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Characterization of fungi in office dust: comparing results of microbial secondary metabolites, fungal ITS region sequencing, viable culture, and other microbial indices

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Practical Implication

We compared three fungal analytical methods by analyzing floor dust samples collected from an office building for fungi using viable culture, internal transcribed spacer (ITS) sequencing, and secondary metabolites using liquid chromatography-tandem mass spectrometry. We

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found that each of the three measurement methods in our study had its own strengths and weaknesses and produced unique information on fungal contamination. However, if we use only one measurement method for exposure assessment in epidemiological studies, there is always possibility of potentially significant misclassification in fungal exposure. Our study findings suggest that using multiple measurement methods may provide an improved understanding of fungal exposures in indoor environments and that secondary metabolites may be considered as an additional source of exposure.

Running title: Characterization of fungi in office dust

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Abstract

Recent developments in molecular and chemical methods have enabled the analysis of fungal DNA and secondary metabolites, often produced during fungal growth, in environmental samples. We compared three fungal analytical methods by analyzing floor dust samples collected from an office building for fungi using viable culture, internal transcribed spacer (ITS) sequencing, and secondary metabolites using liquid chromatography-tandem mass spectrometry. Of the 32 metabolites identified, 29 had a potential link to fungi with levels ranging from 0.04 (minimum for alternariol monomethylether) to 5,700 ng/g (maximum for neoechinulin A). The number of fungal metabolites quantified per sample ranged from eight

to sixteen (average=13/sample). We identified 216 fungal operational taxonomic units (OTUs) with the number per sample ranging from six to twenty-nine (average=18/sample). We identified 37 fungal species using culture and the number per sample ranged from two to thirteen (average=eight/sample). Agreement in identification between ITS sequencing and culturing was weak (kappa=-0.12–0.27). The number of cultured fungal species poorly correlated with OTUs, which did not correlate with the number of metabolites. These suggest that using multiple measurement methods may provide an improved understanding of fungal exposures in indoor environments and that secondary metabolites may be considered as an additional source of exposure.

Introduction

Characterization of fungi in indoor environments is a methodological challenge because there is no gold standard for the sampling and analytical methods to measure specific fungal agents that are reportedly associated with human health. These agents could involve fungal allergens, inflammatory cell wall components, nucleic acids, proteases, secondary metabolites, as well as microbial volatile organic compounds. Of these secondary metabolites have not been normally considered as biologically significant in regards to health outcomes in indoor epidemiological studies.[1]

Fungal secondary metabolites are usually low molecular weight compounds that are produced during developmental processes.[2] Because fungi are ubiquitous and can grow anywhere under proper environmental conditions, presence of multiple secondary metabolites in indoor environments is inevitable. However, characterization of such metabolites is not well developed, and effects of exposures on occupants' health are still not well understood although protective effects of fungal metabolites or mycotoxins with respiratory health in

building occupants have been reported in some studies.[3, 4] This field has been understudied primarily because of the lack of available methods to simultaneously analyze multiple metabolites in environmental samples.[5, 6]

Sequencing of fungal DNA in indoor environmental samples has been considered a promising platform used to further our understanding of the effects of previously overlooked microbes on indoor occupants' health.[7, 8] However, like secondary metabolite screening methods, DNA sequencing remains an unstandardized approach that can potentially produce a bias toward certain fungal species.[9, 10] Similarly, traditionally utilized viable culture methods are limited to the selection of viable fungal propagules biased toward the Ascomycota that are capable of growth on the selected nutrient medium.[11] Considering the limitations of each of these methods it is important to understand whether results using the different methods would provide consistent information on fungal characterization in indoor environments.

In our study we compared the results of three different fungal measurement methods (viable culture, ITS region sequencing, and microbial secondary metabolites) using 28 floor dust samples (22 samples for ITS sequencing) that were available from a study of a water-damaged office building with a cluster of sarcoidosis and asthma.[12] Dampness and mold-related environmental conditions of the building and occupants' health have been described in detail elsewhere.[12, 13] We examined whether these three analytical methods provide consistent results about abundance or diversity of fungi, and what kinds of fungal secondary metabolites are present in floor dust.

Materials and methods

We collected floor dust samples from 120 rooms or cubicles in the study building that had a history of water incursions. The sampling and dust processing method has been previously described.[12] Briefly, we vacuumed floor dust in a polyethylene filter sock (Midwest Filtration Company, Fairfield, OH, USA) using a standardized sampling protocol. In the laboratory, hair, lint, and other larger objects were removed from each sample and the dust was transferred into a conical tube. Then dust was homogenized by rotating on a 360-degree rotary arm shaker (ATR, Inc., Laurel, MD, USA) at 65 r.p.m. for 2 hours before partitioning into aliquots for various analyses. All dust samples were analyzed for culturable fungi and bacteria, (1→3)-β-D-glucan, and endotoxin as previously described.[12] For the current comparison study, we analyzed 28 of those 120 samples for microbial secondary metabolites and 22 of those 28 (six samples did not have enough dust to run the analyses) for fungal DNA.[11]

Analysis of microbial secondary metabolites

Chemicals and reagents

Mass spectrometry (MS)-grade ammonium acetate and glacial acetic acid, and LC (liquid chromatography)-grade methanol and acetonitrile were purchased from Sigma-Aldrich (Vienna, Austria). Fungal and bacterial metabolite standards were either shared by various research groups or purchased from various commercial sources: Romer Labs® Inc. (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktred-witz, Germany), Axxora Europe (Lausanne, Switzerland) and LGCPromochem GmbH (Wesel, Germany). Reverse osmosis water was further purified through Purelab Ultra System (ELGA LabWater, Celle, Germany). We prepared stock standard solutions of each metabolite by dissolving the solid substance in acetonitrile, acetonitrile/water 1:1 (v/v), methanol, methanol/water 1:1

(v/v) or water. We prepared 34 combined working standard solutions by mixing the multiple individual stock solutions for easier handling and stored at −20°C. The 34 combined working solutions were then mixed to prepare final working standard solution for 509 metabolites (Table S1).

