Recognition, Evaluation, and Control of Indoor Mold

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Chapter 12

Laboratory Analytical Methods

12.1 Direct Microscopy

12.1.1 Source Samples

Direct microscopic analysis of bulk material specimens or tape lift samples is usually intended to determine the presence of actual fungal growth on the material as opposed to settled fungal spores and fragments arising elsewhere. Prior to examination by compound microscopy, the specimen should be examined for obvious evidence of growth in reflected light at 10–40X magnification. Mounts of the specimen should be prepared from suspect locations on the basis of this examination. Furthermore, an effort should be made to prepare representative mounts for comparison from regions of the specimen that appear uncontaminated.

At present there are two widely used methods for preparing mounts for microscopy from bulk specimens: (1) surface peel of the specimen using adhesive tape ("tape lifts"), and (2) direct scrapings or shavings of the specimen using a scalpel.

Adhesive Tape Lifts

Methods for the collection of fungal growth from surfaces using tape lifts to preserve the arrangement of spores and spore-bearing structures have long been used to study fungi *in situ*. A modern modification of this method involves the use of clear adhesive tape to collect surface debris for examination by transmitted light microscopy.[1–4] A number of important mold genera can be determined provisionally by the examination of spores alone (e.g., *Chaetomium, Stachybotrys*). However, reliable identification of most genera requires the examination of both spores and spore-bearing structures in culture. Tape lift sampling is a rapid and practical means of recovering fungal material for microscopy that can be employed in both field collection and laboratory analysis. Furthermore, this method allows for the provisional identification and semi-quantification of the density of fungal growth on a surface. A general laboratory procedure is given in following paragraphs, but see also references 5 and 6 in the list at the end of this chapter.[5,6]

Once prepared, tape mounts deteriorate rapidly as a result of the affect of lactic acid on the tape adhesive. Generally, tape mounts become unusable within 12–24 hr of preparation. Should a more

durable specimen record be required, a portion of un-mounted specimen can be stored in its original transport package in a cool, dark, dry place.

General Analytical Procedure. Use the following steps to analyze tape lift samples.

1. In the laboratory trim a segment of sampled tape small enough to fit beneath a microscope cover slip, gently removing any large surface debris (e.g., soil particles, glass fibers).
2. Position the tape segment adhesive side up on a glass microscope slide. Some laboratories add a drop of a suitable liquid to help fix the tape in place.
3. Some labs add a drop of 95% of ethanol to wet the adhesive or proceed directly to the addition of approximately 10 µL of a suitable mounting fluid such as lactofuchsin[7,8] or lactophenol cotton blue[2,4,6,9] either to the tape or to the center of the glass cover slip. Some laboratories dilute the stains, which is a matter of judgment.
4. Gently apply the cover slip to the adhesive side of the tape. Prepare slide mounts 5–10 min prior to examination to permit sufficient time for staining of adherent fungal material.
5. The entire tape sample first should be scanned to characterize the sample. At least two or three transects of the tape specimen should be examined at a minimum of 400 × magnification but including scans at 1000 X, with the goal being to examine a minimum of 20–50 mm^2 of the physical area of the tape. Results should be tabulated on the putative type of fungus observed as well as the category of fungal element (e.g., spores, vegetative hyphae, mycelial fragments, spore-bearing structures).

Interpretation. In the absence of indoor fungal contamination and under normal circumstances, a majority of the fungal spores encountered in settled dusts and on surfaces in the indoor environment originate from the phylloplane together with some soil fungi. Thus, the observation of a fungal spore on a tape lift sample is uninformative with respect to the origin of the spore. In practice, the presence of fungal structures excluding spores (e.g., vegetative hyphae, mycelial fragments, spore-bearing structures) of a given taxon provides evidence of fungal colonization of the surface sampled. The presence of a large number of spores may be evidence of fungal colonization. The presence of a low number of spores is not evidence of growth.

When a qualified and experienced competent mycologist reports the structures seen and identifies the relevant taxa, this method is useful in indicating whether the suspect fungal growth is vegetative, reproductive, or both. This is important information, because it is normally reproductive growth that has the greatest implications for the quality of indoor air. To understand correctly reported direct exam observations for a tape lift, the investigator must know what various morphological structures represent.

Consider the following example.

- Spores, conidiophores, and hyphae of *Penicillium* detected.
- Spores, conidiophores, and hyphae of *Chaetomium* detected.
- Spores, conidiophores, and hyphae of *Cladosporium* detected.
- Spores of *Stachybotrys* detected.

These data mean that the surface is supporting vegetative and reproductive growth of *Penicillium, Chaetomium*, and *Cladosporium*, but neither vegetative nor reproductive growth of *Stachybotrys* was detected. The disadvantage of tape lift data is that species identification cannot always be done, even, as in this example, in the presence of reproductive structures of *Penicillium*.

The following tape lift report is not useful.

• Ascospores	Trace
• Basidiospores	Trace
• *Chaetomium*	Minor
• *Cladosporium*	Major
• Hyphae	Major
• *Penicillium/Aspergillus*	Minor
• *Scopulariopsis*	Trace

There is no way to tell what structures the analyst saw, except for the hyphae, for which no genus is given. The presence of spores alone is insufficient evidence of growth. The entire objective behind collecting a tape lift is to identify morphological structures indicative of growth, either vegetative or reproductive (e.g., conidiophores).

Scrapings and Shavings

Direct microscopy of scrapings or shavings of a specimen is a convenient way to investigate fungal contamination. This method (1) offers the analyst control over the size of particles collected to form the test material; (2) permits sampling of relatively large areas; and (3) allows the analyst to examine the sample surface as well as subsurface layers of the specimen. This method is a useful approach for detecting residual fungal growth that may have been covered by paint or incompletely cleaned from the specimen surface. Another advantage to the use of scrapings or shavings is that permanent microscopic mounts can be prepared if sample retention is required. Scraping or shaving also allows preparing cultures from scraped materials should this be necessary. This method is useful when tape lift samples fail to collect appreciable surface debris.

Materials such as drywall that contain appreciable amounts of alkaline mineral dusts are best sampled on-site either by adhesive tape lift or scraping. Transport of bulk material samples of these matrices can cause powdering of the surface, which impedes microscopic examination.

Procedure. Use the following steps for collecting and analyzing scraping samples.

1. Scraping samples may be collected in the field using a clean, sharp scalpel to scrape or shave the test surface, collecting the scrapings in a clean tube, closable plastic bag or paper packet. Plastic tubes tend to carry static electric charge and therefore may disperse scraped material. Paper packets, particularly gloss-coated heavy black paper, are useful because fine specimen fragments are easily seen on its surface. This collection method is widely used in medical mycology for the collection of skin scraping

specimens.[10] The collection of bulk specimens in the field for later preparation of scrapings in the laboratory is more desirable because it is less prone to cross-contamination and eliminates the need to dispose of sharps in the field.

2. Solid smooth-surfaced materials including paper products; paint chips; rubberized gaskets; vinyl wall coverings; processed and unprocessed wood; foam board insulation; floor and ceiling tiles; upholstery; clothing; and carpet samples (both pile and underlay) can be transferred into unused sterile petri plates for observation. By examining these samples in reflected light using a stereomicroscope at 10–80 X magnification, fungal fruiting structures (e.g., pycnidia), micro-colonies, mycelium, hyphae, and even large spores (especially from those of the muriform spore producing species [*Alternaria spp., Epicoccum spp., Stemphylium spp., Ulocladium spp.*] can often be identified readily on the sample surfaces.
3. For more detailed microscopic analysis, a small amount of scraped material should be placed in a small drop of mounting fluid such as lactofuchsin[5] or lactophenol cotton blue[2,4,6] (~10 µL) on a clean glass microscope slide. Care should be taken to ensure that particles are ground sufficiently to allow air pockets beneath the cover slip. Pre-wetting the scraped material with a solvent may help to moisten hydrophobic specimens. This can be accomplished by placing the sample in 1–2 drops of 95% ethyl alcohol and allowing the solvent to evaporate almost completely prior to the addition of mounting fluid. Woody specimens or those containing alkaline minerals (e.g., drywall) may be mounted in 10–15 % potassium or sodium hydroxide with 10% glycerol to promote softening and avoid foaming. Incubation of hydroxide preparations at 30–40°C for 30–60 min may assist in the softening of wood shavings or other resistant organic matter. A drop of 0.025% phloxine added to the hydroxide mount helps differentiate hyaline fungal elements, particularly those of basidiomycetes.[11] At least 5–10 min should be allowed prior to examination for staining.
4. A minimum of 20–50 microscopic fields should be scanned at a minimum of 400–600X magnification. The identity of any fungus seen should be noted. Furthermore, the type of structural element should be recorded (e.g., spores, vegetative hyphae, mycelial fragments, spore-bearing structures).

