13 Detection of indoor fungi bioaerosols

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Introduction

The detection of microorganisms in environmental samples, particularly from air, has a long history, greatly pre-dating the analysis of the chemical contaminants that are now generally more familiar to occupational hygiene. Antonie van Leeuwenhoek (1632-1723) was the first to demonstrate the presence of microbial cells in indoor dust, but it was not until two centuries later that Louis Pasteur (1822-1895) famously demonstrated lactic acid fermentation by airborne microbes by introducing air into sterile broth in swan-necked flasks, thereby defeating the spontaneous generation hypothesis of microbial life (Pasteur 1857). Based on the recognition that living microorganisms could travel through the air, Pasteur's methods were rapidly enlisted in the search for agents of human diseases, such as cholera and typhoid. Another early air sampler, the "aeroconioscope" of Maddox (1870), relied on wind pressure to propel air through a cone tapered to a narrow point positioned above an adhesive-coated slide onto which particles were impacted (Cunningham 1873, MacKenzie 1961). Although this device was effective in capturing particles from the air, airborne concentrations could not be determined because the volume of air drawn through the instrument could not be measured.

One of the earliest studies using volumetric air sampling was conducted by Pierre Miquel (1850-1922). Miquel's device collected particles by impaction on the surface of a sticky slide that was situated beneath a narrow orifice through which air was drawn by suction. The suction was derived from the descent of boli of water separated by long bubbles of air in a vertical tube (Miquel 1883, Comtois 1997). Remarkably, Miquel's technique was able to sample a cubic metre of air using only 40 litres of water (Miquel 1883). Even though much of this early work sought to clarify the epidemiology of notorious human diseases like cholera and typhoid, this work actually furthered our understanding of the aerobiology of relatively large bioaerosols, primarily fungal spores and plant pollen.

During the last 50 years, a great number of volumetric air sampling devices have been developed to suit various purposes. These devices along with their uses and limitations have been reviewed in detail (Cox *et al.* 1995, Macher 1999, American Industrial Hygiene Association (AIHA) 2005). As well, a number of excellent references have appeared dealing with approaches to building investigations for

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mold growth and the interpretation of sampling results (AIHA 2005, Flannigan *et al.* 2001, Macher 1999).

Within the last two decades, numerous cognizant bodies and governmental jurisdictions have advised that exposure to environmental molds represents a controllable health risk, and that indoor mold growth should not be tolerated irrespective of its contribution to the indoor airborne spore-load (see Chapter 14). This approach has been favored over the establishment of exposure limits based on air sampling for a number of reasons including the following: (1) widely used air sampling methods typically express levels in units such as colony forming units (cfu) or spores per cubic meter, which are not relevant to dose (typically milligrams of contaminant per cubic meter); (2) mold samples contain complex mixtures of chemical contaminants whose constituents vary qualitatively and quantitatively, and cannot readily be defined a priori; (3) no single sampling approach can adequately evaluate the broad spectrum of known and probably health-relevant contaminants expressed by molds; and (4) much of the current knowledge implicating molds as health effectors is derived from proxy indicators of exposure (Douwes *et al.* 2003). Nevertheless, indoor air sampling remains a useful tool in the search for indoor growth sites, in conjunction with a visual inspection by an experienced investigator.

This chapter is by no means an exhaustive review of fungal air sampling techniques, of which there are many. It is intended as a review the uses and limitations of some of the air sampling methods for molds that have been used commonly for occupational hygiene purposes. We have not included sampling approaches that have only been used in research studies, or investigational techniques to assess personal exposure. Our discussions of new technologies, however, include some recent studies introducing or validating techniques that appear to have considerable practical potential.

Environmental sampling

Virtually all indoor materials, ranging from construction products to furnishings and foods, are susceptible to fungal spoilage under conditions of dampness. A range of methods have been developed to detect the presence of indoor fungal growth. Fundamentally, these methods can be classified into: (1) those that monitor fungal particles in the air by air sampling; and (2) those that characterize fungal growth directly on bulk material specimens.

Fungi normally present in indoor environments

Airborne fungal particles represent a complex mixture consisting of elements that differ in biology, chemistry and morphology. Under normal circumstances where indoor fungal contamination is absent, the fungal particles that predominate in indoor environments arise from three primary reservoirs: (1) the phylloplane; (2) the pedosphere; and (3) the dermatoplane.

Phylloplane fungi comprise an ecologically similar grouping of unrelated, mostly dry-spored taxa that inhabit plant leaf surfaces. A proportion of them are biotrophic to necrotropic parasites, while many others are saprotrophs. In northern temperate regions, phylloplane taxa predominate in the aerospora during the growing season. Owing to their abundance in the outdoor air, aerosols derived from these fungi, including spores, hyphal fragments and cellular debris, filter into indoor environments through normal activity. Characteristic taxa in this category include certain species of the anamorph genera *Alternaria*, *Cladosporium* and *Epicoccum*, as well as powdery mildews, lignicolous, foliicolous, coprophilous and lichenized ascomycetes, most myxomycetes, and most basidiomycetes including rusts, smuts, agarics, brackets and corticioid fungi (excluding *Poria* and *Serpula*).

Fungi originating from soil reservoirs also commonly occur in indoor environments even when indigenous fungal amplifiers are absent. These mostly wet-spored fungi enter buildings on footwear: this is particularly common in North America, and other places where footwear is not removed upon entering buildings. Secondarily, soil containing fungal material may become aerosolized during construction or excavation activities, and these aerosols may be carried indoors in the same way as phylloplane fungal materials are, that is, passively in air currents or via human movements or actively through the actions of mechanical systems. Strictly speaking, fungi associated with soil as a reservoir are not necessarily indigenous to soil as a habitat. They may originate elsewhere as saprotrophs or necrotrophs and accumulate over time in the soil "spore bank". Taxa common in this category include species of the anamorph genera *Acremonium*, *Beauveria*, *Chromelosporium*, *Clonostachys*, *Fusarium*, *Gliocladium*, *Myrothecium*, *Phoma*, *Trichoderma,* and *Penicillium* subgenera *Biverticillium*, *Furcatum* and *Aspergilloides*.