Sample Preparation

To quantify metabolites, 30 mg dust aliquots were extracted with 400 µl of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) for 90 min on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany). The extract was then diluted using 400 µl of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v) and 5 µl of the extract were injected into LC without further treatment.[14]

LC-MS/MS parameters

We screened 509 target microbial secondary metabolites with high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) [QTrap 5500 LC-MS/MS System, Applied Biosystems, Foster City, CA, USA] equipped with TurboIonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). The chromatographic method, and chromatographic and mass spectrometric parameters were previously described.[14]

Analysis of fungal DNA

Genomic DNA extraction

Using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA), we extracted genomic DNA (gDNA) from 22 dust samples (5 mg) as previously described.[11, 15] Briefly, 5 mg of dust was added to a reinforced tube containing 300 mg of glass beads (212–300 µm; Sigma-Aldrich, St. Louis, MO, USA) and 350 µl of Roche tissue

lysis buffer. The tubes were vortexed and processed in a bead mill homogenizer for 30 sec. After centrifugation, the supernatants were transferred to new microcentrifuge tubes and incubated with 20 µl of CelLytic B Cell Lysis Reagent (Sigma-Aldrich) for 30 min at 37°C. We added Roche binding buffer (200 µL) and proteinase K solution (40 µl), and incubated the tubes for 10 min at 70°C. Isopropanol (100 µl) was added and the extracts were washed and eluted according to the manufacturer.

Fungal rDNA amplification, cloning, and sequencing

Fungal ITS regions of the DNA were amplified, cloned, and sequenced as previously described.[11] Briefly, ITS regions were amplified using polymerase chain reaction (PCR) with the Fun18Sf forward and ITS4 reverse primers. The ITS amplicons were then cloned into the pDRIVE vector followed by transformation of ligated plasmids into chemically competent *Escherichia coli*. We cultured positive clones (n=48 per sample unless fungal DNA yield was low) to prepare glycerol stocks in 96-well plates for Sanger sequencing analysis (Genewiz, Inc., South Plainfield, NJ). Resultant sequences were clustered into operational taxonomic units (OTUs) using a 97% similarity cutoff and representative sequences of each OTU were searched against the National Center for Biotechnology Information database to identify the fungal species.

Culturable fungi, (1→3)-β-D-glucan, endotoxin and culturable bacteria analysis

We sent dust aliquots for analyses of cultivable fungi and bacteria to EMLab P&K (Cherry Hill, NJ, USA). Serially-diluted sample aliquots were spread onto malt extract, dichloran glycerol 18, and cellulose agars for fungi, and trypticase soy agar (TSA) with 5% sheep blood for total bacteria, colistin nalidixic acid (CNA) agar for the selection of Gram-positive bacteria, and MacConkey agar for the selection of Gram-negative bacteria and incubated for 7–10 days.[12] Fungal species were identified by examiners with expertise in identifying fungi based on morphological and physiological characteristics as previously described.[12] We used

Limulus amoebocyte lysate assay to analyze for (1→3)-β-D-glucan and endotoxin as described previously.[16, 17]

Statistical analysis

We evaluated Pearson correlations among metabolites; between metabolites with greater than 20% prevalence and culturable fungi, bacteria, (1→3)-β-D-glucan, or endotoxin; and among the number of OTUs, number of cultured species, and number of metabolites. We computed kappa statistics to evaluate agreement in identification between culturing and DNA sequencing for fungal genera with at least 20% prevalence in either culturing or DNA sequencing. We performed all statistical analyses with SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and considered P-value < 0.05 as statistically significant.

Results

Microbial metabolites

We identified 32 metabolites (out of 509 tested for listed in Table S1) from 28 samples but seven of the 32 metabolites were found in only one sample (Figure 1). The number of metabolites identified per sample ranged from nine to eighteen, with an average of 15. Of the 32 metabolites, cyclo(L-Pro-L-Tyr), usnic acid, asperglaucide, emodin, and averufin were detected in all samples. Lotaustralin, skyrin, brevianamide F, 3-nitropropionic acid, integracin B, neoechinulin A, citreorosein, integracin A, linamarin, alternariol monomethylether, and rugulusovin were found in more than 50% of samples. Of the 32 metabolites, three (linamarin, lotaustralin, and chloramphenicol) are likely from non-fungal sources such as plants or bacteria. Among the remaining 29 metabolites some may be exclusively associated with fungi, some with fungi and plant, and some with fungi and bacteria. The number of potential fungal metabolites identified per sample ranged from eight

to sixteen (average=13). The level of metabolites ranged from 0.04 ng/g as a minimum level of alternariol monomethylether (MME) to 5,700 ng/g as a maximum of neoechinulin A (Figure 2). The levels of five potential fungal metabolites that were most frequently found ranged from 21.4 to 1,300 ng/g for cyclo(L-Pro-L-Tyr); 15.8 to 590 ng/g for usnic acid; 6.2 to 4,200 ng/g for asperglaucide; 1.2 to 120 ng/g for emodin; and 0.4 to 4.7 ng/g for averufin.

Some metabolites were weakly to moderately correlated with each other (Table 2), and the strongest correlations were found between brevianamide F and cyclo(L-Pro-L-Tyr) ($r=0.92$, $p<0.05$) and integracins A and B ($r=0.82$, $p<0.05$). Significant negative correlations were also observed between asperglaucide and 3-nitropropionic acid ($r=-0.51$, $p<0.05$), and linamarin and asperglaucide ($r=-0.40$, $p<0.05$).

