Applied Occupational and Environmental Hygiene
Volume 18(7): 1–5, 2003
1047-322X/03 $12.00 + .00
DOI: 10.1080/10473220390192845

Analytical Instrument Performance Criteria

Application of a Fluorometric Method for the Detection of Mold in Indoor Environments

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Mold growth on building materials poses a risk to the health of building occupants. Available guidance from public health and occupational health agencies recognize the need for immediate remediation of mold-affected building materials when the potential for occupant exposure exists, regardless of the species present.[1−3] However, specific guidance on appropriate methods to detect sources of fungal growth have not been provided. Ordinarily, deference has been made to experienced consultants using professional judgment.[2,3] With the absence of validated bioaerosol sampling methods capable of *consistently* detecting fungal growth in buildings, and the recognition that most methods underestimate actual concentrations in the room air as well as the occupant exposures, investigators often find interpretation of bioaerosol sampling results difficult.[1,4] An air sample may be collected to detect and estimate bioaerosol concentrations or to estimate bioaerosol exposures associated with disturbance of a suspected source, but is not very useful in detecting the presence or absence of fungal growth in a building.[1]

Due to the multiple factors involved in bioaerosol release, transport, sample collection, and analysis, the ability to detect sources of fungal growth indoors is often questionable. Nevertheless, many consultants continue to rely primarily on air samples for diagnosis of building environments and clearance of post-remediation sites. A glaring problem with the use of air samples for post-remediation clearance testing is that low bioaerosol concentrations cannot be used to demonstrate the absence of fungal growth sources on building surfaces. Moreover, a positive sample (i.e., one with a high spore count and/or predominance of one mold species) *m*ight indicate a mold problem.

However, if mold damage is present, it still must be located by visual inspection and surface sampling.[1] Many investigators and researchers have found that, even with massive amounts (>100 square meters) of visible mold growth in buildings or heating, ventilation, and air-conditioning (HVAC) systems, air sampling of fungal spores may not reveal the presence of the source.[5] The great variability in fungal spore air concentrations combined with the episodic nature of spore release makes air sampling for fungal spores susceptible to false negative results.[1,4] To test the efficacy of mold remediation (i.e., removal of mold from building surfaces), the best, most reliable, and most highly predictive sample may be a surface sample.

Background

There are two basic approaches currently used for measuring mold on building surfaces. One approach is based on cultivation of viable mold present on a surface, and the second approach focuses on determining the biomass of mold present on surfaces.

Cultivation of mold samples obtained from building materials is widely used but is not well suited for quantification of fungi. Cultivation methods were originally developed for quantification of bacteria, which are unicellular organisms. Fungi, however, are multicellular organisms consisting of 95 to 97 percent multicellular coherent growth structures (mycelia), and 3 to 5 percent propagules for dispersion (i.e., spores). When cultivation methods are used for quantification of fungi, each fungal spore gives rise to one colony count, whereas the multicellular hyphal network is dramatically underestimated. Thus, cultivation methods for quantification of fungi have been shown to be mainly a measure of sporulation.[6] Measurement of glucan or ergosterol has traditionally been used to estimate the total fungal biomass on a surface.[1]

Microscopic examination of adhesive tape samples can be used to differentiate between in-situ microbial growth from accumulated spores and fungal debris. The presence of predominately one type of microorganism may indicate growth. In addition, the presence of hyphae and spore-bearing fungal structures, rather than a mixture of fragmented hyphae and spores, may indicate the presence of fungal growth on the sampled material. However, measuring fungal growth using microscopy is semi-quantitative at best, owing to obscuring of fungal elements by extraneous debris.[1]

Due to the necessity of laboratory analysis, long culture times, and the need for a trained microscopist to analyze samples, most traditional mycological methods have proven unsuitable for field analysis. In the course of a building investigation, to locate, identify, and delineate fungal growth sources, a field analysis method would save time and enable building managers to make informed risk management decisions. After development and implementation of remediation measures, a field analysis method would allow the space to be returned to use soon after completion of cleaning. If post-remediation sample results do not

meet clearance criteria, then fast analysis would allow re-cleaning of failed areas with minimal cost due to remobilization of crews and equipment.

