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228 Midway Ln, Suite B
Oak Ridge,TN 37830
865.813.1700
www.assuredbio.com

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Molecular Entrapment to Capture Airborne Mold Spores

By Edward A. Sobek, Ph.D.

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Introduction

Indoor fungal contamination by mold species has been linked to asthma, infection and a variety of health related diseases. Mold impacts occupants directly through exposure; however, the cost in terms of decreased worker productivity, health care, remediation, insurance premiums, and litigation is staggering.

Biodiversity experts estimate that 1.4 million species of fungi are present on earth. Of that, only 100,000 species have been identified. Furthermore only a small percentage (<0.001%) have been associated with the indoor environment—less than 100 species. The American Industrial Hygiene Association's EMLAP program requires that laboratories identify twenty-eight spore types for direct exam accreditation, while the U.S. E.P.A identifies 36 species important to indoor environments. One would assume that with such a small number of molds being problematic indoors that air sampling methods would be refined to capture spores efficiently. However, the opposite is true. Poor spore capture efficiency is the greatest challenge facing industrial hygienists, CIECs and trained mold investigators. Occupants and stake holders suffer because problems are not identified. Mold related costs will continue to rise and occupant health will decline until mold contamination can be correctly diagnosed.

The mold species that do occur indoors have adapted to occupy a unique ecological niche that fosters little competition but an abundance of resources. The defining biological characteristics of indoor molds are (1) an asexual lifecycle, (2) the abundant production of microscopic spores called conidia, and (3) the ability to colonize and degrade a variety of indoor substrates

including wood based products, glues, polymers, and fabrics.

Problem Statement

Inefficient capture of airborne mold spores has resulted in excessive false negative test results throughout the indoor air quality industry. Moreover, outdoor to indoor comparisons are meaningless unless spores are identified to the species level. Broad categories, such as Aspergillus-Penicillium like, comprise many species and several genera. It is impossible to determine if the same species inside is derived from the outside or is colonizing substrate indoors.

Previous Options

Inertial impaction technology was designed to capture airborne particles of uniform size, shape and density. Such particles include minerals and fibers, which have predictable inertial properties. Likewise, inertial impaction devices are adjusted to ensure that the particle of interest exits the air stream and is captured onto a two dimensional adhesive surface. Microbial propagules, being of biological origin, are not uniform even within a species. The majority of microbial propagules remain in the air stream as it travels through the inertial impaction device. Microbes have evolved over millions of years to travel in air streams, and exit under flow conditions that maximize the species success. Success equates to landing on a suitable substrate, germination, colonization and reproduction of new propagules. This must happen before the propagule becomes non-viable due to adverse environmental conditions.

AssuredBio Solution

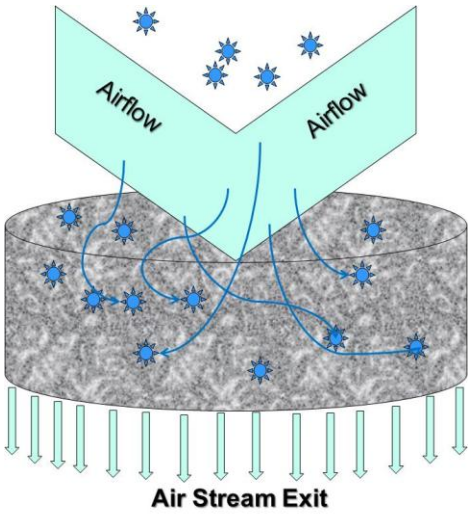
A novel approach was required to efficiently capture microbial propagules in a reproducible manner—an approach that incorporates the basic tenants of biological systems including surface area and adhesion.

Regardless of complexity, biological organisms must maintain seamless function at the cellular and molecular level. A myriad of biochemical reactions must occur in precise sequences. To maintain efficient function organisms have evolved complex cellular morphologies to maximize surface area and facilitate biochemical reactions. Consider, for example, the lining of the small intestine where nutrients are absorbed into the blood stream. The intestine surface is composed of specialized epithelial cells. To the naked eye it may look flat and smooth; however, microscopic examination reveals a surface of convolutions (valleys and hills). The convolutions maximize surface area for nutrient absorption by providing a one hundred thousand fold increase over a flat surface. Starvation and death would result rapidly without that excess surface area.

Adhesive proteins comprise the foundation cement that holds organisms together at the cellular level. These proteins are the biological glue that binds muscle fibers into functional bundles, the framework upon which the cellular matrix is built. Without it

we would literally fall apart. The combination of surface area and protein adhesion were combined and served as the model from which the novel microbial capture technology, 3-D Molecular Entrapment, was invented.