Fungal DNA sequencing, cultivable fungi, other microbial indices

We previously reported 216 different fungal OTUs from 22 samples[11] and the number of different fungal OTUs per sample ranged from six to twenty-nine, with an average of eighteen (Figure 3). Of the 216 fungal OTUs identified, 160 were able to be discerned to genus level, with 119 of these identified to species level. The top five most prevalent fungal species identified with DNA sequencing included *Ustilago syntherismae* (77.3%), *Aureobasidium microstictum* (72.7%), *Pithomyces chartarum* (72.7%), *Epicoccum nigrum* (54.5%), and *Ustilago striiformis* (45.5%). Species belonging to the genera, *Cryptococcus* (59.1%), *Aspergillus* (50%), and *Mucor* (36.4%) were also frequently detected. Other detailed findings of DNA sequencing have been previously published.[11]

Geometric mean (GM) of total cultivable fungi in 28 dust samples was 36,000 CFU/g with large variability (geometric standard deviation, GSD=9.12). We identified 37 fungal species and the most frequently cultured included *Penicillium chrysogenum* (92.9%), followed by *Cladosporium sphaerospermum* (82.1%), *Epicoccum nigrum* (78.6%), *Phoma*

coelomycetes (67.9%), and *Aureobasidium pullulans* (53.6%) (Figure 4). Of these, *P. chrysogenum* (GM: 6,000 CFU/g), *P. coelomycetes* (3,600 CFU/g), and *C. sphaerospermum* (2,700 CFU/g) were found in higher concentrations than the other two species (<1,300 CFU/g). The number of species per sample identified by viable culture ranged from two to thirteen, with an average of eight. GMs for *Penicillium* and *Aspergillus* species were 7,500 CFU/g (GSD=9.15), and 3,600 CFU/g (GSD=4.34), respectively.

(1→3)-β-D-glucan (a fungal cell wall component) and endotoxin (a component of the outer membrane of Gram-negative bacteria) were detected from all 27 samples analyzed (one sample did not have enough dust for the analyses) and their GMs were 38.8 μg/g (GSD=2.39) and 65.1 EU/mg (2.42), respectively. The concentration of total bacteria (GM=190,000 CFU/g, GSD=9.86) in all 28 samples was much higher than that of total cultivable fungi. The GMs of Gram-negative and positive bacteria were 3,200 CFU/g (GSD=3.75) and 46,000 CFU/g (GSD=19.33), respectively.

Comparison of results from different methods

Total cultivable fungi and *Penicillium* were well correlated with averufin (r=0.60 and 0.54, respectively; p-values<0.05), neoechinulin A (0.61 and 0.57), and asperglaucide (0.78 and 0.54) (Table 3). Measurements of glucan were also correlated with averufin (0.43), skyrin (0.54), and lotaustralin (0.52) but these correlations were weaker than those of cultivable fungi. Total bacteria were also significantly correlated with averufin (r=0.51). Asperglaucide was significantly correlated with *Aspergillus* species (r= 0.58, p-value<0.05). Both endotoxin (r=0.39) and Gram-positive bacteria (0.55) were significantly correlated with asperglaucide. While cultivable Gram-positive or negative bacteria were not correlated with dipeptide cyclo(L-Pro-L-Tyr), endotoxin was significantly correlated (r=0.50).

From 22 samples analyzed by both viable culture and fungal DNA sequencing, we found four fungal genera that were exclusively identified from culturing but not from DNA sequencing. These included *Acremonium*, *Paecilomyces*, *Ulocladium*, and *Eurotium*. In addition, the yeast *Sporobolomyces* was cultured in 21.4% of samples, but DNA sequencing identified *Sporobolomyces foliicola* in only one sample. On the other hand, there were at least 90 fungal genera that were not cultured using three selected media (malt extract, dichloran glycerol 18, and cellulose agars) but were identified using DNA sequencing. Of these 90 fungal genera, *Ustilago*, *Cryptococcus*, *Trichosporon*, and *Rhodotorula* that belong to phylum Basidiomycota were the most prevalent. We examined the agreement using kappa statistics in identification of fungal genera between culturing and DNA sequencing methods within the same sample.[18] Except for *Aspergillus*, *Epicoccum*, and *Pithomyces* that showed moderate to weak agreement (kappa=0.27, 0.14, and 0.11, respectively), the agreements for other fungal genera detected using the two methods were poor (kappa<0.07). Within the same sample, the genera *Alternaria*, *Botrytis*, *Chaetomium*, and *Fusarium* were detected by one or other of the two methods, but not by both methods. *Penicillium* was detected in all samples by the culture method but only in 36.4% of the samples by DNA sequencing (kappa statistics could not be calculated due to 100% culturing of *Penicillium*). For *Cladosporium*, all of the samples (18.2%) that were positive with DNA sequencing were also positive with culture method; however, the majority (79%) of the samples in which *Cladosporium* was detected with the culture method were not detected using DNA sequencing. Fungal genera that were more frequently identified by culturing than DNA sequencing include *Aspergillus*, *Chaetomium*, *Cladosporium*, *Epicoccum*, *Penicillium*, and *Phoma*.

The average number of OTUs identified per sample (average=18) was significantly ($p<0.001$) higher than that of cultured fungal species (average=8) (Figure 3). The number of cultured fungal species per sample was weakly correlated with the number of fungal OTUs

(r=0.28). The number of fungal secondary metabolites per sample was not correlated (r=0.09) with the number of fungal OTUs. However, the number of fungal secondary metabolites per sample was weakly correlated with the number of cultured fungal species (r=0.24). These correlations were not statistically significant (p-values>0.1).

Discussion

We compared three measurement methods for fungal propagules (HPLC-MS/MS for secondary metabolites, viable culture, and DNA sequencing), to better understand and interpret the discordance of the resultant datasets. Our study showed that each method has unique features in measuring dustborne fungi and each method could complement each other. Analysis of 509 secondary metabolites using HPLC-MS/MS indicated occupants' potential exposures to previously overlooked fungal metabolites; however, it would be challenging to associate these metabolites with specific fungi and simultaneous exposures to them with potential health effects. While traditional viable culture analysis identified 37 fungal species (on average eight per sample) that were only culturable on our selected media, it also provided useful information on viability of fungi including opportunistic pathogens- e.g., *Aspergillus fumigatus, Paecillomyces varioti, P. lilacinus,* and *Fusarium solani*.[19, 20] In contrast, ITS region sequencing provided much broader information on fungal flora (119 identifiable fungal species and, on average, 18 fungal OTUs per sample), but the overall method influenced by sample extraction, choice of primers for PCR, length of DNA amplicons in sample extract, and difficulty in discriminating ITS sequence homology may result in underestimation of certain amerospore producing fungal genera placed in the phylum, Eurotiales. These findings indicated that the limitations of each method may result in potentially inaccurate assessment of occupants' fungal exposure if each measurement method was used individually.