Interpretation. Like tape lift sampling, the observation of aggregated fungal hyphae and/or reproductive structures with or without associated fungal spores provides conclusive evidence of past or present fungal growth on the test substrate. Fungal content of scrapings or shavings are not readily quantifiable by direct microscopy. Thus, these specimen types are suitable only for qualitative or semi-quantitative analysis.

12.1.2 Spore Counting

This section describes appropriate analytical methods applied to spore trap samples collected during mold assessments and investigations. Fungal spores and hyphal fragments are counted and identified in spore trap analysis. Consequently, spore trap methods generally yield higher levels of fungal structures than culture-based methods because of the presence of airborne fungal structures that are nonviable, dormant, or unable to grow on the media used. Sometimes the differences can be large.

Spore trap methodology typically cannot identify fungi to species level. Airborne fungal spores collected for spore counts can be provisionally identified only to genus or group level. Culture- or DNA-based methods are required for species identification.

As described in Chapter 11, a number of samplers or devices, such as Zefon Air-O-Cell®, Burkard®, Allergenco®, Laro®, Cyclex-D®, Buck®, Rotorod, and several types of filter cassettes are available for collecting airborne fungal spore samples for direct microscopic identification and enumeration. The procedure involves collecting airborne fungal spores and structures (e.g., conidiophores and hyphal fragments) on a filter or on a sticky surface applied to a glass slide. Results are expressed as fungal spores (sometimes structures) per cubic meter. This yields information on total airborne fungal structures including both culturable and non-culturable fungal spores. Such information is important for any allergy-related health concern, because fungal spores and fragments can be allergenic whether they are culturable or non-culturable (including some that do not sporulate on culture media, such as most basidiomycetes and obligate phytopathogenic fungi). However, most fungal spores can be categorized provisionally only to genus or group level by this method.

Miller[12] considered the skill of analysts in counting and identifying fungal structures, especially for small colorless spores, to be extremely important. At the same time, there are currently no standard protocols for laboratory analysis and no numeric standards or guidelines for the interpretation of laboratory results of airborne fungal structures. Although an organization has taken the effort to develop methods for laboratory analysis, it is not very likely that exposure thresholds and guidelines will be developed in the near future. Without standards and guidelines the current approach to the interpretation of the results relies on comparisons of results from indoors versus outdoors, complaint versus non-complaint areas, or both.

In the laboratory, the sample has to be first stained and mounted on a microscopic slide. The microscopic counting of spore trap samples is done at 400X, 600X, or 1000X magnification counting selected microscopic fields or the entire deposit area. Detailed descriptions on the protocols involved in the preparation of slides and microscopic counting of spore trap samples are presented elsewhere.[6,13]

Interpretation of Airborne Fungal Spore Results Derived by Microscopic Counting Method

A detailed process for result interpretation is discussed in this section. Background information, on-site observations, and history of the case and building (such as water damage history and humidity problems) are very important in the final interpretation and conclusion. Information collected during the field study and sampling should be incorporated. Floor plans or blue prints should be used to correlate with the data. This may allow better

correlation of results with locations. Users of results derived from such sampling and analytical methods must be aware of the limitations of such sampling and analytical methods. Most important, the identification of fungal spores and structures is presumptive at best.

The process is divided into several steps.

1. Use of a large database of airborne fungal spores derived from the total spore counting method, if available, may define ambient spore populations with the particular kind of building (house, school, office building, etc.) and can serve to screen results. However, use such data with caution and only when you are sure you understand how the data were collected and analyzed. Only data collected using the same samplers, identical flow rates, sampling media, and sampling times should be directly compared. In general, these data should not be used to evaluate health risks.
2. Compare total counts from indoors, outdoors, complaint, and non-complaint (if possible) areas. In general, indoor counts should be lower than those of outdoors. Residential buildings, warehouses, schools, and buildings with many entrances and operable windows, and buildings with heating, ventilation, and air-conditioning (HVAC) systems with no filtration may have airborne fungal counts higher than or as high as those outdoors. The results of non-complaint areas would typically be expected to be lower than those of complaint areas, if fungal growth is an issue.
3. Compare fungal spore taxa, indoor versus outdoor, and complaint versus non-complaint areas. Generally, fungal spore taxa from indoors and outdoors and complaint and non-complaint areas should qualitatively be similar. However, in a large building such as a convention center or a 30-story office building, indoor fungal spore taxa may not always reflect what is outdoors because of air dilution or time lapse as a result of large air spaces in these buildings. In an airtight and mechanically ventilated building, indoor fungal taxa may include a collection or accumulation of outdoor fungi over several or many days.
4. Compare the set of data from complaint-area samples to determine which fungal spores are consistently detected. Evaluate the entire set of data from complaint area samples to determine whether the complaint area has a consistent presence of certain fungal spores. For example, if 10 complaint area samples are collected and all samples have *Chaetomium* at low levels, this suggests that the fungus may be near to or at the location.
5. Look for marker or signature fungal spores. Some fungal spores, if correctly identified and detected indoors, may be associated with water damage. They are spores of *Chaetomium, Stachybotrys, Memnoniella, Ulocladium*, and *Eurotium*. *Chaetomium, Stachybotrys, Memnoniella*, and *Ulocladium* are moisture-loving fungi. They are usually associated with water damage or chronic condensation problems. *Eurotium* is a genus of xerophilic fungi. Its confirmed presence indicates persistent high relative humidity, poor ventilation, and condensation problems. Spores and structures of these fungi may also come from outdoors, although the probability is low. *Aspergillus/Penicillium*-like (or *Asp/Pen*-like) spores are common indoors and outdoors if samples are taken at ground level.[14] If the prevalence of *Aspergillus/Penicillium*-like spores indoors is consistently higher than for those outdoors, then the difference may be indicative of a water-damaged environment.
6. Consider seasonal and geographical effects of airborne fungal spores. Indoor fungal growth may become dormant during the winter heating season unless there are persistent leaks or water sources to sustain growth. Therefore, low airborne fungal spore levels in winter do not suggest a "clean or healthy" environment. Fungi in temperate and sub-arctic climates have well-defined summer peak seasonal patterns.[6,15–17] The seasonal patterns in tropical areas can be significantly different.
7. Relate complaints, field observations, and laboratory results to determine whether fungal contamination and growth occurs in the building or complaint area. Remember that moisture and water are the critical factors in indoor fungal growth. If there is fungal growth, there must be a moisture or water problem nearby.
8. Understand the ecology and background of the fungal spores identified. Some fungi grow in high water activity conditions. The detection of *Chaetomium, Memnoniella, Stachybotrys*, and *Ulocladium* spores may indicate current or previous wet conditions. Another group of fungi, such as species of *Eurotium*, occur indoors, are moderately xerophilic, and grow under more humid conditions. The presence of these fungi often suggests condensation problems.

Common Airborne Spores and other Fungal Structures

The identification of fungal spores and structures is presumptive, because fungal identification is based on structures associated with sporogenesis (how spores are produced). Identifications based on spores alone may include many unrelated spore types. For example, smuts may include spores of smuts, microfungi, myxomycetes, and others. The reliability of such identification depends largely on the laboratory analysts and their supervision.

12.2 Culture Assay

Culture assay can be performed for air, bulk, and surface samples. Bulk and surface samples are analyzed either through direct or dilution plating as described in following sections. Air samples collected directly on culture media using impactors do not require any additional sample processing in the laboratory, except to subculture when necessary to identify to the species level. Air samples collected by impingers or filter samplers usually are processed through dilution plating. Air, bulk, and surface samples are similar in that the same principles for the selection

of agar media, incubation, and colony identification apply for all three types of culture assay.

12.2.1 Dilution Culture versus Direct Plate

Once the material has been evaluated for active growth using non-culture methods, it can then be sampled by direct and/or dilution plating methods.

Direct Plating

Direct plating is a generalized method by which samples are processed to provide information on the species diversity and composition associated with a sample. Direct plating allows confirmation of the presence of a species and /or source(s) of suspected microbial growth, so that the material can be remediated. Direct plating is recommended for determining actively growing species that may be present.[6] Although direct plating is a valuable method used to assess biological contamination diversity, fungal overgrowth is common, especially when dust and soil samples are direct plated.

Dust, other solid particles, porous, and/or fleecy samples for direct plating analysis can be weighed and then crumbled directly over previously poured and set culture plates. If there is sufficient material, some 5–15 pieces of material should be crumbled over the whole medium surface.