Research conducted in the framework of the European Union (EU) Environment and Climate (EEC) Program (project no. EV5V-CT940-034) led to the development of a fluorometric detection method for fungal enzymes.[7] In these studies, a reproducible correlation was found between a certain enzymatic activity (â-N-acetylhexosaminidase) and the amount of fungal biomass in a complex environmental sample (soil). Further research by the manufacturer (MycoTec, Copenhagen, Denmark) found a strong correlation between the activity of the enzyme â-N-acetylhexosaminidase and the fungal biomass ($R^2 = 0.935$; $p < 0.001$). The methodology was further developed for use in building surveys under the framework of the Danish research program, Mold in Buildings; the work was supported by the Danish Government and private companies. The resulting technology and the descriptions of the contamination categories were published in the proceedings of the international conference Healthy Buildings 2000.[8]

Building investigations attempting to ascertain the presence and extent of fungal growth sources usually require a test method capable of differentiating fungal growth from visibly stained materials.[1] Surfaces not supporting fungal growth can often resemble those having fungal growth, and some wet building materials do not support fungal growth because of high pH or the absence of adequate nutrients. Dependence on visual assessments alone or the presence of water damage as the determinant of fungal-damaged materials can result in misclassification of surface conditions in both directions.

When water, adequate nutrients, and tolerable temperature and pH are present, germination of ubiquitous mold spores can occur in a few hours.[1] If conditions remain favorable for mold growth for a few days, fungal biomass density can increase by a million-fold. It is this explosive increase in fungal biomass that differentiates surfaces supporting fungal growth from those with just-deposited spores.[8] Culture-based detection methods can often be overwhelmed by high spore concentrations (frequently found in house dust), causing investigators to misclassify a surface as one that is supporting fungal growth.

By using enzyme activity as a surrogate for fungal biomass, a fluorometric detection method can be used to determine whether a building material is supporting mold growth. Also, the extent of mold-affected material(s) and the efficacy of cleaning after remediation efforts can be monitored. This article describes the fluorometric detection method currently employed in the MycoMeter-test, and its application in building investigations and post-remediation inspections. The MycoMeter-test method is a fast, sensitive method for determining the presence of mold growth. This fluorometric test is also useful for assessment of the efficacy of mold contamination removal.

Supplies and Instrumentation

Equipment

The MycoMeter-test method (MycoTec, Copenhagen, Denmark) utilizes a field-portable, hand-held fluorometer (18.4 cm [L] × 8.9 cm [W] × 4.4 cm [H], ~500 g; *Pico*fluor Hand-Held Dual Channel Fluorometer; Turner Designs, Sunnyvale, CA). The fluorometer ultraviolet (UV) excitation and emission wavelengths have been optimized for the fungal detection method. The accessory equipment, including automatic pipet, thermometer, timer, and sample tube racks, is carried in a customized field carrying case (see Figure 1). Chemical standards are used to calibrate the fluorometer, and a procedure is described in the instrument handbook to measure blank values for each sample, thereby providing a simple technique for background correction.

Reagents, Sample Preparation, and Analysis

The method involves collecting a swab sample from a 9-square-centimeter (cm^2) area with the aid of a supplied self-adhesive template. Before collecting the sample, a sterile swab is wetted with a supplied wetting agent containing biostatin (this is added to prevent growth of fungi on the swab for at least 7 days). Manufacturer studies have also determined that a sample can be stored for up to seven days without significant loss of enzyme activity (if present). The treated swab is used to collect a surface sample, the area of which is defined by the template.

Up to 20 samples can be simultaneously analyzed, starting with a 30-minute (approximate) extraction in a synthetic enzyme substrate (4-methylumbelliferyl-labeled enzyme substrate). Due to the effect of temperature on enzyme activity, the precise extraction time is adjusted based on the measured temperature. After the required extraction time, 100 microliters (μL) of the sample extract is transferred to a developer solution in a 10-mm by 10-mm plastic cuvette. Each sample cuvette is then placed in the fluorometer and read for approximately five seconds.

