# Update on Sampling and Analysis of Mold and Other Indoor Biocontaminants

Wei Tang, Ph.D., QLab, Cherry Hill, NJ

*Did you know that "ERMI" cannot be found on EPA's mold website? (www.epa.gov/mold)*

Many indoor biological contaminants can cause health effects in human. Common building related biological contaminants include fungi (mold), bacteria, and cell components or byproducts of living organisms (allergens, endotoxins, glucans, mycotoxins, MVOCs, etc.) All of these biological and also chemical agents should be considered to be the potential causes. Further investigation should be conducted if initial mold growth investigation does not reveal an indoor mold growth problem.

Laboratory analysis of samples containing these agents involves complicated procedures and also requires good sample quality. Selecting sampling devices and procedures as well as a quality laboratory greatly affect the quality of the analytical results. This presentation will discuss the important difference in sample quality and laboratory procedures.

**Terminology:**
Organisms (fungi, bacteria): total (non-viable and viable), non-viable, viable (culturable and non-culturable)
Sample (air, surface, bulk): culturable, non-culturable
Methods: direct microscopic examination, culture method, PCR analysis, ELISA, bioassay, GS/MS

**Example 1:** Allergenco-D is a spore trap that collects a non-culturable sample. It can be submitted to a laboratory to be analyzed for total fungi by using direct microscopic examination, but the sample can not be analyzed for culturable fungi by using the culture method.
**Example 2:** Swabs collect culturable sample. It can be submitted to a laboratory to be analyzed for culturable fungi by using the culture method and total fungi by using direct microscopic examination.

**Accuracy and Precision:**
Accuracy (bias)
Precision (variance)

**Example 1:** The 70 millions and 3 years old dinosaur fossils

## I. FUNGI

Fungi (mold) samples can be collected from different matrices (air, surface, bulk) in indoor environments by using different kinds of devices (spore traps, tapes, swabs, etc.) and analyzed for total fungi, culturable fungi, or fungal DNA by using direct microscopic examination, culture method, or PCR, respectively.

## 1. Total Fungi by Direct Microscopic Examination

Fungal biomass (spore, yeast, hyphae and fruiting reproduction structure) can be observed under a microscope (200x to 1,000x) directly after mounting samples onto a glass slide for observation. Stains (lactofuchsin, lacto phenol cotton blue) are used to make biological matter with stained color more easily recognized. The background needs to be free of interference, e.g. heavy debris or opaque collection media.

### (1) Air Samples

A. Sampling

Air samples are collected in occupied space mostly to do indoor and outdoor reference (or indoor reference) comparison. Indoor reference collected in non-compliant/non-damaged area is strongly recommended due to the large difference between indoor and outdoor air. Available devices are impaction-type spore traps and filter cassettes.

Impaction-type spore trap cassettes have different collection efficiencies. Devices with large $d_{50}$ or small collection area may or may not perform well in field conditions. The overall actual field performances of different devices under all conditions need to be studied further by independent parties. Many other factors also affect the collection efficiencies of those spore traps, e.g. slide overloaded with debris, low temperature, device quality control, etc.

For filtration-type spore traps, Bi-Air cassettes (2 LPM) collect two separate samples that can be easily used for two different analyses individually. SmartCassette is also a filtration-type spore trap. They can both be used for long-term sampling.

B. Laboratory Analysis

Impaction-type devices (Allergenco-D, Mocro-5, Air-O-Cell, BioSlide, etc.) collect spores by impacting particles onto a gel surface through a slit or round orifice. After airborne particles are collected on the gel surface on a glass slide, the gel surface slide is removed from disposable cassette and glued on top of a microscopic glass slide. For reusable impactor, the gel surface is already on a glass slide. It was then stained with dye and covered with a cover slip. For disposable cassettes, preparing the sample with the collection media on the top of the spores is not recommended. It can not be observed under 1,000x magnification and the spores are observed through the collection media, which may sometimes become not clear and interfer with observation.