Settled dust and soil samples and fine particles for plating should be weighed and then separated by sieving through "mesh" series sieves. Alternatively, as an inexact method of separating particulate fractions, samples can be placed in a clean, sterile petri plate and gently shaken horizontally by hand for about 1 min to separate coarse and fine crumbles. The fine crumbles can then be collected aseptically using fine forceps and sprinkled over the surface of the mycological medium. Weighed material can also be collected on a damp, sterile filter paper or cotton swab that is then streaked over the culture medium surface.

An alternative direct plating method is to weigh, wash, and extract the sample in a sterile 0.1% (v/v) peptone water prior to plating, especially if serial plating is to be employed. When extracted to peptone water, the samples are spread over the culture medium surface using a sterile glass rod bent at a right angle some 2 to 3 cm from one end.[6]

For most purposes, nonselective culture media for saprotrophic and xerophilic saprotrophic fungi should be employed. The most commonly employed media for the isolation and growth of building-associated fungi is malt extract agar (MEA) (2% MEA = 20 g malt extract, 20 g agar, 1 L distilled water and dichloran, 18% glycerol agar [DG-18]) for the xerophilic saprotrophs (= 5 mg peptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 1.0 mL dichloran [mixed in 0.2% ethanol], 220 mL glycerol, 0.1 g chloramphenicol, 15 g agar, 1 L d.w.). A wide range of saprotrophic species is supported by 2% MEA, ranging from xerotolerant *Aspergillus spp.* and *Eurotium spp.* to hydrophilic species such as *Acremonium strictum, Stachybotrys chartarum*, and *Trichoderma* spp. MEA also supports growth of yeasts and some bacteria including actinomycetes, unless the medium has been supplemented with a bactericide such as streptomycin sulfate. DG-18 is a more selective medium than MEA and is used for the isolation and growth of xerophilic species such as many *Aspergillus* species, *Eurotium* species, and *Wallemia sebi*, which do not readily grow on MEA, and some of the moderately mesophilic species such as *Penicillium*. More specialized media are sometimes used, but there is less experience with their use. Additional media and their formulations used for the isolation and identification of selective fungi are provided in Samson et al.[18]

After plating, samples normally are incubated upright and in the dark at 25°C for mesophilic species. However, if sample material is from sites suspected of supporting thermo-tolerant species such as *Aspergillus flavus, A. fumigatus*, and *Paecilomyces variotii* (composting facilities, wet pipe wrap from hot water lines), part of the sample should be incubated at 35–40°C. Plates should be incubated for 5–10 days but examined after 3 days for evidence of growth. When they are read, the plates typically are examined using a stereomicroscope to discriminate among the different colonies growing on plates. The number of colonies of each type of fungus should be counted to estimate fungal species dominance and mix in the culture. For culture-based methods, representative material from colonies may be transferred into lactic acid on glass slides for generic or species identifications, or in the case of *Aspergillus, Fusarium,* or *Penicillium* spp. onto specific media appropriate for species identification. Species identifications should be made by comparing gross and fine characteristics of the sample isolates with descriptions in various identification manuals.[19–30] Non-sporulating isolates, which likely cannot be readily identified using any of the previously listed manuals because they lack reproductive structures, typically are examined microscopically to determine whether they support clamp connections along their hyphae. The presence of clamp connections distinguishes basidiomycetous fungi (including wood rot and mushroom species) from ascomycetous fungi. Yeast colonies should be counted.

Final results should provide total colony forming units per sample. They should also provide colony forming units for each species observed and the percentage of each species in the sample.

Dilution Plating

Dilution plating provides the quantity of colony forming units for each species per sample unit (i.e., area, volume, weight). It should be noted that it is biased toward recovery of species with heavy spore production (e.g., *Aspergillus, Penicillium* spp.). These species will be over-represented in the sample results compared with species that do not sporulate (e.g., nonsporulating basidiomycetes) or only lightly sporulate (e.g., *Stachybotrys chartarum*).

The method employed in the processing of the sample for dilution plating reflects the type of material being tested. The starting place for this type of analysis is the use of materials of known volume (cm^3), area ($inch^2$, cm^2), or weight (g). For porous and/or fleecy materials such as fiber glass or cellulose insulation and bulky material such as carpet and ceiling tiles, 1–5 cm^3 samples can be removed using aseptic technique. For paper or

vinyl surfaces, among others, 1–10 cm^2 sections can be cut using sterile scissors and then processed. Alternatively, sterile templates of known area can be superimposed over the sample surface and the circumscribed area rubbed with 0.1% peptone/0.01 Tween® 80 wetted swab or cloth, which in turn can be processed for plating. Tween®80 is a detergent that aids in spore dispersion by reducing spore surface hydrophobicity. Samples such as dust and friable material such as soil or concrete efflorescence are weighed (0.010–1. 000 g) in sterile weigh pans.

All samples for processing are then added to a known volume (5 mL to 1 L) of diluent comprising sterile distilled water + 0.1% peptone + 0.01% Tween® 80. The samples are then extracted using a rotary shaker (3.81 cm throw, 220 rpm) for 20 min[31], vortex homogenizer (highest speed for 30 sec to 1 min), or specimen (tissue) homogenizer[18] (high speed for 30 sec, alcohol and flame sterilized between each sample processed). The sample is then serially plated 1:10 (= 1+ 9) in 0.1% peptone in factors of 10 to the desired dilution, usually to a maximum of $1:10^6$.[6,31] Samples typically are inoculated onto MEA and DG-18 (other media are also used) in duplicate or triplicate, using 0.1 mL of diluent per plate. It is important to use only 0.1 mL and spread it over the medium surface, because overloading the plate with more diluent can result in yeast overgrowth.

After plating, samples are incubated as described previously for direct plating. Plates should be incubated for 5–10 days but examined after 3 days for evidence of growth. When they are read, the plates should be examined using a stereomicroscope to discriminate among the different colonies growing on plates. After colony enumeration, representative colonies should be processed for species identification directly, or subinoculated onto agar media appropriate for species identifications (for *Aspergillus* and *Penicillium spp.* see Pitt[32], Klitch[33], and Klitch and Pitt[25] for *Fusarium spp.* see Nelson et al.[26]). Additionally, the identification of certain terverticillate *Penicillium* spp. may be aided by plating isolates onto yeast extract sucrose agar and creatine sucrose agar media.[18,34] Species identifications should be made by comparing gross and fine characteristics of the sample isolates with descriptions in various identification manuals, as previously discussed.

Final results typically provide total colony number per unit measure, e.g., volume (cm^3), area (cm^2), or weight (g) for each sample for the medium used. They should also provide colony number for each species observed and the percentage representation of each species in the sample. Results from more than one dilution should not be combined. That is, results from replicates of a $1:10^3$ dilution should not be combined with replicates of a $1:10^4$ dilution. Likewise, the results from different media should not be combined. That is, results from MEA media should not be combined with those from DG-18 media.

12.2.2 Controls

For quality control, all direct and serial plates are examined for background contamination. Typically, 10% duplicates plus 10% blanks are used as laboratory controls; they should be processed for all tests.

12.2.3 Interpreting Results from Culture Analysis

Interpreting culture results is only briefly discussed in the following section and in Chapter 13. For additional information the reader is referred to the extensive treatment given to the subject in the *Field Guide for the Determination of Biological Contaminants in Environmental Samples*, 2nd edition.[6]

In normal building materials the most prevalent fungi encountered are phylloplane types. In nature, these are fungi that are associated with leaf surfaces. However, they can also be found on many other outdoor substrates including wood and soil. In buildings, commonly encountered phylloplane genera include *Alternaria, Cladosporium, Epicoccum, Oidiodendron, Stemphylium*, and *Ulocladium*, which are mycelial; and *Aureobasidium, Rhodotorula*, and *Sporobolomyces*, which are cellular (yeasts) fungi.[35] The second major group comprises soil fungi. These include members of the genera *Acremonium, Aspergillus, Emericella, Eupenicillium, Eurotium, Fusarium, Paecilomyces, Penicillium, Phialophora, Stachybotrys, Trichoderma*, and *Verticillium*. They also include the zygomycetous fungi including *Absidia, Mortierella, Mucor, Rhizopus*, and *Syncephalastrum* and nonsporulating ascomycete and basidiomycete (with clamp connections) isolates.[18,36]