Fungal secondary metabolites are commonly low molecular weight compounds that are often bioactive and generally produced during developmental processes associated with or corresponding to sporulation and hyphal development in the fungal life cycle.[2, 19] Therefore, wherever viable fungi grow under appropriate conditions, bioactive fungal secondary metabolites are likely to exist. However, potentially harmful or beneficial effects of inhalation exposures to airborne mixed microbial secondary metabolites are not well understood or researched. The majority of the metabolites identified and quantified in our study might be from fungi, except for linamarin, lotaustralin, and chloramphenicol, which may be exclusively from plant or bacterial sources. Cyclo(L-Pro-L-Tyr) is a cyclic dipeptide (diketopiperiazine) identified in 100% of our samples and can be produced by *Alternaria alternata* or bacteria including *Streptomyces*; but, we only identified *A. alternata* in 10.7% of our samples with culturing and 27.3% with DNA sequencing.[2, 21, 22] However, we cannot exclude a possibility that this metabolite might be also produced by other members of the Pleosporales of which we identified a broad diversity using DNA sequencing.[23] Nine metabolites (3-nitropropionic acid, asperglaucide, chlorocitreorosein, citreorosein, emodin, linamarin, lotaustralin, physcion, and tryptophol) could be derived from plants and fungi.[24-29] Usnic acid and physcion are produced by lichen, a composite organism consisting of a symbiosis between a fungus and an algae or cyanobacterium. The most common fungi in these symbiotic relationships are ascomycetes (*Penicillium*, *Alternaria*, *and Aspergillus* etc.), called ascomycete lichens or ascolichens.[30]

Numerous metabolites possibly originated from Eurotiales genera were placed in the genera *Aspergillus* or *Penicillium.* These secondary metabolites identified in our study included 3-nitropropionic acid, asperglaucide, averantin, averufin, brevianamide F, chlorocitreorosein, citreorosein, emodin, kojic acid, meleagrin, neoechinulin A, nidurufin, physcion, versicolorin C, skyrin, neoxaline, agroclavine, chanoclavin, quinocitrinin A, and

rugulusovin.[4, 24, 31, 32] Some of these (such as 3-nitropropionic acid, asperglaucide, emodin, and neoachinulin A) could be also originated from plants. Averufin, emodin, and asperglaucide were the most frequently identified metabolites in the samples and they could be also produced by certain species of *Aspergillus*, *Chaetomium*, *Cladosporium*, *Mycosphaerelia*, *Penicillium*, and *Phoma*.[32] Averufin (one of the most prevalent metabolites in our study that can be produced by *Aspergillus versicolor* and *A. ustus*) and averantin are intermediate compounds in the biosynthesis pathway of sterigmatocystin and aflatoxin B_1.[4, 32, 33] Sterigmatocystin, a precursor of aflatoxin biosynthesis, can be produced by more than 30 species of filamentous fungi including *A. flavus*, *A. nidulans*, *A. parasiticus*, and *A. versicolor*.[34, 35] Presence of sterimatocystin in house dust samples with water damage has been reported;[4, 35, 36] however, we did not detect sterigmatocystin and aflatoxin B_1 in our samples although we found *A. versicolor* in 32.1% of samples by culturing and 13.6% by DNA sequencing. Aflatoxin B_1 can be also produced by *A. flavus*, *A. nomius*, and *A. parasiticus*[32] Nidurufin found in about 10% of the samples, is the direct precursor of averufin.[4, 37] Versicolorin C is also a biosynthetic precursor of aflatoxins but we found in only about 10% of samples.[32]

Widespread brevianamide F (93% prevalence) can be produced by *Penicillium brevicompactum*, and neoechinulin A (75% prevalence) by *A. ruber* (*Eurotium rubrum*), or *A. amstelodami* (*E. amstelodami*).[32] Of these fungi, we only found *E. amstelodami* in 14% of the dust samples using viable culture. A Japanese study has isolated asperglaucide, one of the most prevalent metabolites in our study, from the organic extract of the xerophilic fungi *A. restrictus* and *A. penicillioides*.[38] However, we only detected *A. penicillioides* from one sample with culturing and 13.6% of samples with DNA sequencing. Integracin B was more prevalent than A (93% versus 71%), and they are produced by the endophyte, *Cytonaema* species[39] and *Cytospora* species[40] which were not identified in our samples. Alternariol

MME, which has been also found in tobacco, grain sorghum, and pecans, are produced by *Alternaria tenuis*, *A. dauci*, and *A. cucumerina*[32] and they were not identified in our samples. Although the prevalence of secondary metabolites potentially associated with *Penicillium* and *Aspergillus* were generally high, DNA sequencing revealed a broad spectrum of fungi placed in the phyla Ascomycota as well as the Basidiomycota. The current analyte panel contains many fungal secondary metabolites derived from commercially available standard materials that are predominantly represented by taxa placed in the phylum Ascomycota. Although our data provided further insight into the diversity of secondary metabolites produced by traditionally studied fungal species such as *A. versicolor*, *A. ustus*, and *E. amstelodami*, the ITS region sequence analysis also identified many Ascomycota and Basidiomycota species whose secondary metabolite profiles may remain relatively uncharacterized. This suggests that the discordance between secondary metabolites and ITS region sequencing could be due to a lack of secondary metabolite coverage of understudied Ascomycota (e.g., *Pithomyces chartarum*) as well as Basidiomycota species (e.g., *Ustilago syntherismae*).