Counting 100% of the impaction trace of an Air-O-Cell, Allergenco-D, or similar devices in less than 30 min is not recommended. Counting a representative portion of the trace *carefully* is better than counting 100% of the trace at accelerated speed. Counting 100% of the impaction trace in the same time period required to carefully read 25 to 30% of the trace will misidentify spores

because the average time spent to observe each spores is significantly decreased.

Advanced procedure of differentiating indoor and outdoor Cladosporium and Aspergillus/Penicillium-like airborne spores performed by a Ph.D. microbiologist/mycologist can provide more information for data interpretation. Cladosporium and Aspergillus/Penicillium-like spores can be commonly found in both indoor and outdoor air. Further identification of those spores based on taxonomy is not possible for direct microscopic examination. When outdoor air has predominately Cladosporium herbarum-like spores and indoor air has predominately Cladosporium cladosporioides-like spores in similar concentrations, it is an indication of a possible indoor growth of Cladosporium. However, there is no way to indicate this difference when they are being grouped and reported together under the same name. Same fungal colony produces spores with identical or similar microscopic morphology. Spores with different microscopic morphology are most likely originated from different fungal colonies. A good approach is to differentiate those two groups of airborne fungal spores based on their microscopic morphology and chemical reactivity. Cladosporium are sorted into 3 groups and Aspergillus/Penicillium-like spores are sorted into 9 groups. When indoor and outdoor Cladosporium and Aspergillus/Penicillium-like spores are differentiated into several groups, possible difference in indoor and outdoor distribution can be shown. Otherwise, when the concentrations of those common dominant airborne fungal spores are observed both in indoor and outdoor air in similar concentrations, regular spore counting results can not indicate any difference.

C. Data Interpretation

Accurate identification of spores is crucial to the data interpretation for indoor and outdoor (or indoor reference) comparison. Identification of loose airborne fungal spores is often presumptive to the best even by well-trained and highly experienced analysts. Data quality of results produced in a mass-production, low-price laboratory is often questionable. Several studies have revealed the inconsistency of results of side-by-side spore traps analyzed by several commercial labs. Therefore, it's important to choose a quality laboratory using a more advanced protocol rather than a quick and basic procedure that can be done in less time by under-trained staff.

Many approaches have been used to compare indoor and outdoor fungal spores to aid the investigation of possible indoor fungal growth including inside to outside concentration ratios (I/O >1 and I/O >10), rank order, complicated statistical analysis, and diversity comparison. Airborne spores in indoor environment are settling and being filtered out constantly. If the indoor fungal growth is hidden and the concentrations of airborne spores in contaminated air are not very high, comparing only the concentrations will most likely fail to identify the source. However, the percentage of some spores in contaminated indoor air

should show significant elevation. It's recommended for laboratory to report percentages of spores identified as well as the concentrations.

An easy and effective way to interpret air sample results is to group them by their importance. Common dominant airborne spores (ascospores, basidiospores, Cladosporium spores, and Aspergillus/Penicillium-like spores) can be used for concentrations and percentages comparisons. Their sizes are mostly less than 8 µm and they stay airborne much longer than larger spores. Comparing to outdoor air, significantly lower diversity of basidiospores observed in indoor air based on microscopic morphology could be an indication of indoor mushroom growth.

The presence of water-damage indicators (Stachybotrys, Chaetomium, Ulocladium, etc.) is important. They require high water activity ($A_w$) to grow (minimal $A_w$: 0.89-0.90). The presence of their spores even in low numbers is an indication of possible water-damage history. For laboratories that can not identify Ulocladium and group it together with Pithomyces, it is recommended to supplement their analysts' training with a competent Ph.D. mycologist for the identification of Ulocaldium. The rest of the spores usually come from outdoors if no indoor mold growth is disturbed. They don't usually signify an indoor source unless the concentration is more than 100 to 300 spore/$m^3$ and significantly higher than the outdoor count.

Differentiation of indoor and outdoor Cladosporium and Aspergillus/Penicillium-like spores is helpful when they are both observed in high number in indoor and outdoor air.