Direct Plating

As indicated previously, culture results should be interpreted together with the results of nonculture analyses. Failing this, direct plate results often, but not always, can be interpreted by comparing the phylloplane fungal loads with the soil-dwelling fungal loads. A rule of thumb is "fungal growth is likely if the ratio of fungal colonies of all species compared with the sum of the total counts corresponding to phylloplane fungi is about an order of magnitude or greater."[6,31] In general, moldy materials support growth of different mesophilic and hydrophilic species than unaffected ones. Of course, there are exceptions to this guideline. One such exception can include bathroom surfaces suffering from condensation wetness problems resulting in growth of nearly pure *Cladosporium sphaerospermum* colonies. Mesophilic species include *Alternaria alternata; Aspergillus fumigatus; A. niger; A. ochraceus; Cladosporium spp.; Epicoccum nigrum; Paecilomyces variotii; Penicillium aurantiogriseum; P. brevicompactum; P. chrysogonum; P. crustosum; Scopulariopsis brevicaulis; S. candida;* and *Ulocladium chartarum*.[36] Representative hydrophilic species include *Acremonium strictum; Botrytis cinerea; Chaetomium globosum; Gliocladium roseum; Graphium spp.; Memnoniella echinata; Mucor racemosus; Phoma spp.; Rhizopus spp.; Sistotrema brinkmanii; Stachybotrys chartarum; S. chlorohalonata; Trichoderma spp.*; and *Verticillium spp.*[36–38] The recovery of filamentous indicator species that grow on building materials (see Chapter 1; e.g., *Aspergillus ochraceus, A. ustus, Acremonium strictum, Chaetomium globosum*), or toxigenic (*A. flavus; A. versicolor; Fusarium verticillioides; P. aurantiogriseum; P. brevicompactum; P. viridicatum; S. chartarum; S. chlorohalanata*; and *Trichoderma spp.*) and/or pathogenic (*A. fumigatus*) colonies from direct plate samples can provide clues that the sample material may support fungal growth. Because the result

is equivocal without non-culture analysis, further investigation for confirmation would be required.

Dilution Plating

Dust from normal building environments supports a relatively high culturable fungal spore load. Spore loads range from 10^3 to 10^5 colony forming units (CFU)/g of dust in uncarpeted areas[36] and up to 10^6 CFU/g of dust in carpets.[39] Spore loads exceeding these levels and comprising a predominance of hydrophilic, soil dwelling species (discussed previously) may indicate that the sample material supports fungal growth and requires remediation using appropriate abatement procedures.

12.2.4 Units for Reporting Culture-Based Results

The results of laboratory analyses of dilution cultures usually are expressed in mass-relevant units (e.g., colony-forming units per gram). Mass-based measures of fungal bioactivity, however, have notable shortcomings.

- Heavy spore-producing species are over-represented relative to predominantly mycelial taxa. This is particularly a concern when the goal of sampling is to establish the predominant components of the fungal flora of a specimen as a function of biomass. For example, a culture of a specimen of rotten wood may detect moderate or even high levels of mold, such as *Aspergillus* or *Trichoderma* yet fail entirely to detect a wood rot basidiomycete (e.g., Poria) that may be solely responsible for the apparent damage. Many wood rot basidiomycetes grow predominantly in mycelial forms and thus are mostly non-sporulating. By contrast, a tiny colony of mold on the surface of a sample may generate vast numbers of spores in great disproportion to its contribution to the fungal burden of the specimen, making a mass-based approach to biomass estimation misleading. Mass-based units, however, are useful in evaluating the potential for bioaerosol release from a contaminated material.
- Mass-based levels of cultured fungi (e.g., colony-forming units per gram) are greatly influenced by the proportion of inert mass contained in the specimen. Bias relating to inert mass content is particularly important in fibrous or fragmentary specimens. For example, the inclusion of a large amount of biologically noncontributory mass (e.g., the gypsum core of drywall) in a sample matrix causes an artificial reduction of the apparent microbial content of a specimen if mass-based measures are used. This effect has potentially misleading consequences. In duct dust, for example, inorganic particles often comprise a substantial portion of the mass fraction. Such particles, however, are rarely associated with appreciable biological activity. Duct cleaning measures tend to remove larger debris and fibrous matter with greater efficiency than small particles, such as fungal spores. The comparison of pre-/post-cleaning levels of culturable fungi in duct dust by mass-based units, counter-intuitively, reveals an apparent rise in fungal burden following cleaning.[40] However, this result is consistent with an overall reduction in dust mass within a duct, necessitating a much larger sampling area for the collection of a sufficient mass of dust for post-cleaning analysis. Area-based measurements of fungal burden are better suited for these applications (e.g., colony-forming units per square centimeter).
- As explained in Sussman[41], spores have characteristic half-lives, which can be of great help in understanding the results of culture-based samples.

12.3 DNA Diagnostics

12.3.1 Background

The advent of polymerase chain reaction (PCR) during the 1980s revolutionized DNA diagnostic technology, facilitating a wide range of practical applications including the detection and characterization of microbiological contaminants in the built environment. DNA-based sampling methods make use of the unique nature of nucleic acid sequences in microbial cells as a means to identify and quantify contaminant burden in air and bulk samples.

A number of DNA-based methods have been employed successfully in research applications relating to the characterization of biological populations.[42,43] However, a primary interest in the use of these techniques by the research community has been the discrimination of differences below the species level in an effort to understand ecological or evolutionary processes affecting populations of a particular species of interest. For the most part, the complexity and cost of these methods may exceed the needs and means of the industrial hygienist.

12.3.2 MSQPCR

Mold specific quantitative PCR (MSQPCR) is a DNA-based technology for detecting and quantifying molds/fungi in environmental samples.[44]

Mold Sampling and DNA Extractions for MSQPCR

Sampling. Most samples taken by industrial hygienists can be analyzed by MSQPCR. Most commonly used sample types for MSQPCR are dust samples and filter-based air samples. However, bulk samples, scrapings, sticky tape, or similar samples also can be analyzed. For example, *Cladosporium* conidia were recovered from Zefon Air-O-Cell® with acetone and then analyzed by MSQPCR.[45]

For MSQPCR, air sampling is accomplished using a polycarbonate filter with a 0.45 µm pore size in any filter holder. A sampling time of at least 6 to 8 hr is recommended, even longer if possible. MSQPCR analysis does not have any upper limitation on sampling times. Air samples collected at a rate of 3.5 L/min for 8 hr were found to be sufficient for MSQPCR analysis. This procedure has been used in hospital studies.[5,46] Disposable filter holders are sent to the laboratory, or the filters are recovered under aseptic conditions from non-disposable holders and sent in sterile containers for analysis.

Dust samples have been collected by a number of different methods that appear to produce comparable results.[47,48] The dust sample is mixed and sieved to <300 µm before analysis.

Mold DNA Recovery from Samples. A "bead beating" method has been developed for disrupting mold/fungal cells.[49] For dust samples, 5 mg is added to a screw cap tube containing small glass beads. The tubes are shaken, and mold DNA is then recovered from the supernatant.[50] Air samples are handled in the same way, except in this case the entire filter is added to the tube for extraction.[5,51]

Details concerning the theory of MSQPCR and its use for quantitative measurements of input target DNA sequences are presented elsewhere.[52,53] Reliable performance of the MSQPCR analysis requires the right instrumentation, mold specific assays, uniform analytical conditions, and the appropriate calibrator and internal reference cell suspensions.

The MSQPCR method requires the production of calibrator and reference cell suspensions. The calibrator is a standard with a known concentration of spores that must be prepared for each species. These are used to develop standard curves for use in quantification. The reference cell suspension is an exogenous internal standard against which the data is evaluated. A known concentration of a reference spore suspension is added to each sample tube before extraction. This creates both a check on the extraction and also acts as a carrier for the DNA of the other species present in the sample.

Environmental samples may contain inhibitors that affect either DNA extraction efficiency or the MSQPCR reactions themselves. In either case, the effect will show up as anomalous reference assay results. To adjust for these effects, serially diluted aliquots of DNA extracts from a sample can be analyzed. The dilution at which the inhibition disappears can be used to then calculate a result.[50]

Media and field blanks should always be taken at the time of sampling. Standard samples, consisting of DNA extracts from stock cell suspensions of a designated strain for each assay, are analyzed in each analysis and serve as either calibrators or positive controls. The sample and calibrator are amended with equal amounts of the reference mold prior to DNA extraction. As described previously, the assay results for the reference organism can be used to normalize test sample assay results for matrix-related variations in DNA extraction efficiency and/or DNA degradation in the samples during storage.

Records are maintained of both target and reference organism cycle threshold results obtained for each positive control or calibrator sample in each run. Each time a series of assays is performed on a particular sequence detector instrument, the calibrator results are compared with previous results from the same assay for evidence of deviations. If such a situation is indicated, the data are examined for evidence of reduced performance of both target mold and reference mold assays, which would suggest either an instrument problem or a problem with the components of the assay. In such instances instrument diagnostic information for the run is examined, and/or the assays are performed again with new lots of the universal components.