The European HITEA study reported loads (pg/cm^2) of 30 metabolites identified in 675 settled dust swab samples from schools using the same HPLC-MSMS method as ours. They found geographical differences in metabolite profiles and higher number of metabolites (with higher loads: >1.0 or 10 pg/cm^2) in water-damaged (index) schools compared to reference schools with emodin, enniatin B, and physcion being the most prevalent metabolites (< 23% for index schools). In the present study, we identified emodin in all samples and physcion in 10.7% of the samples, but found no enniatins.[41] The LUKAS2 birth cohort study in Finland also reported 42 different metabolites from 90 residential building samples with the same analytical method and the prevalent ones included chloramphenicol, alternariol, alternariol MME, averufin, 3-nitropropionic acid, brevianamide F, skyrin, emodin, physcion, tryptophol, sterigmatocystin, enniatins, moniliformin, and monocerin.[4] The concentrations of all

metabolites in their study ranged from 10 to 2,900 ng/g, which is less variable than ours (0.04–5,700 ng/g). Another Finnish study of nine moisture-damaged homes found 28 different fungal metabolites in 69 samples (building materials, vacuum cleaner dust bag, vacuumed floor dust, settled airborne dust samples) with the same method. Of the 28 metabolites, emodin, physcion, meleagrin, enniatin B, beauvericin, equisetin, and sterigmatocystin were found in more than 20% of samples.[1] The concentrations of all metabolites in their study ranged from 0.05 to 3,100 ng/g. It appeared that the metabolite profiles in European buildings with moisture damage may vary from those identified in our study building in the United States, but that some of the most prevalent metabolites were commonly identified. In our study, the dust samples had been stored for about 7.5 years at -80ºC, which might have affected the profile of secondary metabolites; however, effects of storage in the freezer on detection of secondary metabolites are not known.

DNA sequencing identified a much broader diversity of fungi than viable culture; however, there was poor agreement between the two methods. Ascomycota genera including *Aspergillus*, *Chaetomium*, *Cladosporium*, *Epicoccum*, *Penicillium*, and *Phoma* are frequently found in damp indoor environments.[42] In our samples, these fungi were more dominantly identified by culturing than DNA sequencing. Our study finding is expanding the previous report that species within the order Eurotiales, specifically, *Penicillium* and *Aspergillus* species, were dominant fungi recovered by culturing but not by DNA sequencing.[43] In addition, we found that within the same sample, *Alternaria*, *Botrytis*, *Chaetomium*, and *Fusarium* were never identified by both methods (i.e. only detected by one or other of the two methods). These discrepancies might have resulted from the limitations associated with each method. Viable culture does not support the growth of non-viable spores, dead cell fragments, and live cells and fragments that are not culturable within the selected media and time frame, or may select fast growing and fastidious fungal species. There is also possibility of

misidentification of fungi by even trained mycologists. Green and colleagues[11] previously demonstrated that *Ustilago syntherismae* was the most abundant fungal ITS region sequence identified as well as many other Basidiomycota species that represented 41% of all identified sequences in these samples. We propose that traditional culture may not support the growth of these Basidiomycota species and this could account for some of the reported discordance as well. Further, ITS region sequencing analysis may have revealed many additional fungi that were not viable in the dust samples and could account for the lower proportion of Eurotiales identified. In contrast, sample extraction method, primers used for PCR, length of DNA amplicons, and difficulty in discriminating ITS sequence homology in DNA sequencing could also lead to underestimation of certain fungal species, especially those placed in the order Eurotiales.[11] In addition, DNA sequencing does not discriminate between viable and non-viable cells as well as cell-bound and cell-free DNA.[44]

Rittenour and colleagues[15] compared three commonly used DNA extraction methods in terms of sensitivity and susceptibility to PCR inhibitors in dust for three fungal species with similar sized amerospores including, *Aspergillus versicolor*, *Rhizopus microsporus*, and *Wallemia sebi*. They found that DNA extraction efficiency differed by extraction method and fungal species and that the dust-specific extraction method significantly affected the fungal diversity detected using ITS region sequencing. Bellemain and colleagues[9] also showed that various fungal ITS region primers for PCR amplification produced widely different proportions of amplified sequences, especially under strict PCR conditions. They also found that some primers had a taxonomic bias that favored certain fungal taxa (some favored ascomycetes and others basidiomycetes). In addition, the primer mismatches with the target sequence occurred differentially across the fungal taxa under the relaxed PCR conditions. The shorter DNA amplicons (number of base pairs) was also more favorably amplified than longer ones, which resulted in taxonomic bias (more ascomycetes than basidiomycetes)

especially when ITS2 region was targeted.[9] High similarity in sequences within the ITS2 region between *Aspergillus* and *Penicillium* and among species within *Alternaria* and *Cladosporium* in both ITS regions could also lead to inability to identify to species level.[45, 46] Taking these limitations into consideration, continued studies are required to further develop and standardize this rapidly evolving field of analysis although ITS region sequencing enables the identification of viable and non-viable fungal diversity.

In conclusion, each of the three measurement methods in our study had its own strengths and weaknesses and produced unique information on potential fungal exposures. Furthermore, these methods complemented each other. On the other hand, if we use only one measurement method for exposure assessment in epidemiological studies, it should be noted that there is always possibility of potentially significant misclassification in fungal exposure. The ideal approach would be to use multiple measurement methods for various microbial agents to better understand real exposure situations.

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Table 1. Metabolites identified in dust samples, retention time, and MS conditions for fragmentation

Analyte	Retention time (min)	*m/z* precursor ion	DP (V)	*m/z* product ions[a]	Collision energy (V)[a]	Cell exit potential (V)[a]
3-Nitropropionic acid	3	118	-65	46	-16	-3
Agroclavine	6.1	239.1	86	183.2/208.2	27/27	10/12
Alternariolmethylether	12.8	271.1	-95	256/227	-32/-50	-13/-9
Apicidin	12.8	622.4	-135	462.2/209	-32/-68	-11/-9
Asperglaucide	11.6	445.1	136	194.1/224.1	19/29	8/10
Averantin	15.3	371.1	-90	295/256	-56/-50	-15/-13
Averufin	15.9	367.1	-90	295.1/284	-52/-44	-11/-11
Brevianamid F	7.4	284.1	86	130.1/103.1	31/75	8/16
Chanoclavine	5.7	257.1	76	168.1/226.1	27/17	10/14
Chloramphenicol	7.8	320.9	-80	151.9/256.9	-24/-18	-7/-13
Chlorocitreorosein	12.3	318.9	-165	255/226	-40/-48	-9/-9
Citreorosein	11.3	285	-30	241/211	-50/-62	-13/-15
Cyclo (L-Pro-L-Tyr)	6.0	261.1	66	107/136.1	49/25	10/8
Emodin	14.3	269	-100	224.9/240.9	-38/-38	-11/-13
Integracin A	15.6	627.3	-135	347.2/287.2	-38/-64	-13/-11
Integracin B	15	585.3	-155	305.2/261.2	-38/-60	-11/-9
Kojic acid	3.2	143	86	113/69	31/23	10/10
Linamarin	3.6	306.1	-50	161/188.1	-20/-16	-5/-7
Lotaustralin	5.4	320.1	-40	161/188	-22/-16	-17/-7
Meleagrin	7.6	432.1	-75	332.1/177.1	-23/-35	-13/-7
Neoechinulin A	9.6	322	-75	253.1/139	-30/-52	-5/-11
Neoxaline	7.2	434.1	-65	346/403.1	-40/-22	-9/-11
Nidurufin	13.5	383.1	-95	284.1/256	-50/-50	-10/-10
Physcion	15.4	283	-95	240/212	-36/-50	-11/-11
Quinocitrinine A	7.4	271.2	61	214.1/199.1	37/51	8/8
Rugulosuvine	8.7	334.1	101	130/103	31/81	6/8