**(2) Wall Cavity Air Samples**

A. Sampling

Remediation process often discovered more hidden mold growth behind dry walls that initial investigations missed. Sampling of wall cavities air could have helped to indicate possible hidden mold growth. The analysis has not been very successful due to high debris (gypsum from drill the dry wall) loading on gel surface in spore traps, which reduce collection efficiency dramatically and interfere with microscopic analysis. MoldSense™ Wall Cavity Sampling Protocol and filter cassettes have been suggested for better results for wall cavity air sampling.

B. Laboratory Analysis

The analysis of wall cavity air is very similar to that of occupied space air. Recognizing spores from gypsum debris is a difficult task for samples collected without using MoldSense™ Wall Cavity Sampling Protocol. A significant amount of spores will bounce from the trace loaded with debris and could spread into an area 6 times larger than the original impaction trace, which significantly increases time needed for analysis. Some laboratories may only read a very small portion of trace in order to save analysis time.

C. Data Interpretation

Wall cavity air samples are collected to investigate a possible source. Spore concentrations greater than 5,000 spores/$m^3$ for species with easily airborne spores could indicate that. Spores of some water-damage indicators like Stachybotrys don't become airborne easily, therefore the criteria would be lower.

**(3) Surface Samples**

A. Sampling

It is impossible to identify a mold growth (IICRC Condition 3) or surface contaminated with settled spore (IICRC Condition 2) without using a microscope. Therefore, surface sampling is used to confirm possible mold growth and verify remediation results. Available devices are tapes and swabs.

Tape can lift fungal structure from a dry surface, just like lifting a fingerprint. It preserves part of the structure needed for identification. Counting spores on tape-lift is not recommended because the results are inconsistent. Swab samples can be used to quantitatively measure total and culturable fungi on the surface. All regular swabs need to be stored between 2 to 8ºC after sampling and during transport to the labs (within 24 to 48 hours) to prevent spore germination and yeast multiplication.

Collecting large area (4" x 4") of sample by using foam-tip swabs moistened with Sporicidin are recommended for Post-remediation Verification (PRV) surface sampling of fungal biomass (spores, yeast, hyphae). Tape and fiber-tip swabs have very limiting collection efficiency and collection area. Aggressive air sampling method can be used to measure "aerosolizable surface fungal biomass" for PRV.

B. Laboratory Analysis

Bio-Tape and similar devices are very easy to prepare in the lab. It can be observed under microscope directly after applying stain and placing a cover slip. Scotch tape is prepared by cutting a piece of tape and mounting it on a glass slide with stain and a cover slip. Scotch tape longer than 2 inches will need to be subsampled. The subsample of tape sample should be at least 1 square inch.

It is not recommended to do quantitative analysis on tape-lifts. The collection efficiency of tape-lift may vary from less than 1% to greater than 90%, depending on the surface conditions. The distribution of fungal biomass on the tape is highly heterogeneous. It can not be "mixed" or homogenized in the lab, therefore, counting a small portion of the tape is not representative and the data is not qualified to be a quantitative measurement.

The sample collected on swabs is extracted in the lab. Proper mixing is important to ensure that the subsample is still representative. After mixing the suspension, a portion of it will be mounted onto a glass slide and observed under microscope. Identification will be limited since the spores are loose and do not associate with

their identifiable structure anymore, just like airborne loose spores collected in spore traps.

Fungal biomass is carefully analyzed under a microscope. Observation of fungal spore, yeast, hyphae and fruiting structure must be all reported. Spores observed alone may only indicate an IICRC Condition 2 and not Condition 3.

C. Data Interpretation

It is important to analyze the samples and report their level of fungal biomass to help consultants to determine the IICRC S520 Conditions. Observation of hyphae and structure is important for identifying fungal growth and need to be reported. Spores observed alone may only indicate an IICRC Condition 2 and not Condition 3. Semi-quantitative measurement for the observed fungal biomass collected on tapes is the recommended method for this purpose.