In each experiment, negative control samples containing all reagents but no cells also are processed and analyzed to evaluate cell or DNA contamination. If contamination is indicated, further analyses are performed to determine whether the contamination is occurring in the assay reagents or during DNA extraction. If the assay reagents are contaminated, the assays should be repeated with new lots of reagents. If it is determined that contamination has occurred during DNA extraction, new samples from the same source are extracted and analyzed, if they are available. If such samples are not available, measured cell quantities in the original test samples are adjusted by subtracting estimated target cell equivalents determined to be present in the negative control samples. Care must be taken in interpreting these results, however, because it cannot be assumed that all DNA extracts in a given experiment are equally contaminated. Parallel analyses of method negative control samples, containing extraction buffer only, and positive control conidia suspensions are recommended.

Data Interpretation

The same principles can be used to interpret the air sample data as described in Chapter 13. For dust samples, a relative moldiness index (RMI)[47,52,54] was developed to simplify the interpretation of MSQPCR data. The theory is that the higher the RMI value, the higher is the "relative moldiness" of the sampled environment.[55,56]

12.3.3 Advantages and Disadvantages

As with all available methods, using PCR detection of nucleic acids has strengths and weaknesses. Strengths include

1. potentially high sensitivity when this is useful or needed, particularly in samples containing a lot of extraneous material;
2. greater control of standardization between analysts;
3. identifications with only a moderate level of analyst training in mycology; and
4. rapid analysis turnaround.

Even so, a number of challenges exist with respect to the performance reliability of PCR-based methods in general, in addition to limitations inherent to traditional microscopic and culture-based techniques. In part, these relate to the youth of molecular biology as a scientific discipline as well as the perennial lack of basic knowledge on microbial diversity. In all cases, fungal cells that are absent of nucleic acids (e.g., a proportion of dead spores) as well as acytoplasmic spores and cellular fragments cannot be detected by PCR methods. MSQPCR analysis also limits study to just those species selected for investigation and is more expensive than more traditional methods.

12.3.4 Quantitative Uncertainties

Reliable isolation and purification of target nucleic acids is crucial to the success of PCR. The isolation of nucleic acids from fungal cells first requires the disruption of the fungal cell wall, a dense, insoluble, fibrous matrix consisting primarily of chitin in addition to glucans and other polymers that vary in composition depending on species. It remains technically unfeasible to achieve identical rates of DNA recovery among all species that are likely to be sampled.

PCR-based methods are highly sensitive to contamination. Endogenous contaminants such as melanins (pigmented

polyphenolic compounds) and polysaccharides may impair DNA recovery and thus impede PCR. Exogenous contaminants, such as environmental pollutants, may be co-extracted with DNA and inhibit PCR.[57] Furthermore, extraneous fungal contaminants introduced post sampling may generate a false positive result.

The use of nucleic acids as a proxy measure for fungal biomass presumes a predictable quantitative relationship between the two measures. Although this association seems intuitive, the predictive power of nucleic acid as an estimate of biomass is poor at best given the vast variability of nuclear characteristics known to exist among species, and in some cases, even within a single species.

12.3.5 Qualitative Uncertainties

Taxonomic Knowledge Gaps

Although the more-or-less traditional application of morphological concept of species remains roughly accurate in many common organisms, particularly animals such as mammals and insects, species delimitation is not nearly so clear in fungi. One test that is routinely applied to discern a species involves the investigation of whether two individuals can mate and produce viable offspring. The ability to do so is confirmatory that the two individuals belong to the same biological species. This test fails on a number of levels in fungi. Not all fungi are able to beget sexual offspring. Some, particularly certain mold species, appear entirely to have abandoned sexual enterprise for asexual (vegetative) propagation.

The promised taxonomic insight of new technologies such as secondary metabolite chemistry, immunology, and molecular genetics has further clouded the boundaries of species. Identifications for many stable species that, for years, had been based on traditional morphological concepts have recently been revealed to consist of multiple morphologically indistinguishable "cryptic species," many of which may possess unique biological properties, such as specific toxins, substrate utilization patterns, and stable genetic differences.

Mycologists are presently struggling with finding productive, minimally disruptive ways of dealing with cryptic taxa. Unfortunately, in many cases this may mean dividing up familiar species and adding new, unfamiliar names. In the coming decades the consequences of these decisions are likely to be profound and could well shake the foundations of fungal biology with ripple effects felt widely in many user communities.

Species Concept and Target Sequence Specificity

Traditional morphologic-based taxonomic schemes (e.g., genus and species designations) tend to lump together groups of organisms that are closely similar in appearance. The tendency of this approach is to report only one species, despite the fact that the organism observed may be a mixed entity. By contrast, DNA-based methods report only the fraction of this group that corresponds precisely to the sequence motif sought. In practical terms this means that you are able to find only the species for which you are looking. Organisms that are taxonomically close but differ in the target sequence will be missed.

Unfortunately, the knowledge of biodiversity, population structure, and genetic variation necessary to provide a basic level of technical reliability for DNA-based diagnostic methods is lacking for most fungi. On the other hand, nucleic acid methods are considerably more reliable than traditional approaches, in which only minute amounts of material, indistinct spores, or hyphal fragments are available. Careful consideration of the species concept of the target organism is crucial to ensure appropriate analytic sensitivity (e.g., primer-probe specificity) of the method to achieve the analytical goal.

Developmental Strains and Gene Databases

During the developmental process of all DNA diagnostic methods, there necessarily is heavy reliance on the use of fungal strains from culture collections and genetic databases to discover unique sequence motifs on which the designs of primers and probes may be based. Fidelity of these strains and gene sequences to the identification provided is key to the development of a robust and accurate diagnostic method. Identifications provided in culture collections and online gene databases are, for the most part, only as reliable as the depositors of these materials. Not surprisingly, the species names provided for a great number of fungal strains in culture collections and gene sequences in online databases are incorrect. Thus, technical developers of DNA diagnostic methods must ensure that the resources they use in the design of target sequences are correct. Methods developed using only one or a few characterized strains for each species are of low reliability. The use of greater numbers of strains per species in the development phase greatly improves the consistency of the method.

12.4 Mycotoxins

Mycotoxins are low molecular weight (generally 300–700 daltons) fungal metabolites that can affect human and animal health. They are members of a very large and diverse group of natural products (often referred to as secondary metabolites) produced mainly by fungi, bacteria, and plants. The known mycotoxins are quite diverse and defy a general classification, either in their health effects or chemical structures. Furthermore, they tend to be idiosyncratic in that the individual mycotoxins often are restricted to a few (or even only one) genera of fungi, and within these genera only a few species of the fungus may produce the specific class of mycotoxin. This is further complicated by the mycotoxin production behavior of the various strains (isolates) of any given fungus. For example, the aflatoxins are produced by six species of *Aspergillus*, but only *A. flavus* and *A. paraciticus* are of any significance in the context of mycotoxin hazards. For *A. flavus*, only about half of the isolates produce aflatoxins, and this percentage varies geographically.[58] Finally, it should be mentioned that some fungal species are not stable with respect to toxin production, and that handling them incorrectly in the laboratory may result in their ceasing to produce mycotoxins. An extreme example of this is *A. oryzae*, which is used to ferment soybeans into soy sauce. From a genetic

analysis it appears that *A. oryzae* is a domesticated version of *A. flavus* and possesses the necessary genetic machinery to produce aflatoxins and yet does not seem to do so in practice.[58]

12.4.1 Mycotoxins in the Indoor Environment

Most of our experience with mycotoxins has been in agriculture from which most of our knowledge about these toxins is derived. In an agricultural environment the route of exposure is nearly always through ingestion; however, when comparisons have been made, mycotoxins administered to laboratory animals via inhalation have been as much as an order of magnitude more potent than when they are administered orally.[59] There are five important agricultural mycotoxins: aflatoxins, ochratoxin A, fumonisins, deoxynivalenol (DON), and zearalenone.[60] These mycotoxins are produced by various species of *Aspergillus*, *Penicillium*, and *Fusarium*, but as noted, mycotoxin production is restricted to only a few species within these genera. Aspergillus and Penicillium species are also among the most important fungi found in moldy buildings, but the particular species associated with those buildings are different from those found on moldy food and feed, and *Fusarium* fungi are rarely encountered in indoor environments. Table 12.3 lists the most important indoor fungi and their toxins.