Skyrin	15.2	537.2	-100	469.2/175	-46/-66	-11/-9
Tenuazonic acid	9.6	196.1	-100	139/112.1	-28/-32	-7/-5
Tryptophol	7.8	162.2	81	117.1/143.1	33/35	6/14
Usnic acid	14.9	343.1	-65	328/259	-28/-28	-13/-9
Versicolorin C	13.8	339.1	-90	311.2/283.1	-35/-45	-10/-10
Xanthotoxin	9	217	101	202/174	35/43	18/6

DP: Declustering potential; V: voltage; *m/z*: mass to charge ratio; a: quantifier ion/qualifier ion

Table 2. Pearson correlation coefficients among microbial metabolites in dust

	Averufin	3-nitropropionic acid	Neoechinulin A	Asperglaucide	Brevianamide F	Skyrin	Citreorosein	Emodin	Alternariol MME	Integracin A	Integracin B	Usnic acid	Rugulusovin	Lotaustralin	Cyclo (L-Pro-L-Tyr)
3-nitropropionic acid	-0.13	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Neoechinulin A	0.10	-0.11	–	–	–	–	–	–	–	–	–	–	–	–	–
Asperglaucide	0.53**	-0.51**	0.32*	–	–	–	–	–	–	–	–	–	–	–	–
Brevianamide F	-0.21	0.19	0.03	0.09	–	–	–	–	–	–	–	–	–	–	–
Skyrin	0.45**	0.002	-0.06	0.37*	-0.04	–	–	–	–	–	–	–	–	–	–
Citreorosein	0.06	0.32*	-0.13	-0.23	0.04	0.31	–	–	–	–	–	–	–	–	–
Emodin	0.16	0.12	0.002	0.12	0.36*	0.12	0.36*	–	–	–	–	–	–	–	–
Alternariol MME	0.41**	0.12	-0.21	0.09	-0.17	0.38**	0.11	0.02	–	–	–	–	–	–	–
Integracin A	0.33*	-0.05	-0.08	0.23	-0.36*	0.40**	0.12	0.14	0.21	–	–	–	–	–	–
Integracin B	0.39**	0.13	-0.06	0.21	-0.24	0.49**	0.08	0.17	0.52**	0.82**	–	–	–	–	–
Usnic acid	0.14	0.33*	0.14	0.15	-0.20	0.58**	0.20	0.01	0.42**	0.46**	0.60**	–	–	–	–
Rugulusovin	-0.04	-0.34*	0.04	0.30	0.43**	0.17	-0.004	0.07	-0.02	0.02	-0.07	0.04	–	–	–
Lotaustralin	-0.03	0.59**	0.22	-0.26	0.19	0.22	0.30	0.12	0.21	-0.16	0.02	0.31	-0.10	–	–
Cyclo (L-Pro-L-Tyr)	-0.17	0.06	-0.12	0.04	0.92**	-0.02	0.04	0.30	-0.13	-0.37*	-0.26	-0.24	0.44**	0.02	–
Linamarin	-0.02	0.60**	0.05	-0.40**	0.14	0.04	0.36*	0.10	-0.08	-0.15	0.04	0.16	-0.19	0.68**	0.10

** p-value<0.05; * 0.05<p<0.1

Table 3. Correlation coefficients between microbial metabolites and other microbial indices in dust

Metabolite	Total fungi (n=28)	Glucan (n=27)	Total *Penicillium* (n=28)	Total *Aspergillus* (n=21)	Total bacteria (n=28)	Endotoxin (n=27)	Gram negative (n=16)	Gram positive (n=23)
Averufin	0.60**	0.43**	0.54**	0.29	0.51**	0.35*	0.37	0.53**
3-nitropropionic acid	-0.34*	0.24	-0.23	-0.26	-0.09	0.12	-0.29	-0.25
Neoechinulin A	0.61**	0.14	0.57**	0.22	-0.11	0.03	-0.17	0.10
Asperglaucide	0.78**	0.26	0.54**	0.58**	0.35*	0.39**	0.33	0.55**
Brevianamide F	0.10	0.33*	0.03	0.16	0.08	0.48**	-0.31	-0.11
Skyrin	0.27	0.54**	0.15	0.15	0.37*	0.49**	0.11	0.25
Citreorosein	-0.17	0.25	-0.13	-0.17	-0.05	0.16	-0.49*	-0.16
Emodin	0.25	0.36*	0.26	0.07	0.02	0.27	-0.09	-0.18
Alternariol MME	0.06	0.37*	-0.12	-0.03	0.37*	0.32	0.39	0.29
Integracin A	0.30	-0.01	0.30	0.18	0.31	-0.01	0.08	0.34
Integracin B	0.30	0.25	0.24	0.05	0.39**	0.22	0.28	0.37*
Usnic acid	0.10	0.34*	0.01	0.11	0.14	0.32*	0.27	0.09
Rugulusovin	0.18	0.22	-0.04	0.34	0.24	0.38*	0.18	0.10
Lotaustralin	-0.05	0.52**	-0.03	0.02	-0.31	0.08	-0.12	-0.34
Cyclo(L-Pro-L-Tyr)	-0.02	0.22	-0.09	0.05	0.14	0.50**	-0.27	-0.13
Linamarin	-0.21	0.17	-0.11	-0.22	-0.14	-0.03	-0.22	-0.19