Exposure limits and the condition of "normal fungal ecology" are both extremely difficult to define. Therefore, it has not been an easy task for consultants and remediation contractors to agree on PRV acceptance criteria. If a normal fungal ecology condition cannot be established, it's recommended to select PRV acceptance criteria from the range of 1,000 to 10,000 counts/in$^2$. This is based on the concept of "How clean can this material be by cost-effective means and methods?" To select final acceptance criteria within the range, many factors listed below need to be considered.

a. mold growth size, location, building material
b. building type, age, usage, geographic location
c. occupant health conditions
d. building air movement
e. budget limitations

The criteria can even be lower or higher than the range if there are valid considerations.

**(4) Bulk Samples**

A. Sampling

Bulk samples are collected when tape-lifts and swabs can not efficiently collect fungal biomass. Plastic bags and plastic tubes are commonly used to store bulk samples (loose surface dust or large pieces). Care needs to be taken not to disturb or damage the mold growth on the sample during storage and transport since plastic bag does not provide physical protection. Loose bulk material (e.g. dust) can be collected using a micro-vac method. A polycarbonate filter in a cassette with a sealable cap on the inlet is recommended.

B. Laboratory Analysis

Small amounts of loose bulk samples (dust) can be mounted onto a glass slide for observation most of the time. When subsamples of a large bulk sample can not be observed directly, three tape-lift subsamples are taken to be representative of the whole bulk piece submittted. Similarly, swab subsamples

can be taken and then extracted to a liquid solution and mounted onto a glass slide for observation with limited identification. See previous section on surface samples (I.1.(3)B) for more analysis information.

C. Data Interpretation

Please see previous section on surface samples (I.1.(3)C) for interpretation.

**2. Culturable (Viable) Fungi by Culture Method**

Culturable fungi can grow on agar media plates if the media is suitable for them. Fungal colonies are observed after 6 to 7 days of incubation. Fungi which are viable but not culturable on the media selected will not grow and will not be detected. Culture method samples can provide more detailed and accurate identification if analyzed by a competent Ph.D. microbiologist/mycologist. This method is especially useful when high numbers of Aspergillus/Penicillium spores are observed in both indoor and outdoor air.

**(1) Air Samples**

A. Sampling

Andersen N-6 impactor, whose $d_{50}$ is 0.57μm, is an excellent tool for airborne fungi and bacteria sampling. Care needs to be taken not to contaminate the culture plates while handling them. Sterilization of the impactor is required. BioCassette is easy to use and does not require cleaning between sampling. It has a short shelf life like all other agar plates. Filter cassettes (PC filter membrane recommended) can be used to collect airborne fungal spores. Viability of spores may decrease over prolonged sampling periods. Samples need to be stored between room temperature and 40ºC after sampling and until arriving at the analytical lab (within 48 hours). Using blue ice packs is not recommended unless the temperature during transport will exceed 40ºC. Blue ice packs can easily cause condensation to occur on the agar surface and spread out one yeast or bacteria micro-colony into many different colonies. It's recommended to wrap the plates with surgical (paper) tape and place them inside of cardboard box to be separate from the blue ice pack. When it is necessary to use a blue ice pack, ship the package in a thermo-insulated box.

B. Laboratory Analysis

Andersen agar plates received are incubated for 6 to 7 days. Longer incubation times may be required for slower growing fungi. For filter cassettes and other devices, spores collected on the filter membrane or other media are extracted into an aqueous solution. Spore suspensions are inoculated onto agar plates for culture analysis. Dilution can be made if necessary, which is an advantage over Andersen-type impactors. Colonies that appear after incubation are identified and counted. Both macroscopic and microscopic morphological characteristics are observed.

C. Data Interpretation

Culture methods can provide more detailed and accurate identification. Culturing is especially useful when high numbers of Aspergillus/Penicillium spores are in both indoor and outdoor air. Results from analysis of fungi on agar plates that are either (a) overloaded with fungi/bacteria or (b) occupied by spreading fungi (Mucor, Rhizopus, Trichoderma) may not be representative because slow growing fungi may not be able to form colonies. Culture methods provide much better fungal identification, which is very beneficial for comparing indoor and outdoor samples. However, non-culturable (non-viable) fungi will be overlooked. Concentrations of indoor airborne culturable fungi higher than 250 to 1,000 CFU/$m^3$ are generally considered to be atypical. Comparison to outdoor air (or an indoor reference) is required. Please see pervious section on airborne total fungi (I.1.(1)C) for more data interpretation information.