Comparison Method

Although no "gold standard" test method exists for the determination of surface fungal growth, microscopic analysis of adhesive tape on glass slides can provide a reasonable surrogate as an indicator of growth conditions on a surface. In order to assess the validity of the fluorometric test method as a screening tool to detect the growth of mold on building surfaces, paired samples were collected for microscopic analysis. Clear adhesive tape (3M Company, St. Paul, MN) was applied to the surface directly adjacent to the swabbed area. The tape was then slowly removed and placed onto a standard glass slide. Paired adhesive tape samples were sent to one of three different laboratories for microscopic analysis using light microscopy after staining to improve visibility of fungal elements.

Method Performance

During development of the MycoMeter-test, filamentous fungi were

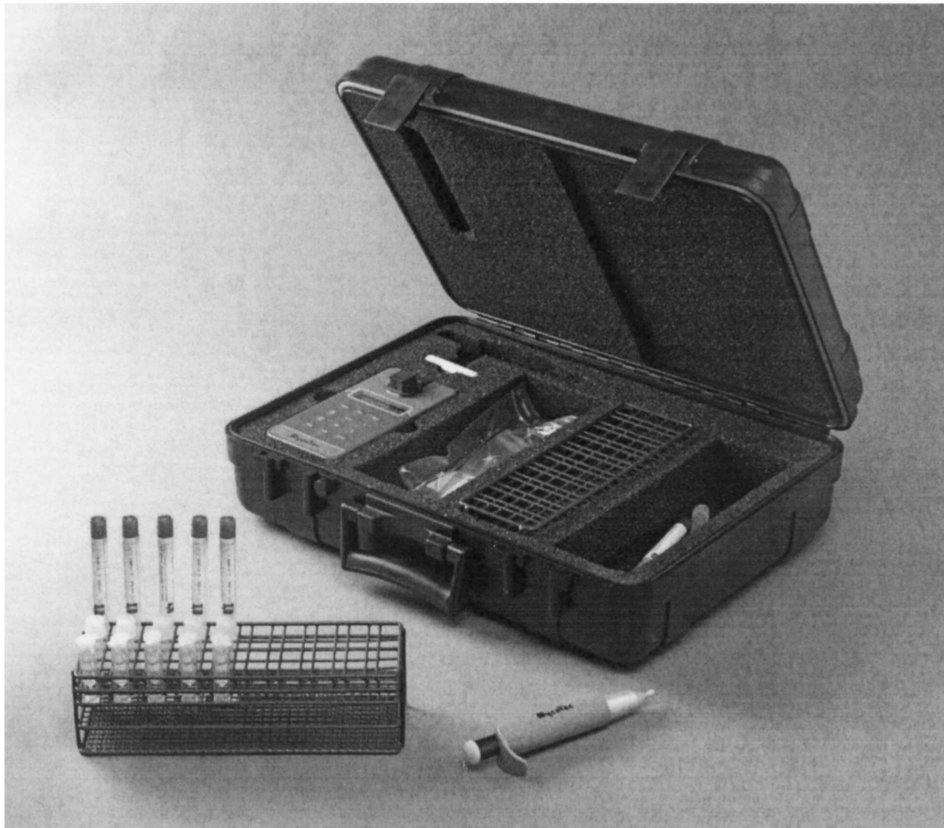

FIGURE 1
*Pico*fluor Hand-Held Dual Channel Fluorometer with accessory equipment and carrying case.

screened for â-N-acetylhexosaminidase activity. All 43 species of filamentous fungi tested to date have been found to exhibit this enzyme activity. The fluorometric analysis does not distinguish the genera or species of fungi, but it does provide an excellent screening test for the growth of mold on building surfaces. It is acknowledged that using microscopic analysis of tape-lift samples as a "confirmation" of mold growth may introduce error due to subjective interpretations by the microscopist.