Only presented are metabolites of which prevalence is greater than 20% among 28 samples. * 0.05<p-value<0.1; ** p-value<0.05

Table 4. Agreement in identification of common genus between DNA sequencing and culture method in 22 dust samples

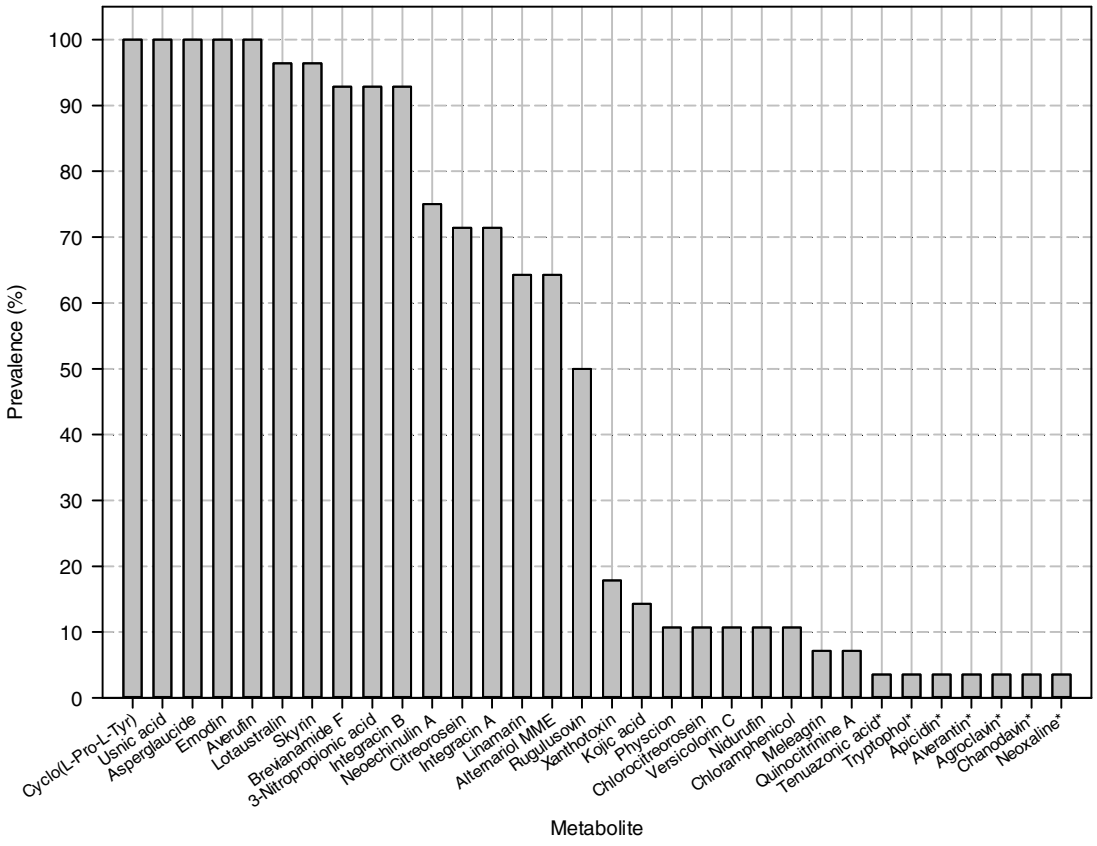
Genus*	Kappa statistics**	Total detection % (no. of samples)		% of common detection	% of exclusive detection	
		DNA	Culture		DNA	Culture
Alternaria	-0.08	27.3 (6)	4.6 (1)	0	27.3	4.6
Aspergillus	0.27	50 (11)	77.3 (17)	45.5	4.6	31.8
Aureobasidium	0	72.7 (16)	50 (11)	36.4	36.4	13.6
Botrytis	-0.08	18.2 (4)	4.6 (1)	0	18.2	4.6
Chaetomium	-0.08	4.6 (1)	27.3 (6)	0	4.6	27.3
Cladosporium	0.07	18.2 (4)	86.4 (19)	18.2	0	68.2
Epicoccum	0.14	54.6 (12)	77.3 (17)	45.5	9.1	31.8
Fusarium	-0.12	13.6 (3)	9.1 (2)	0	13.6	9.1
Mucor	-0.09	40.9 (9)	27.3 (6)	9.1	31.8	18.2
Penicillium§	–	36.4 (8)	100 (22)	36.4	0	63.6
Phoma	-0.06	22.7 (5)	68.2 (15)	13.6	9.1	54.6
Pithomyces	0.11	72.7 (16)	13.6 (3)	13.6	59.1	0

* Genus which was detected in more than 20% of samples by either culture or DNA sequencing method was only included in this analysis.

** Kappa score: no agreement (<0); slight agreement (0.01-0.20); fair agreement (0.21-0.40); moderate agreement (0.41-0.60); substantial agreement (0.61-0.80); almost perfect agreement (0.81-0.99)[1].

§ All samples were positive from culture method. Therefore, Kappa score could not be calculated.

Figure 1. Prevalence of metabolites in floor dust (n=28)



* Only one among 28 samples was above LOD.