**(2) Surface Samples**

A. Sampling

Swab with a fairly short handle in a protective tube is recommended for sampling. It can easily collect a mold sample from a surface. Before sampling, it's recommended to pour all liquid out except what is necessary to keep the swab moist. A defined area (1 to 4 $in^2$) is swabbed in three (horizontal, vertical, and diagonal) directions while rolling the swab to collect as much sample as possible from the surface. All swabs need to be stored between 2 to 8ºC after sampling and until arriving at the lab (within 24 to 48 hours).

B. Laboratory Analysis

Fungal biomass will be extracted from the swabs. After mixing the suspension and making dilutions, a portion of it will be inoculated onto agar plates. Although it's a small portion of the original sample, proper mixing during serial dilution ensures that it is still representative. See previous section (I.2.(1)B) for identifying fungal colonies.

C. Data Interpretation

Concentrations of culturable fungi on indoor surfaces higher than 10,000 to 100,000 CFU/$in^2$ are generally considered to be atypical.

**(3) Bulk Samples**

A. Sampling

See sampling for total fungi (I.1.(4)A).

B. Laboratory Analysis

Fungal biomass will be extracted from the bulk material. After mixing the suspension and making dilutions, a portion of it will be inoculated onto agar plates. See previous section for identifying fungal colonies (I.2.(1)B).

C. Data Interpretation

Concentrations of culturable fungi in indoor bulk samples higher than 25,000 to 200,000 CFU/g are generally considered to be atypical. Concentrations of

culturable fungi in household dust higher than 5,000 to 25,000 CFU/ft² are generally considered to be atypical.

**3. Fungal DNA by PCR**

PCR is a useful analytical tool to detect specific known organisms, e.g. avian pathogens. If only a limited group of species is being detected, it's recommended to have other analyses performed at the same time to cover all organisms of concern. ERMI is suitable for research study but needs further validation before it can be used as an investigation tool for individual cases.

## II. BACTERIA

Common bacteria associated with building-related illness or symptoms are gram negative bacteria, fecal coliforms, enterococci, Legionella, mycobacteria, and thermophilic actinomycetes. The main analytical methods for bacteria are culture-based. Several PCR analyses are also available for selected organisms.

### 1. Bacteria (General, Gram Negative)

Sampling procedures for general culturable bacteria are similar to that of culturable fungi. Different media plates for bacteria (TSA, Blood Agar, R2A) can be used. Antifungal antibiotics are recommended to be in the ingredients of the media. Andersen impactor, swabs, and bulk sampling are the available options. Preparation and analytical procedures for general culturable bacteria are similar to those for culturable fungi. An elevated concentration of gram negative bacteria is a sign of possible indoor bacteria growth due to water damage. Although it's usually unnecessary, further species identification can be done by fatty acid analysis.

### 2. Fecal Coliforms and Enterococci

Swabs and bulk sampling are used to sample possible indoor contamination of fecal matter. Samples need to be stored between 2 to 8ºC after sampling and until arriving at the lab (within 24 hours). Samples are inoculated onto selective media which inhibit the growth of unwanted microorganisms. Color change of media will indicate the presence of the target organisms. Presence of fecal coliforms and enterococci in the indoor environment is an indication of fecal matter contamination.

### 3. Legionella

Non-potable water (cooling tower, water fountain, etc.), potable water (faucet, shower head, etc.) or swab samples are taken during Legionella investigation or routine checkup. CDC and ISO methods can be used to prepare samples and analyze the results. The incubation time is 7 to 10 days. Action (facility maintenance, occupant safety) needs to be taken for significant amounts of Legionella bacteria in potable water and high levels of them in non-potable water.

