.7 (106–387) **	9,020.8 (6,080–12,894) ***	0.021 (0.011– 0.034) *
	S. Ohio	232.5 (192–272) n=6	59.6 (18 - 151)	123.3 (89–185)	0.483 (0.148–1.484)
Winter	New Orleans	1,241.1 (707– 2,097) n=3	520.5 (405–652)	615.8 (251–1,358)	0.845 (0.480–1.614)
	S. Ohio	1,128.2 (1,002–1,219) N=3	338.0 (104–722)	525.0 (333 - 792)	0.644 (0.132–2.163)
New Orleans all[a]		6,574.5 (707–15,854) N=12 ***	247.0 (106– 652)	4,610.9 (251–12,894) ***	0.054 (0.011–1.614) *
S. Ohio all		393.6 (192–1,219) n=9	106.3 (18–722)	199.8 (89–792)	0.532 (0.148–2.163)

Note: values present geometric mean (range); asterisks present the significance level of statistical difference (*:P<0.05; **: P<0.01; ***: P<0.001), and n is the total number of samples.

[a]: New Orleans all (or S. Ohio all) include data both in summer and winter.

LIST OF APPENDIXES

APPENDIX A: COPIES OF PEER-REVIEWED PUBLICATIONS RESULTED FROM THE Ph.D. STUDY.

A1: **A new field-comparable methodology for the collection and analysis of fungal fragments.** Seo, S.C., S.A. Grinshpun, Y. Iossifova, D. Schmechel, C.Y. Rao, and T. Reponen. *Aerosol Sci. Technol.* 41: 794-803.

A2: **Aerosolization of particulate (1→3)-β-*D*-glucan from moldy materials.** Seo, S.C., T. Reponen, L. Levin, T. Borchelt, and S.A. Grinshpun. *Appl. Environ. Microbiol.* (submitted).

A3: **Size-fractionated (1→3)-β-*D*-glucan concentrations aerosolized from different moldy building materials.** Seo, S.C., T. Reponen, S.A. Grinshpun. *Atmos. Environ.* (submitted).

A4: **Fungal fragments in moldy houses: A field study in homes in New Orleans and southern Ohio.** Reponen, T., S.C. Seo, F. Grimsley, T. Lee, C. Crawford, and S.A. Grinshpun. *Atmos. Environ.* **41(37)**: 8140-8149.

A1:

A new Field-Comparable Methodology for the Collection and Analysis of Fungal Fragments

Seo, S.C., S.A. Grinshpun, Y. Iossifova, D. Schmechel, C.Y. Rao, and T. Reponen. *Aerosol Sci. Technol.* 41: 794-803.

A2:

Aerosolization of Particulate (1→3)-β-*D*-glucan from Moldy Materials

Seo, S.C., T. Reponen, L. Levin, T. Borchelt, and S.A. Grinshpun. *Appl. Environ. Microbiol.* (submitted).

A3:

Size-Fractionated (1→3)-β-*D*-glucan Concentrations Aerosolized from Different Moldy Building Materials

Seo, S.C., T. Reponen, L. Levin, and S.A. Grinshpun. *Atmos. Environ.* (submitted).

A4:

Fungal Fragments in Moldy Houses: A Field Study in Homes in New Orleans and Southern Ohio

Reponen, T., S.C. Seo, F. Grimsley, T. Lee, C. Crawford, and S.A. Grinshpun. *Atmos. Environ.* 41(37): 8140-8149.

APPENDIX B: LIST OF OTHER PUBLICATIONS (NOT INCLUDED IN THE Ph.D. DISSERTATION) AUTHORED/CO-AUTHORED BY Mr. SUNG-CHUL SEO.

B1. Peer-reviewed publications

1. Seung-Hyun Cho, **Sung-Chul Seo**, Detlef Schmechel, Sergey A. Grinshpun, Tiina Reponen. Aerodynamic characteristics and respiratory deposition of fungal fragments. *Atmospheric Environment* 39 (2005) 5454–5465.

B2. Conference abstracts

1. **Sung-Chul Seo**, Sergey A. Grinshpun, Yulia Iossifova, and Tiina Reponen. A New Field Comparable Method for the Collection of Fungal Fragments. *American Industrial Hygiene Conference and Expo* 2005, Anaheim, California, USA (Awarded "**The Best Poster**").

2. Tiina Reponen, **Sung-Chul Seo**, Yulia Iossifova, Atin Adhikari, and Sergey A. Grinshpun. New field-comparable method for collection and analysis of (1-3)-β-*D*-glucan in fungal fragments. *7th International Aerosol Conference* 2006, St. Paul, Minnesota, USA.

3. **Sung-Chul Seo**, Tiina Reponen, and Sergey A. Grinshpun. (1-3)-β-*D*-glucan contents in submicrometer sized particles released from moldy building materials. 7th Annual Symposium of the National Institute for Occupational Safety and Health supported Education and Research Center Pilot Project 2006, Cincinnati, Ohio, USA.