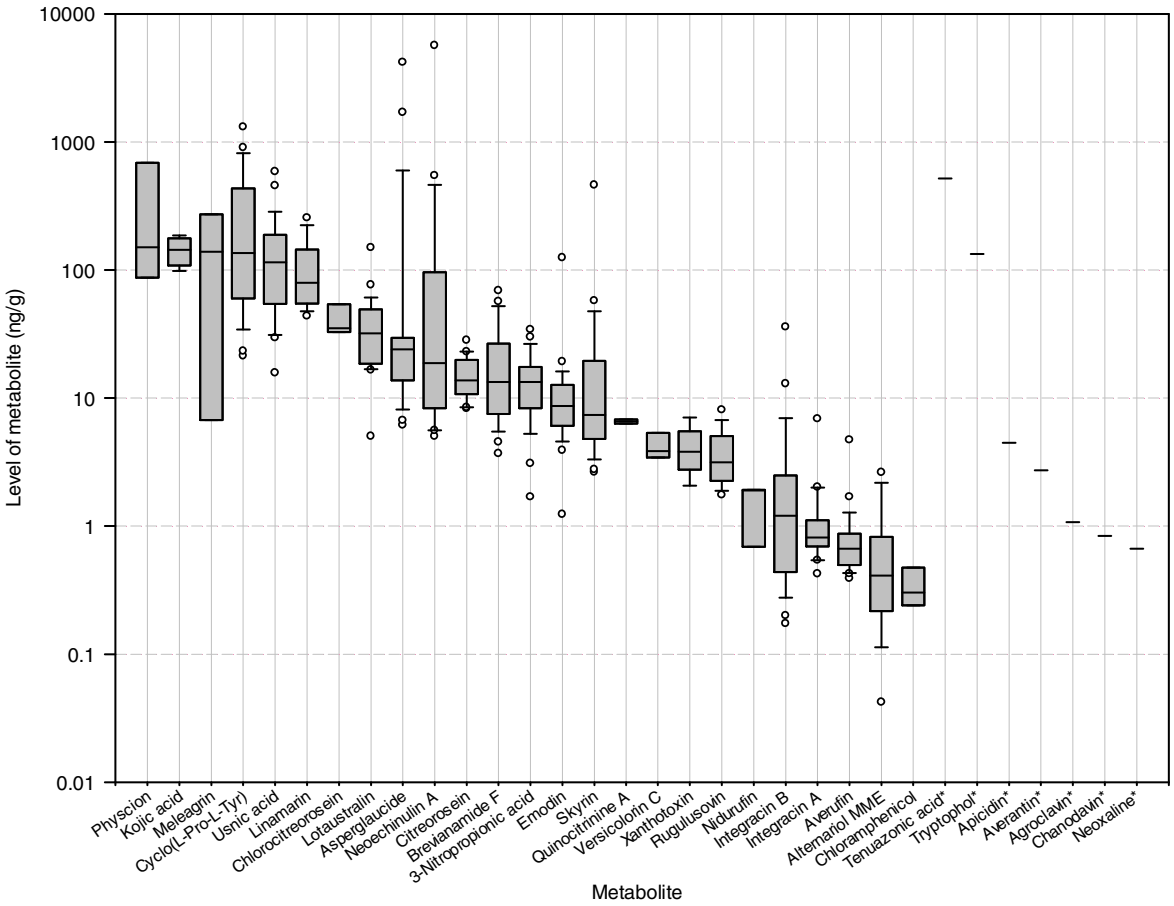
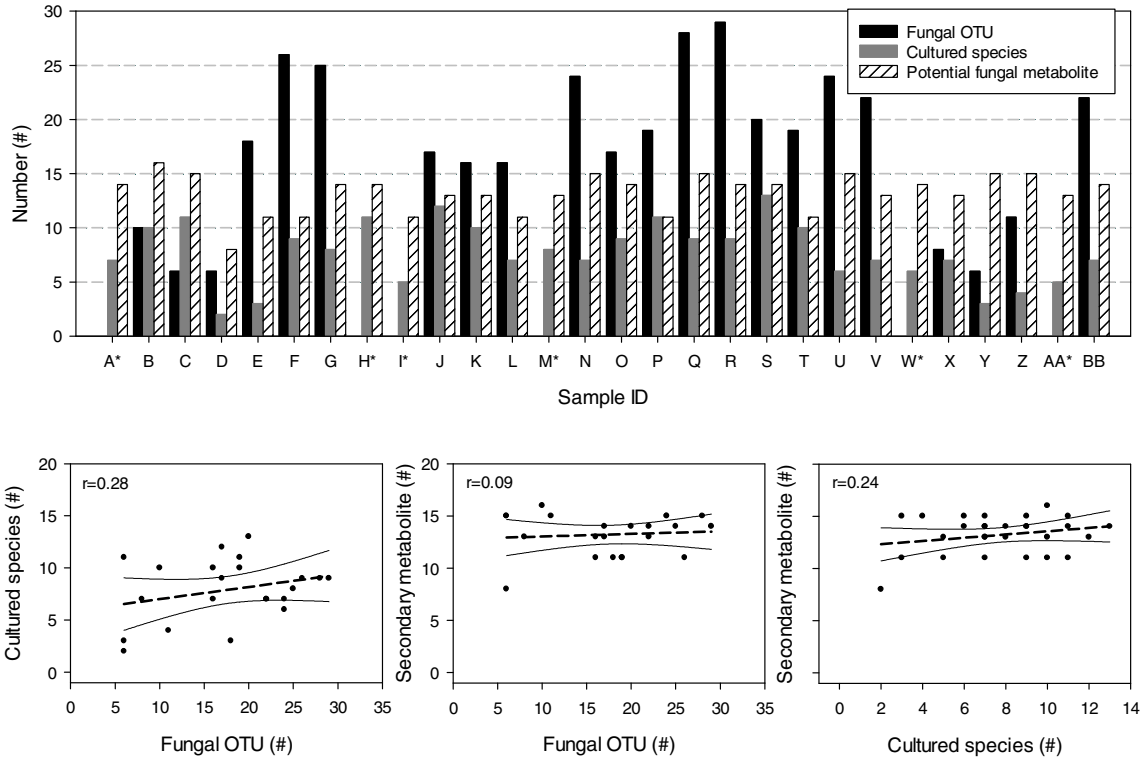


Figure 2. Levels of metabolites in floor dust. Alternariol MME=Alternariol monomethylether.

* Only one among 28 samples was above LOD.

Figure 3. The number of fungal OTUs, cultured species, and secondary metabolites for each analyzed sample and the correlation between them.



In the first bar graph: * Samples were not analyzed for fungal DNA due to insufficient quantity of dust available for the analysis.

Figure 4. Prevalence (%) and average level (geometric mean CFU/g: colony forming unit per gram of dust) of each fungal species identified in 28 dust samples with culture. Fungi with no error bars in the geometric mean bar graph indicate that they were identified in only one sample. The upper limit of *Acremonium strictum* is off-scale (5.5×10^7 CFU/g)