3-D Molecular Entrapment consists of drawing air through a biologically active, fibrous substrate that has depth width and height.



The substrate is coated with a biomixture that contains adhesive proteins. The proteins are receptive to microbial propagules such as spores and cells. When air flow rate parameters are met, the biomixture coating forms thin sheets that protrude into the gaps between the fibers. The phenomenon creates tremendous surface area. The probability that a propagule in the airstream will contact the biomixture is increased significantly. When contact occurs, the receptive proteins in the biomixture adhere to the propagules surface disrupting its natural aerodynamic properties. Microbial propagules immediately become unstable in the airstream. The result leads to further protein adhesion and premature exiting of the airstream and entrapment within the fibrous matrix. Once entrapped, propagules do not need to be removed from the substrate for analysis. Instead they remain tightly bound and their DNA is extracted for PCR analysis.

Molecular Entrapment Provides Superior Capture Efficiency

In a study that included 31 locations comprised of both commercial and residential properties, molecular entrapment showed a 95% increase in capture efficiency over inertial impaction. Table 1, below, was compiled from side by side samples collected via molecular entrapment and inertial impaction.

Table 1. Capture efficiency comparison of inertial impaction (spore trap) to molecular entrapment (mTrap).

Genus	Sum Spore Ratio (spore trap/mTrap)	
	Sum Spore Ratio	Efficiency Change
Stachybotrys	**0.034**	**97.2%**
Alternaria	**0.003**	**99.6%**
Chaetomium	**0.017**	**98.3%**
Cladosporium	**0.054**	**94.9%**
Aspergillus-Penicillium	**0.013**	**98.7%**

The sum spore ratio provides a metric to evaluate two capture methods for each genus

identified in the study. As the ratio becomes smaller, the difference in capture efficiency between two collection technologies increases. Efficiency change, expressed as a percentage of total spores captured for each genus, indicates the magnitude of efficiency shift when comparing two capture methods.

Reliable Detection of Large and Sticky Spores.

Indoor mold spores range from 2.0µm to 25µm in length. Some spores are dense and sticky like *Stachybotrys*. Others are long and tapered like *Alternaria*. Large spores settle rapidly compared to small round *Aspergillus* spores that may remain airborne for days after release. Sticky spores rely on vectors for transport, such as mites and other small invertebrates, hence they tend to aerosolize poorly.

Detecting low concentration spores indoors is essential for a quality indoor air investigation, since spores like *Stachybotrys* are known to produce potent mycotoxins that accumulate in the spore rather than the growing colony. Exposure to toxic molds often results in recommendations that include erecting specialized containments to protect occupants in commercial buildings or occupant relocation to a hotel from residential homes. Molecular entrapment captured significantly greater concentrations of large and sticky spores (ANOVA, F=5.369, P=0.021). On average, molecular entrapment captured ten to one thousand times more spores than inertial impaction (Table 2). Moreover, greater than two thirds of inertial impaction samples did not detect large or sticky spores.

Table 2. Average spore concentrations, expressed in cubic meters of air sampled, captured by molecular entrapment (mTrap) and inertial impaction (spore trap)

Genus	Sum Spore Ratio (spore trap/mTrap)	
	Sum Spore Ratio	Efficiency
Stachybotrys	**0.034**	**97.2%**
Alternaria	**0.003**	**99.6%**
Chaetomium	**0.017**	**98.3%**
	(n = 138)	

Direct Species Comparison among Samples.

Inertial impaction spore traps are analyzed using AIHA EMLAP Direct Microscopic Examination. Direct exam requires the use of a microscope with 40x to 100x power objectives. The spore trap has a 2-D rectangular or circular zone containing an adhesive. Captured spores will be located on the adhesive zone. The zone is centered on a glass cover slip that is removable. The cover slip is mounted on a microscope slide and viewed under the microscope by a trained analyst. The analyst is proficient in identifying and counting spore types. Spore types including *Stachybotrys* and *Alternaria* are identified with confidence to genus since they are large and have distinct

morphologies. However any 2.0µm to 5.0µm small round to ellipsoidal spore is typically lumped into the broad category called Aspergillus-Penicillium like spores. This group is a broad catch-all category that includes many species and multiple genera. In any given sample the true concentration of *Aspergillus* or *Penicillium* spores remains unknown.

The spore concentration is tallied for each category and converted to spores per cubic meter of air based on the liters of air sampled. The final results are delivered to the inspector in a report format. Most investigators collect multiple indoor samples per location and an outdoor sample for comparison. The consensus among inspectors is that greater concentrations indoors than outdoors of a particular spore category is suggestive of mold replication occurring indoors. No current standard exists for the required difference in spore concentration between indoor and outdoor that elicits reactionary steps; however, the typical rule of thumb is about 1,000 spores. When the spore category of concern is limited to distinct morphologies like *Alternaria* or *Stachybotrys*, confidence in the report and subsequent actions is high. However, when spore concentrations are elevated in the category Aspergillus-Penicillium like, confidence falls precipitously.