The analysis of data from 17 field investigations (April 2001 through October 2002) is presented in Table I. It can be seen from these data that the MycoMeter-test method has high sensitivity, specificity, and positive predictive value in the detection of fungal growth. Such a screening test can provide a useful tool for building investigations involving mold growth and indoor air quality.

The ideal comparison test method currently available for fungal biomass on a surface is one for ergosterol.(1) Results of in vitro studies to compare detected ergosterol results with measured enzyme activity have been submitted for publication elsewhere, and are currently under review. Although the supporting data cannot be presented here, the correlation between ergosterol content and enzyme activity was found to be strong ($R^2 = 0.9684$; $p < 0.001$).

Interpretation of Monitoring Results

The fluorometric response to fungal enzyme activity is linear. Experimental data from several studies have found the standard deviation of the MycoMeter-test to be ±3 fluorescent counts (FC), and the lower limit of detection is 10 FC.(9)

Empirically derived categories have been established for surfaces that reflect three different physical conditions. The first condition is a visibly clean surface in a well-maintained building without history of mold problems, or newly installed building materials. Sample results from surfaces meeting these criteria were ≤25 FC. Sample results equal to or lower than 25 FC meet Category A criteria and are considered at or below acceptable levels of fungal biomass, but not free of fungal spores. A comparison study with adhesive tape-lift samples analyzed by microscopy demonstrated a correlation with samples having <100 spores or fungal elements per cm^2.(10)

Category A (≤25 FC) indicates the sample contains a mass of mold not above typical background levels on visibly clean surfaces without fungal growth.

TABLE I
Sensitivity and specificity of fluorometric screening test for mold growth

Screening test for mold growth	Confirmation of mold growth (by microscopy): Mold growth confirmed	Mold growth *not* confirmed	Row total
Fluorometric test results			
Positive (MycoMeter values > 450 FC[A])	24 (a)	0 (b)	**24**(a+b)
Negative (MycoMeter values ≤ 450 FC)	3 (c)	55 (d)	**58**(c+d)
Column total	**27**(a+c)	**55**(b+d)	**82** total samples
Screening test statistics	Ratio	Percentage	
Sensitivity $= \frac{a}{a+c}$	$= \frac{24}{27}$	=88.9%	
Specificity $= \frac{d}{b+d}$	$= \frac{55}{55}$	= 100%	
Positive predictive value $= \frac{a}{a+b}$	$= \frac{24}{24}$	=100%	
Negative predictive value $= \frac{d}{c+d}$	$= \frac{55}{58}$	=94.8%	

[A]FC = fluorescent counts.

The second physical condition is a visibly dusty indoor surface. Sampling of 127 dusty surfaces found that 96 percent of the sample results were below 450 FC. Although Category B surfaces have an elevated level of mold present, they have a significant probability of not supporting active fungal growth. It is still a judgment call if these surfaces require remediation, but simple dust removal procedures such as vacuuming or wiping will likely reduce fungal biomass levels to Category A.

Category B ($25 < X \leq 450$) *indicates the sample contains a mass of mold above typical background levels. This may be due to accumulated spores in dust deposits, or may indicate old desiccated mold damage that is no longer actively growing.*

The third physical condition is a mold-damaged material with surface growth. When mold growth was induced in the laboratory or found to be present on building surfaces, sample results were above 450 FC.[9] When macroscopic mold colonies are visible on a surface sample, results typically range well above 1000 FC, but growth can easily be detected by the MycoMeter-test method long before it is visibly evident. Category C results are often found one to three meters beyond visible mold growth on water-damaged drywall, and correspond well with water-damaged areas.

Category C (>450 FC) indicates the sample contains a mass of mold high above typical background levels due to growth on the surface.