Few of the mycotoxins listed in Table 12.3 have been found in mold contaminated buildings[63], possibly because no one has actually attempted to determine their presence. Several of the more common indoor fungi have been grown on building material in the laboratory and shown, in many cases, to produce mycotoxins, though the levels of production are tied to the relative moisture of the material.[64] Mycotoxins also have been detected in mold-contaminated buildings, usually from bulk samples and less often from air samples, because this sampling usually provides only small amounts of material to analyze. However, the route of exposure to indoor mycotoxins is most certainly via inhalation, and thus, air sampling data are the most important.

Dermal exposure is possible but there is little evidence that this results in symptoms. An exception to this statement is dermal exposure to *Stachybotrys*, a mold that can produce potent skin irritants: the trichothecenes.

In certain occupational environments (e.g., grain elevators and compost handling facilities)[65] where airborne spore levels can be quite high, mycotoxins are more readily detected in the air samples collected on filters. The use of filter sampling allows collection of mycotoxins with high efficiency, because a large portion of airborne fungal particles are fungal fragments considerably smaller than the fungal spores[66], and these fragments have been shown to contain mycotoxins.[67] Recently, mycotoxins also have been detected in indoor air samples collected into liquid by a SpinCon® sampler and into polyurethane foams by a modified Andersen impactor.[68] Finally, the ultimate measure of human exposure to mycotoxins would be that for the mycotoxin biomarkers in those exposed. There are only a few such biomarkers for mycotoxins, for instance, aflatoxins[69], ochratoxin A[70], and DON.[71] In some cases it is the toxin (or protein-bound toxin) itself: ochratoxin A[72] and aflatoxin B1[69] in blood. In other cases, it may be a well-characterized metabolite: the glucuronide of DON in urine[71] or a DNA adduct of aflatoxin B1.[69] However, for the vast majority of mycotoxins, not only do we lack characterized biomarkers, we also have little information on the pharmacokinetics or metabolism of these compounds in animals — and even less in humans. It is important to remember that even if biomarkers are found in an individual, it might be impossible to distinguish between routes of exposure, e.g., mycotoxins which are present in foods and feeds like aflatoxins.

Table 12.3 Fungal Metabolites[a]

Fungus	*Mycotoxins*
Aspergillus sydowii	aspermutarubrol, sydowinins, sydowic acid
A. unguis	**sterigmatocystins**
A. ustus	austamide, austdiol, austins, austocystins, kotanin
A. versicolor	**sterigmatocystins**, versicolorins
Eurotium herbariorum	echinulins, neoechinulins, flavoglaucins, auroglaucins
Paecilomyces variotii	viriditoxin
Penicillium citrinum	kojic acid, citrinin, tanzawaic acids
P. aurantiogriseum sensu lato	auranthine, penicillic acid, verrucosidin, **nephrotoxins**
P. brevicompactum	mycophenolic acid, brevanamides A, B
P. chrysogenum	roquefortine C, meleagrin, chrysogine
P. commune	roquefortines, cyclopiazonic acid
P. corylophilum	citreoisocoumarinol
P. decumbens	brefeldin A
P. fellutanum	citrinin, citreoviridin
P. glabrum	trypacidin, citromycetin, asterric acid
Cladosporium cladosporioides	asperentin, cladosporic acids
Chaetomium globosum	**chaetoglobosins, chaetomin**
Memnoniella echinata	trichodermol, trichodermin, dechlorogriseofulvins, memnobotrins A and B, memnoconol, memnoconone
S. chartarum sensu lato[b]	**macrocyclic trichothecenes**, atranones, trichodermol, trichodermin, dolabellanes, stachybotrylactones and lactams, stachybotrydials
Trichoderma harzianum	**alamethicins**, emodin, suzukacillin, trichodermin
Wallemia sebi	walleminols A and B

Source: [a]Adapted from References 37 (mycotoxins found in food) and 61 (mycotoxins that could be produced by mold occurring in indoor environments). Metabolites in boldface are of higher potency.
[b]See Reference 62.

12.4.2 Chemical Analyses for Mycotoxins

One never submits environmental samples for mycotoxin analysis without a clear picture of the specific fungi present, including genera and species. The reason for this is that knowledge of the fungal pattern gives the investigator insight as to which

mycotoxins may be present and thus, for which mycotoxins to test. Mycotoxin analyses are expensive, and large samples are needed for mycotoxin analyses. These samples may be derived from air sampling, settled dust, or scrapings from building materials that have obvious mold growth. Analyses of the latter most consistently give positive results for the presence of mycotoxins. Settled dust and air samples invariably contain a multitude of organic compounds unassociated with molds, which interfere with the analysis for any mycotoxins that may be present.

The bulk samples collected should be on the order of grams for settled dust and tens to hundreds of milligrams for scrapings off the mold-contaminated areas. Air sampling for mycotoxins is more problematic, because one would rarely obtain quantities of this sort with normal air sampling procedures. Thus, the air sampling (for example, sample collection on Teflon® filters) should be conducted for long periods of time, much longer than those normally employed for collecting biological data. This may present practical problems for the investigator.

Samples should be air-dried if necessary and stored in glass containers to avoid contamination by plasticizers found in some plastic containers. Problems with plasticizers can be minimized by using hard plastics such as those used in filter cassettes.

Only a few commercial laboratories offer mycotoxin analyses, and even in these cases, there are only a limited number of mycotoxins for which they will provide analyses. Laboratories should provide a list of compounds for which they can provide analyses, indicating the limits and the range of errors in their analyses and providing their laboratory standard operating procedures (SOPs). The companies should have on hand the mycotoxin standards and be able to provide a description of their good laboratory practices.

Although it is beyond the scope of this book to give details of the analytical procedures, it is useful for the practitioner to understand the overall process. The sample is extracted with an aqueous organic solvent, most commonly methanol or acetonitrile. Although the water in the extraction solvent may prevent complete extraction of the mycotoxins, it is desirable to have 10–20% water in the solvent to minimize the interfering contaminants that are always present. The extract is concentrated to dryness, taken up in an organic solvent, and passed through a "cleanup column." This is the first of two chromatographic methods designed to first separate the majority of the contaminants from the mycotoxin analytes and then in a second chromatographic step, to separate and analyze for the mycotoxins themselves. This second step is a high-resolution process designed not only to separate the mycotoxins from each other but also (ideally) to completely separate the mycotoxins from the interfering compounds.

The two high-resolution chromatographies used in practice are thin-layer chromatography (TLC) and high-performance liquid chromatograph (HPLC). TLC was used commonly from the 1960s through the middle of the 1980s, and many standard TLC protocols were developed for the mycotoxins found in food materials. However, HPLC has supplanted TLC as the preferred technique because of its tremendous resolving power and sensitivity. This is particularly important for analysis of indoor mycotoxins, because, if present, they are found in low levels. Modern HPLC analyses typically are coupled techniques[73,74], that is, the peaks separated by HPLC are subjected to both ultraviolet (UV) spectral analysis (best performed with a photo diode array (PDA) detector that provides UV spectral data that can be compared with those of the standard mycotoxins) and mass spectrometry (MS) analysis. The latter provides critical proof of structure for the mycotoxin(s), because individual mycotoxins have unique MS patterns.

Of course, this is the ideal case. In practice there can be complications. The HPLC may not always provide clean separation, and both the PDA and MS analyses for HPLC peaks made up of two or more components may give results difficult to interpret. Varying the conditions for the HPLC analysis (different column and/or different solvent systems) will often solve the problem. A solution to this problem could be to employ HPLC-PDA-MS-MS. The use of tandem MS (MS-MS) allows the investigator to subject an MS peak, believed to be to the result of a specific mycotoxin, to further MS analysis. Performing this additional MS analysis on one or more mass peaks in the initial mass analysis usually guarantees positive identification of the mycotoxin.[73]

Not all laboratories offer mycotoxin analytical services on which you can rely. Practitioners are urged to consult the many publications of AIHA, including Chapter 10 in this one, to assess the qualifications of those offering their services.

12.5 Microbial Volatile Organic Compounds

Fungi have been shown to release a variety of volatile organic compounds during active growth[75–79] as a direct result of their metabolic processes. Microbial volatile organic compound (MVOC) sampling is described in Chapter 11. The present chapter focuses on the analytical methods for the analysis of MVOCs.

12.5.1 MVOC Sample Analysis

Many methodologies may be used to collect and analyze MVOCs from indoor environments. The most practical approach involves the collection of volatile species on a sorbent media, followed by high temperature (thermal desorption) or liquid (solvent desorption) analyte removal from the collection medium. The investigator must determine the most appropriate MVOC collection and analysis protocol to follow based on laboratory instrumentation availability, technician skill, and the availability of sorbent collection media. To date, there are no "standards of practice" for MVOC collection and analysis, yet there is agreement that gas chromatography followed by mass spectrometric detection provides the most information on the presence of MVOCs in sampled indoor environments.