Aspergillus and *Penicillium* species are documented in the scientific literature as the most common mold colonies associated with water intrusion indoors. Recent U.S. E.P.A studies support these finding and identify the most common water intrusion *Aspergillus* and *Penicillium* species. However, direct comparison between indoors and outdoors is confounded in inertial samples because of the broadness of the Aspergillus-Penicillium like category. Many outdoor fungal species produce spores that fit the Aspergillus-Penicillium category. Investigators have little or no confidence in accepting that the Aspergillus-Penicillium like spores indoors are of the same species or even genus as the outdoor spores. For example, indoor Aspergillus-Penicillium like spores may comprise 500 spores of *Aspergillus fumigatus* and 2,000 spores of *Penicillium brevicompactum*, while outdoor concentrations comprise 150 spores of *Aspergillus ustus* and 3,350 spores of *Thysanophora penicillioides*, which is cosmopolitan in soil and on conifers. The direct exam report indicates that there is a greater concentration of Aspergillus-Penicillium like spores outdoors than indoors, and the difference is greater than 1,000 spores. Interpretation of results leads to the false conclusion that no indoor mold replication is occurring when in fact it is.

Analysis of molecular entrapment samples relies on differences in spore DNA not morphology. DNA provides superior differentiation at the species level, and is even capable of detecting strains within a species if so desired. The capture matrix is removed from the capture cassette and the entire substrate is processed in a set of chemical reactions that extracts the DNA

from the entrapped spores. Precision DNA probes and high fidelity enzymes are combined with the sample DNA into heat resistant multi-well plates. All components are dispensed into wells by dispensing robots. Finished plates are loaded into state-of-the-art thermocyclers that are controlled through a computer interface. The thermocyclers heat and cool according to programmed specifications that maximize enzyme efficiency and DNA replication. Probes specific for species bind with sample DNA only if the DNA of that species is present. Once bound, the enzyme drives replication of the target DNA. Every replicon produced releases fluorescent energy that is detected by sensors in the thermocycler. The light is quantified and applied to a set of algorithms that provide species identification and the spore concentration for each species present in the sample. The algorithms incorporate multiple replicates of known spore concentrations for each species detected beginning with a one log concentration and progressing through a six log concentrations. Together the concentration gradient forms a calibration curve, one for each species reported. Each sample set includes positive and negative controls. The end results deliver spore concentrations for species that investigators can use with high confidence for determining the magnitude and location of mold contamination indoors. Action levels and risk factors can be determined from the sample data. Toxic or pathogenic species are identified providing a wealth of information that can be given to physicians treating exposed patients and to remediators to ensure appropriate containment of contaminated locations

Implementation

Currently, molecular entrapment technology is being used to determine if species are present that the U.S. E.P.A had identified as significant indoor water intrusion molds. In total, 36 species are included in the analysis. A report is generated that provides the spore concentration and prevalence. Graphs and charts provide comparison among samples including indoor and outdoor samples. The analysis includes 19 species within the genera *Aspergillus* and *Penicillium*. This allows for direct comparison among indoor samples and outdoor samples.

Sample collection is conducted using a cassette containing the molecular entrapment medium. The cassette is trademarked as the mTRAP and includes an adapter for standard ¼" tubing to fit a standard rotary pump and rotameter setup that many inspectors currently use.

The recommended flow rate of the pump is fifteen liters per minute for a minimum sampling duration of ten minutes. Each cassette is packaged in a holder. The mTrap is labeled with serial number and a field to write the sample description. It is recommended that several samples are collected indoors depending on size and complexity of the site. In addition, one outdoor sample is recommended for comparison of mold species concentrations between indoor and outdoor environments. Elevated concentrations of water intrusion species indoors warrant action to contain affected areas and prevent continued exposure to occupants.

Summary

Inertial impaction is tied to direct microscopic exam analysis, which is a morphology based spore identification method that fails when morphologies converge as within the Aspergillus-Penicillium like spore category. Furthermore, important water intrusion molds are underrepresented relative to their airborne concentrations. Species like *Stachybotrys* pose significant health risks to exposed occupants because of mycotoxins and allergenic properties. Molecular entrapment technology overcomes the problems associated with inertial impaction by providing direct comparison among samples collected indoors and outdoors at the species level. Significant gains in capture efficiency using molecular entrapment allows inspectors to detect species that occur in low concentrations because of the ecological niches that molds inhabit indoors.

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