These empirically derived categories illustrate how sample results can be interpreted. A sensitive test method capable of detecting mold growth (even amounts that are otherwise undetectable by visual inspection) is now available for the inspection of mold damage in buildings (refer to Table I). The *positive predictive value* (the probability that a surface has growth) of the screening method, given a positive sample result, is estimated to be 100 percent. The *negative predictive value* (the probability of a surface not having fungal growth) of the screening method, given a negative sample result, is estimated at about 95 percent. The *sensitivity* (the probability of a positive screening test result if there is actually fungal growth) of the method is estimated at near 90 percent. Lastly, the *specificity* (the probability of a negative screening result if growth is truly absent) of the method, has an estimated value of 100 percent.[11]

Applications in Building Investigations

The MycoMeter-test method has a variety of applications in the investigation and remediation of mold in buildings. During building diagnostics, the MycoMeter-test method is a powerful tool to screen for mold growth (i.e., contamination). The fluorometric detection method can differentiate fungal growth from staining and discoloration that are often indistinguishable with the unaided eye. Areas with a history of water damage, condensation, or high humidity can be sampled to determine if fungal growth has actually occurred. The ability of the MycoMeter-test to quickly demonstrate the presence of active fungal growth on building and HVAC surfaces enables the investigator to advise building occupants on appropriate actions without delays associated with laboratory analysis. At this initial stage of a building investigation, knowing whether a surface is supporting fungal growth can enable the investigator to test hypotheses and make risk management decisions that limit exposure and initiate appropriate remedial actions (if necessary).

Prior to beginning mold remediation, the MycoMeter-test can be used to determine the extent or "delineate" the surfaces and building systems with fungal contamination. Because fungal contamination can often extend for some distance beyond the visible colonies, the investigator should empirically determine the extent of fungal growth and the limits of necessary remediation. These screening results can then be used in conjunction with visual inspection and moisture meter measurements to define the scope of work for the remediation contractor. In this manner, remediation projects can be well-defined, and a substantial reduction in change orders should result.

Cleaning methods are not usually 100 percent effective, and some materials are not cleanable. The extent of fungal growth within porous and semi-porous

building materials can impact cleaning efficacy. Therefore, it is wise to measure the effectiveness of cleaning procedures on representative materials before attempting to clean the entire building. The fast sample turn-around of the MycoMeter-test, and its compatibility with most building materials enables a remediation contractor to determine if the cleaning procedure chosen for each material is effectively removing mold by performing test cleanings on small areas. Test cleanings have proven extremely useful when cleaning mold growth from wooden trusses or wall studs.

The extent of fungal invasion can range from superficial to a depth of several millimeters. Visual assessment often cannot differentiate between these two conditions, before or after cleaning. It is advisable to sample several of the "worst" areas after cleaning and sanding have been attempted to assess the amount of residual fungal biomass. A "cleanability" assessment provides confidence in the remediation procedures, and demonstrates that the materials may be salvaged, or that the procedures are not effective, before too much time and money are expended.

The application of the MycoMeter-test method that takes greatest advantage of its fast sample analysis is post-remediation testing of contaminated building materials. Measuring surface contaminant levels after cleaning and abatement of mold-contaminated building materials allows verification that cleaning efforts were effectively carried out. By sampling representative materials after remediation, the consultant provides verification that the remediation contractor met the scope of work. Use of the MycoMeter-test method removes much of the subjectivity in "visual" clearance criteria that are often used. The on-site analysis of samples provides results within one hour, enabling the remediation contractor to re-clean failed areas or disassemble containments and move on to the next remediation area. After remediation, it is only recommended that sample results in Category A, described above, be accepted.

Both laboratory and field data have proven the MycoMeter-test method to be a fast, accurate, and reliable method to determine the presence of fungal growth on building materials. The on-site analysis of samples provides unprecedented data availability, enabling investigators to determine if mold is growing, and to what extent. With this information, investigators can make responsible and informed decisions to limit exposure and remediate sources of growth and moisture without unnecessary delays due to laboratory analysis.

Acknowledgments

Microscopic analyses were conducted by Robert Scarry, Rogina, Inc., Lakeland, Florida.; Lori Streit, Unified Engineering, Aurora, Illinois; and Aerotech Laboratories, Phoenix, Arizona.

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