Thermal Desorption/Gas Chromatography/MS

An internal standard (toluene-d8 is suggested) is injected onto each tube before analysis via thermal desorption/gas chromatography (GC)/MS analysis, according to the analytical laboratory's SOPs. The tubes are sealed according to the instructions provided by the

manufacturer of the particular thermal desorption instrument, which is connected to a gas chromatograph equipped with a mass spectrometer detector and a capillary column. A chromatographic separation must be programmed to ascertain that closely related compounds (e.g., 3-methyl-1-butanol and 3-methyl-2-butanol) do not co-elute (a polar column such as WAX is recommended for MVOC analysis). The analytical laboratory may create a five-point working calibration curve for MVOCs[79–81] using authentic standards (Table 12.4). Moreover, instrumental limits of detection must be determined by the analytical laboratory prior to sample analysis. Mass spectral analysis may be performed in the total ion chromatogram (TIC) acquisition mode or in the selected ion monitoring (SIM) mode for more specific analysis. SIM analysis may be used to uniquely identify MVOCs of interest. The analytical laboratory must ensure the analytical system's performance according to their SOPs prior to analysis of any samples.

Table 12.4 Compounds to be used for GC/MS Calibration

1-Butanol	3-Isopropyl-2-methoxypyrazine
1-Octen-3-ol	Limonene
2-Ethyl-1-hexanol	3-Methyl-1-butanol
2-Ethylhexanal	3-Methyl-2-butanol
2-Heptanone	3-Octanol
2-Hexanone	3-Octanone
Hexanal	2-Pentanol

Note: This list is not meant to be exhaustive; additional compounds may be included if found in many samples.

Solvent Desorption/GC/MS

Once samples are submitted to the laboratory for analysis, the collection tubes are taken out of the refrigerator and allowed to come to room temperature before being desorbed for analysis. Solvent desorption should be performed in accordance with manufacturer's guidelines or the analytical laboratory's SOP. Samples must be analyzed within a week of preparation.

Prior to analysis a suitable internal standard (toluene d8 is recommended) is introduced into each sample vial and mixed well. Analysis may be performed on a well-maintained gas chromatograph equipped with a mass spectrometer detector and a capillary column (a polar column such as WAX is recommended for MVOC analysis). Mass spectral analysis may be performed in the TIC acquisition mode or in the SIM mode for more specific analysis. SIM analysis may be used to uniquely identify MVOCs of interest. The GC/MS run conditions described for thermal desorption analysis may be investigated for solvent desorption sample analysis, yet the laboratory needs to ascertain that the elution of the solvent does not interfere with the elution of the analytes of interest. Chromatographic conditions should be adjusted as necessary to assure proper analytical separation of the MVOCs.

12.5.2 Data Analysis

After both of the previously described analytical methods, semiquantitation of additional identified compounds may be performed based on the mass spectrometric response of the internal standard. The semiquantitation may be done by means of response factor calculations:

$$C_{Unknown}\ (\mu g/m^3) = [(Area_{Unknown}/Area_{I.S.})^* \text{ I.S. ng}]/X_L$$

where Area represents chromatographic area counts, I.S. ng indicates the injected amount of internal standard onto the column (in nanograms), and X_L is the total sampled air volume (in liters). The precision of the analytical system should be monitored by calculating the RSD (relative standard deviation) of the internal standard's chromatographic response; RSD values less than 15% may be deemed acceptable. When RSD values exceed the 15% threshold value, the analytical system should be thoroughly checked for leaks and contamination, followed by recalibration of the system.

Mass spectral interpretation should be based on a match to an authentic standard or best match in conjunction with manual interpretation with reference to the NIST and WILEY mass spectral libraries when available.

12.5.3 Data Interpretation

Although the presence of MVOCs in indoor environments suggests that microbial growth is present, it does not unambiguously indicate the existence of growth indoors. The data obtained from MVOC analysis should be compared with data obtained from culture-based bioaerosol sampling, if such sampling was performed, to get a clearer picture of the microbial ecology of the location where MVOC sampling was conducted. This is because some compounds reported to be of microbiological origin may be found in common indoor products. For example, 2-ethyl-1-hexanol is an eight-carbon compound that may be emitted by fungi during their normal growth cycle. However, it is recognized also as an indoor air pollutant emitted from commercial furniture or carpets with a polyvinyl chloride backing.[82] Thus, the presence of 2-ethyl-1-hexanol during MVOC sample collection may not be a direct result of fungal growth in indoor environments.

Moreover, many MVOCs contain carbonyl and aldehyde groups in their chemical makeup, and thus may undergo chemical reactions with other indoor chemical species (e.g., ozone), resulting in different compounds being produced in the indoor setting of interest. Consequently, the absence of some of the indicative MVOCs presented in Table 11.5 does not explicitly suggest the absence of microbial growth indoors. The investigator is encouraged to examine the presence of other volatile organic compounds present in the indoor environment being investigated (and moisture levels) to achieve a more complete picture of the indoor environment's chemistry and the potential for microbiological growth indoors.

12.6 (1→3)-β-D-glucan

Molds are a mixture of different fungal species. Their cell walls are composed of a polysaccharide, (1→3)-β-D-glucan. This substance is present in many different forms depending on the

species of fungus, but a common denominator that also determines the biological activity is the 1-3 binding between the saccharide units. Determination of (1→3)-β-D-glucan gives a quantitative measure on fungal cell biomass (living and dead cells). (1→3)-β-D-glucan is also present in pollen and some bacteria; hence, the values obtained in a given environment are not always specific for fungi. Most of the (1→3)-β-D-glucan in nature is insoluble in water, but there is also a water soluble portion, comprising about 10% of the total.

(1→3)-β-D-glucan is a polyglucose polymer with a main chain and side chain molecules, tied together in a (1→3) -β linkage.[83] These steric characteristics have been reported to determine a variety of biological activities. Other linkage forms such as (1→4) -β or (1→6) -α appear not to have biological activity. After inhalation, (1→3)-β-D-glucan is taken up by macrophages in the airways and broken down slowly over periods of several weeks.

An effect of (1→3)-β-D-glucan is an interference with the immunological defense system of the body.[84] Available information suggests that an altered reaction to agents causing inflammation, an increased reactivity to antigens, and an accumulation of white blood cells in the tissue (granulomas) are important cellular reactions caused by (1→3)-β-D-glucan.

12.6.1 Analysis of (1→3)-β-D-glucan

For environmental measures (1→3)-β-D-glucan can be analyzed in samples of sedimented (floor) dust or airborne dust collected on filters. In clinical applications it can also be analyzed in body fluids such as blood or lung lavage. Environmental samples should be stored in a dry place without sunlight, or in a refrigerator, and can be kept for several weeks or even months. The sample should be extracted with 0.5 M NaOH or heat-treated at 120°C to render the (1→3)-β-D-glucan water-soluble. Without this procedure only about 10% or less of the (1→3)-β-D-glucan present will be detected.

Because fungi are ubiquitous and the method is very sensitive, care must be taken to work with clean utensils. Glucan-free pipettes and plates for samples must be used. Laboratory glassware can be made (1→3)-β-D-glucan free by heating to 270°C for 6 hr or 350°C for 1 hr. Preparations and dilutions are performed in a filtered air box, and care is taken not to stir up dust from surfaces.

There are currently two analytical methods in use for environmental samples, based on different biological principles. The first method uses a derivative of the *Limulus* amoebocyte lysate preparation.[6]

To determine (1→3)-β-D-glucan, a factor C depleted preparation of the amoebocyte lysate is used together with a chromogenic substrate that changes its color as a marker of enzyme activation.[85] The intensity of this color or its development over time are read in a spectrophotometer against a standard (1→3)-β-D-glucan. The plate reader software computes a linear curve for the standard against which the concentration of (1→3)-β-D-glucan in the sample can be calculated.[6]

The sensitivity of the method is very high. Picogram levels can be detected. The sensitivity in a given sample depends on the volume of liquid used for elution of the filter or the sampled material. For air sampling the amount of air drawn through the filter determinates the sensitivity.

Another method to measure (1→3)-β-D-glucan uses antibodies formed by rabbits, which are injected with a (1→3)-β-D-glucan.[86] Blood is drawn from the rabbits, and the antibodies in the serum are purified. An inhibition ELISA (enzyme-linked immunosorbent assay) test presents these antibodies to the sample; antibody-antigen complexes are thus formed and detected. The results are read against a standard (1→3)-β-D-glucan. The test has a considerably higher detection threshold than the *Limulus* assay, and therefore, larger samples such as dust from the floor are required. The antibody test is not commercially available at present.