### 4. Mycobacteria

Metal working fluids are some of the common sources of mycobacteria in occupational environments. Bulk liquid samples are taken for analysis. Liquid samples are inoculated onto selective media. The incubation time is 14 to 21 days or longer. Presence of mycobacteria in metal working fluids may indicate an amplification (growth) and probable cause of infection.

**5. Thermophilic Actinomycetes.**
Air samples are usually taken for the detection of thermophilic Actinomycetes. TSA or Actinomycetes Isolation Agar can be used. The agar plates are incubated at 55°C for 10 to 14 days. The colonies are observed after incubation. Further biochemical testing may be needed for genus identification. Presence of thermophilic Actinomycetes in the living environment of a hypersensitivity pneumonitis patient may indicate the cause.

**6. MRSA**
Methicillin-resistant Staphylococcus aureus (MRSA) are strains of Staphylococcus aureus that are resistant to methicillin and its related antibiotics. MRSA are considered to be carried by humans and do not grow on building materials. Good personal hygiene is a much more effective solution than building disinfection. Most disinfecting chemicals don't have enough residual killing effects for MRSA after initial application. Therefore, the surface can be contaminated again as soon as it is touched by someone who carries MRSA. Organosilane is claimed to have long lasting killing effect after initial application according to laboratory experimental data. The actual effectiveness under field conditions needs to be verified by further research studies.

## III. CELL COMPONENTS AND BYPRODUCTS

IAQ investigations may also involve the sampling and analysis of biological components and byproducts, like allergens, mVOCs, endotoxins, glucans and mycotoxins. The methods employed include immunoassay, chromatography, and chromogenic in-vitro bioassay.

**1. Allergens**
Dust vacuumed from carpet, bedsheet, etc. is collected for allergen analysis. Filter cassettes, Dustream Collector or similar devices can be used. Allergens in dust samples are extracted and suspended in solution. ELISA kits (Indoor Biotechnologies, Inc.) can be used for analysis. For dust mite allergens (Der p 1, Der f 1), a concentration higher than 2 µg/g in dust is a concern. For dog (Can f 1) and cat (Fel d 1) allergens, a concentration higher than 1 µg/g in dust is a concern.

**2. Endotoxins and Fungal Glucans**
Endotoxins are Lipopolysaccharides in the outer cell membrane of gram negative bacteria. Fungal glucans are polysaccharides in the fungal cell wall. Endotoxin-free filter cassettes and tubes are used to collect endotoxin in air and water,

respectively. Polycarbonate filter cassettes are used for glucans sampling of air. Bioassay using Horseshoe crab (Limulus amoebocyte) lysate (LAL) is used for analysis of endotoxins and fungal glucans. An indoor/outdoor ratio is commonly used for endotoxin results interpretation.

**3. MVOCs**

Microbial volatile organic compounds (MVOCs) are volatile secondary metabolites of microorganisms (fungi, bacteria, etc.). MVOCs are collected in indoor air to investigate possible causes of odors and/or other health complaints potentially caused by these compounds. MVOCs in indoor air can be collected as whole air sample using stainless steel sampling vessel (e.g. SUMMA canister) or as adsorbed compounds on the media inside of sorbent tubes. Both samples can be sent to labs to be analyzed using GC/MS. Whole air sample is more suitable for noticeable amount of MVOCs (odors). Although sorbent tubes can detect lower concentration of MVOCs due to higher collection volume, the appropriate sorbent needs to be selected based on possible type of MVOCs or target organisms prior to sampling. If the sorbent type is not suitable for the target compounds, the result will be poor.

**4. Mycotoxins**

Mycotoxins are secondary metabolites of fungi which are toxic to humans and/or animals. To investigate possible causes of health complaints, mycotoxins in (a) airborne fungal cells (whole or fragments) and particles of growing substrate (paper, dust, etc.) and/or (b) mold growth in indoor environment including the growth substrate are collected for analysis. Polycarbonate (PC, 0.2 μm pore size) filter membrane cassettes and other high-volume aerosol collector (SpinCon, Sceptor Industries) can be used to sample airborne mycotoxins. Collection from a large volume of air at a low flow rate is recommended. GC/MS is used for analysis.