4. Tiina Reponen, **Sung-Chul Seo**, and Sergey A. Grinshpun. β-glucan Content of Fungal Spores and Fragments Released from Different Building Materials. *American Thoracic Society Conference* 2007, San Francisco, California, USA

5. **Sung-Chul Seo**, Tiina Reponen, Faye Grimsley, Carlos Crawford, Yulia Iossifova, Taekhee Lee, Detlef Schmechel, Carol Rao, and Sergey A. Grinshpun. Laboratory and field

evaluation of a new method for the sampling and analysis of fungal fragments. *American Industrial Hygiene Conference and Expo* 2007, Philadelphia, PA, USA.

6. Tiina Reponen, **Sung-Chul Seo**, Faye Grimsley, Taekhee Lee, Carlos Crawford, and S.A. Grinshpun. Laboratory and field characterization of (1-3)-β-*D*-glucan in aerosolized fungal fragments. *European aerosol conference*, 2007 (Accepted).

7. Tiina Reponen, **Sung-Chul Seo**, Faye Grimsley, Taekhee Lee, Carlos Crawford, and S.A. Grinshpun. Field-testing of a new methodology for characterizing fungal fragments in moldy buildings. *17th Annual Meeting of the International Society of Exposure Analysis,* October, 2007, Durham, NC, USA (Accepted).

APPENDIX C: PROTOCOLS OF EXTRACTION AND CLEANING

C1. Extraction of samples.

C2. Cleaning of the Fragment Sampling System.

C1. Extraction of samples

C1.1. After-filter

- Take off the polycarbonate filter from the filter cassette.
- Transfer the filter into a pyrogen-free sterile tube (Pyrotubes®, 13 × 100 mm borosilicate glass tube; Associates of Cape Cod, East Falmouth, MA).
- Add 2 ml of pyrogen-free water (LAL Reagent Water®; Associates of Cape Cod, East Falmouth, MA) containing 0.05% Tween 80.
- Soak the filters in the tube (inside the Biosafety cabinet) for 60 minutes to enhance the extraction.
- Mix the filter and extraction solution by 2-min vortexing and 10-min ultrasonic agitation.

C1.2. Collection Cups

- Add extraction solution (pyrogen-free water containing 0.05% Tween 80) into the collection cup:

  $PM_{2.5}$ cup - 9 ml; $PM_{1.0}$ cup - 2 ml.

- Cover with a metal cap.
- Prepare a 1000 ml-beaker with 50 ml of tap water.
- Put a collection cup with extraction solution into the beaker (Figure App.-1).
- Move the beaker with the collection cup into ultrasonic cleaner.
- Do ultrasonic agitation for 10 min by ultrasonic cleaner.

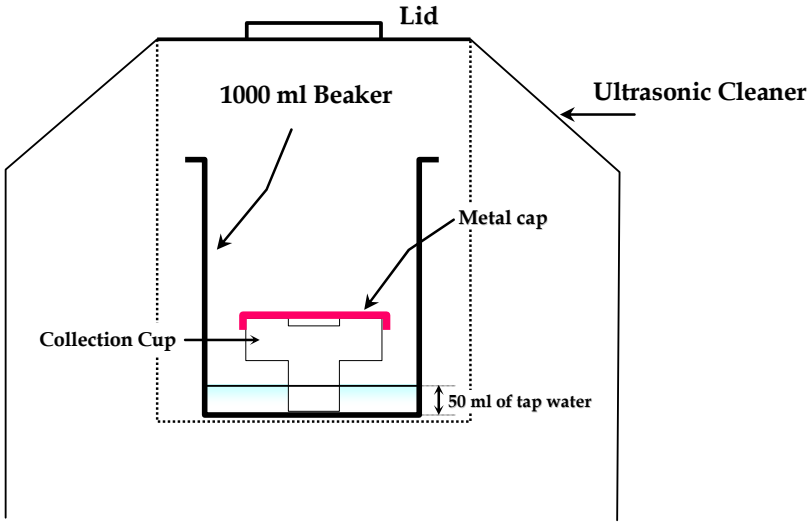

FIGURE App.-1. Preparation for extraction in the ultrasonic bath.

C2. Cleaning of the Fragment Sampling System

- Dissemble metal parts and rubber-O rings from the Sharp-Cut cyclone sampler.
- Wash with soap and tap water for 10 minutes.
- Ultrasonic cleaning with soap solution for 10 minutes.
- Rinsing with tap water for 10 minutes.
- Clean in Ultrasonic bath with 2-Propanol (alcohol) for 10 minutes.
- Dry in the Biosafety Hood for 30 minutes.
- Heat all metal parts in the Oven (Model 725G; Fisher Scientific) at 250 ºC for 60 minutes.