12.6.2 Interpretation of Results

The *Limulus* test is very sensitive and as such has a large variability. The end value of the enzyme color reaction in one of the commercial standard kits is compared to a specific (1→3)-β-D-glucan (usually pachyman or curdlan). Values for (1→3)-β-D-glucan obtained from different kits, using different standards or lots of standards and certainly from different laboratories, should thus be looked on as operational rather than definitive.

Based on experience from analysis of endotoxin using the *Limulus* method, the variation within a laboratory can be expected to be relatively small, but between laboratories it can be large. Thus, it is necessary for the laboratory to have access to a database on measurements both in contaminated and uncontaminated environments.

There is at present only limited information on (1→3)-β-D-glucan levels in the environment. Among workers collecting household waste, airborne levels ranged from 10.8–36.4 ng/m^3.[87] Indoors in houses with mold problems, airborne levels up to 51.7 ng/m^3 were found[88], and in floor dust values ranged from 7.3 to 83.4 ng/mg. There are at present no guidelines for (1→3)-β-D-glucan, but effects on the immune system and on circulating white blood cells have been seen at levels around 20 ng/m^3.[84,88]

12.7 Immunochemical Methods

A number of different immunochemical assays are available for evaluation of the quantity of allergens, including fungal allergens, in environmental samples. The specificity of these methods allows for the detection of specific agents of disease (e.g., allergens) rather than measuring particles that may carry various amounts of the agent. The analysis is technically straightforward and can be used to test large numbers of samples; hence, these techniques are valuable in research studies that try to elucidate the relationships between health effects and exposure to particular allergens. Although the use of immunoassays in building investigations has been limited, a few commercial laboratories are now offering a small number of fungal allergen assays.

Immunoassays, including ELISA, take advantage of the remarkable specificity of antibodies for the protein (or part of a protein) for which they have been produced. Antigens

(immune-stimulating proteins) that are known to be associated with allergy in humans can be used to generate antibodies in laboratory animals. These antibodies can be harvested for use in the assay. When an allergen is used to immunize laboratory animals, they produce an array of antibodies specific to different components of the allergen (epitopes). This mixture of antibodies, generated by multiple B cells of the animal, is called polyclonal antibody. Alternatively, antibodies generated by a single B cell and specific for a single epitope can be produced by the fusion of the B cell with an immortal cell line to create a hybridoma. This clone can then produce large amounts of consistently homogeneous monoclonal antibody. Use of either antibody type allows for the quantification of individual allergenic proteins in environmental samples.

Settled dust samples or high-volume air samples in filter cassettes can be used for immunoassays. For settled dust samples collected by vacuum, care must be taken to sample each site or building in a similar and standardized way to compare results (see Chapter 11).

Because quantities of dust in air are typically much lower than that which settles to the floor, large volumes of air must be sampled to obtain an assayable quantity. Hard floors are also remarkably free of accumulated dust, and it is often difficult to obtain assayable quantities with the same standard sampling procedure as that for carpeted areas.

Dust collected by vacuum sampling is sieved through a course sieve (0.250–0.500 mm) to retrieve the fine dust fraction (although it is also possible to simply remove large chunks of debris). The fine dust fraction from air or dust is weighed so that final results can be expressed in units per gram of dust. Approximately 50–100 mg of dust is required for the analysis. Proteins associated with dust are extracted by suspending 50 mg of fine dust in 1 mL of borate buffered saline extraction fluid. The suspension is agitated for 2 hr at room temperature, and dust particles are then removed by centrifugation. The supernatant can be analyzed immediately or kept frozen at −20°C.

Two types of ELISA are used for the detection of allergens in environmental samples: inhibition and sandwich. The two types of assays differ on the type of antibody used for detection.

Inhibition (or competition) assays, coat ("bind") standard allergen to the bottom of microtiter plates. Unbound allergen is then washed away. A dilution series of the environmental sample is combined with antibody specific for the bound antigen and applied in a duplicate series of wells in the microtiter plate. For the allergen to be compared with a concentration of known allergen, the allergen standard is also serially diluted, mixed with antibody, and pipetted into allergen-coated wells. Wells also are used for negative controls. In this assay the higher the ratio of antibody to allergen in the sample, the more antibody is bound to the allergen on the plate. Excess allergen and antibody are then washed from the plate. Detection of bound antibody occurs through an enzyme-conjugated second antibody that is specific for the first antibody. Again, excess antibody is washed from the plate. Measurement of allergen and antibody complexes occurs by application of enzyme substrate, which elicits a color change in proportion to the presence of bound antibody. The amount of color is inversely proportional to the amount of allergen in the sample. The color change is quantified using a spectrophotometer, and concentrations of allergen in the environmental sample are determined by comparison with the allergen standard curve.

The sandwich assay is used with monoclonal antibodies. In this assay a monoclonal antibody is first bound to the microtiter plate. Unbound excess antibody is washed away. A dilution series of environmental samples is applied along with allergen standards and controls. Once allergen and antibody have had a chance to combine, the excess is washed from the plate. Bound allergen is detected through the use of a second monoclonal antibody that is specific for a different epitope of the allergen. Alternatively, a polyclonal antibody is used. Again, excess antibody is washed from the plate. The second antibody is enzyme-linked, and quantification of allergen and double antibody sandwich complexes occurs by application of the substrate followed by measurement of colorimetric changes with a spectrophotometer and comparison with allergen standards. Unlike the inhibition assay, the amount of color change is directly proportional to the amount of allergen in the sample.

Because of the high specificity, immunoassays typically also have excellent sensitivity and are capable of detecting even very low levels of allergen (in the range of tens of nanograms per gram of dust). However, notable differences exist for the lower limit of detection between laboratories as a result of differences in processing methods.

Currently there are only a few commercially available fungal immunoassays. These use monoclonal antibodies to the major allergens of either *Alternaria alternata* (Alt a 1) or *Aspergillus fumigatus* (Asp f 1). A few research laboratories also have developed polyclonal assays to *Alternaria* and *Cladosporium*, but these are not readily available. In addition, a number of immunoassays have been developed for detection of other allergens important in the indoor environment.[6] These are summarized in Table 12.5.

Table 12.5 Summary of Allergens for which ELISA Assays are Commonly Performed

Source	*Species*	*Allergen*	*Antibody Type*[a]
Fungi	Alternaria alternata	Alt a 1	mAb, pAb
	Aspergillus fumigatus	Asp f 1	mAb
Dog	Canus familiaris	Can f 1	mAb
Cat	Felis domesticus	Fel d 1	mAb
Mouse	Mus musculus	Mus m 1	pAb
Mite	Dermatophagoides farinae	Der f 1	mAb
	Dermatophagoides pteronyssimus	Der p 1	mAb
Cockroach	Blatella germanica	Bla g 1	mAb
		Bla g 2	mAb

[a]mAb=monoclonal antibody; pAb=polyclonal antibody.

12.7.1 Advantages and Limitations

Immunoassays provide sensitive methods to detect very specific allergens. However, because of their specificity these assays provide no information on total allergen level. For example, the Alt

a 1 assay is not expected to provide information on the other putative *Alternaria* allergens, and it would be unwise to assume that they vary concomitantly. Nor is any information provided on the plethora of other fungi that are inevitably present in any environmental sample. The limited number of fungal assays restricts our ability to assess more generalized fungal levels with these methods.

A distinct advantage of using monoclonal antibodies in immunoassays is the ability to quantify allergen levels against World Health Organization standard allergen extracts. However, as a result of the involved process required to produce them, monoclonal antibodies are much more expensive. On the other hand, polyclonal antibodies have the advantage that they are more tolerant to changes in the allergen, such as denaturation, that could occur over time in dust accumulations. These assays may provide a better assessment of allergen level.

Immunoassays, although specific and sensitive, do exhibit an intra-assay variability of 3–8%.[31] When variations in replicate sites, extraction procedure, intra-assay, and linearity with the standard curve are also taken into account, the variability can rise to as much as 20% for some allergens.[31] In addition, carpet sprays and powders can inhibit the reactions, add to the fine dust weight, and generate spurious results. Assay results are generally considered to be site-specific (i.e., living room) and should not be generalized to other areas of the home or given as a general home measure, although some correlations have been demonstrated.

Settled dust cannot be related to airborne exposure by any constant ratio. Allergen levels derived from settled dust have this same limitation. Therefore, it is currently not possible to associate absolute levels of recovered fungal allergen with onset of disease or symptoms. Nevertheless, these techniques do provide a useful method of associating allergen levels with particular environmental conditions (e.g., measures of dampness, housing characteristics) to highlight conditions that might indicate a potential risk of exposure.

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