Human skin is an underestimated but a rich source of fungal material in the indoor environment. In a study by Pitkäranta *et al.* (2008) of indoor dusts analysed via sequence analysis of rDNA clone libraries 14% of all clones recovered represented *Malassezia* yeasts (*Exobasidiomycetidae*), fungi growing on human skin where they are often associated with tinea versicolor, dandruff and seborrhoeic dermatitis (Gupta

et al. 2001). The abundance of these fungi in indoor environments is not surprising in light of their high prevalence in human populations. The results of this report suggest that *Malassezia* species from human sources may be non-trivial contributors to dust-borne beta-(1,3)-D-glucan. Dermatophytic fungi, by comparison, have been reported from indoor air and dust but are uncommonly recovered in microbiological investigations of indoor air and dust (MacKenzie 1961, Gupta and Summerbell 2000, Summerbell 2000).

Lastly, a number of fungi are known to proliferate in dry indoor habitats, such as settled dust. They do so under normal ambient conditions without superfluous moisture, but their growth is enhanced by dampness. Typical indoor xerophilic taxa include members of the *Aspergillus versicolor* group, *Aspergillus penicillioides* and *Wallemia sebi* (AIHA 2005, Samson *et al.* 2010, Miller 2011). Examination of normal household floor dust often reveals mite fecal pellets that consist largely of comminuted fungal hyphae and conidia that, when cultured, yield *A. penicillioides* and other xerophiles (Van Asselt 1999). In light of such findings, Van Bronswijk and others have suggested that xerophilic molds are important elements of the indoor dust ecosystem, providing nutritionally enriched grazing materials for dust microarthropods such as pyroglyphid mites (Van Bronswijk and Sinha 1973, Van de Lustgraaf 1978, Hay *et al.* 1993, Van Asselt 1999). The observation of low background levels of these and other xerophilic taxa in indoor air and dust is of ambiguous significance and does not necessarily reflect abnormal indoor fungal growth.

Bioaerosols

The atmosphere is replete with fine particles of different size, shape, composition and origin, which are constantly sent aloft by disturbance and sedimented out by gravity. This suspension of airborne particles, known as an aerosol, contains considerable biologically-derived material. Fungal particles and pollen together account for up to 10% of the total mass (Womiloju *et al.* 2003). The term "bioaerosol" refers more narrowly to the fraction of total aerosol comprising particles immediately originating from biological materials or processes of biological origin. Particulates arising from the combustion of fossil fuels are not considered bioaerosols despite the biological origin of the parent fuel. Bioaerosols are generally interpreted to include whole cells, cell fragments, and non-volatile chemicals arising directly from biological processes, such as proteins and carbohydrates.

Origin and release

The liberation of fungal bioaerosols is accomplished by a range of mechanisms including both active and passive release. Active release refers to adaptive means of propagule aerosolization, via forces arising from a burst of energy. Ballistic spore discharge, for example, is seen in basidiospore release in the gilled mushrooms, bracket fungi and corticioid fungi, while forcible ascospore discharge is observed in the perithecial and pseudothecial ascomycetes. Ballistoconidium release occurs in the mirror yeasts. In conidial filamentous fungi, an important means of active spore discharge involves the disruption of conidia by hygroscopic torsion of conidiophores and conidial chains (Meredith 1973).

Passive release is accomplished by mechanical disturbance in activities such as construction and excavation, in maintenance practices like sweeping and grass mowing as well as in personal activities including walking and sitting. Adhesion of fungal propagules to mist droplets has also been identified as an important aerial dispersal mechanism. Nebulization of water containing fungal debris results in the generation of a mist containing cells and other materials. Subsequent desiccation of mist droplets can cause the accretion of all non-volatile constituents into a composite solid particle, in a process known as "droplet nucleation" (Wells 1934).

The release of fungal propagules from the structures producing them often demonstrates pronounced temporal patterns, ranging in scale from seasonal to diurnal. Phylloplane taxa, for example, show peak airborne concentrations seasonally in accordance with the life cycles of their plant hosts. In northern temperate climates, airborne levels of these taxa substantially decrease during the winter. Many of the same phylloplane species demonstrate diurnal periodicity of propagule release, with peak concentrations detected during the daylight hours and with lower concentrations present during the night-time hours (Gregory 1971). By contrast, the cooler, often damp night-time conditions are associated with the enhanced release of ascospores and ballistosporic yeasts that otherwise only dominate the aerospora following precipitation (Gregory 1971).

Factors affecting bioaerosol dispersion, deposition, collection and recovery

Aerodyamics

The aerodynamic behavior of bioaerosols is governed by intrinsic properties, such as size, morphology, hygroscopy and density under the influence of prevailing environmental conditions. From a kinetic standpoint, the most important property

of a bioaerosol particle is its terminal settling velocity (TSV); this refers to the maximum velocity attained by a particle at the point that gravitational acceleration is offset by viscous resistance of air. In still air, in the absence of thermal or electrostatic disturbances, a 1 μm diameter sphere of unit density has a TSV of 12 cm/hr, whereas the TSV of similar sphere of 10 μm diameter is 100 times greater, 1.2 m/hr (Cox *et al.* 1995).

Particle morphology and density are also important determinants of TSV. For the purposes of estimation of "aerodynamic equivalent diameter" (AED), fungal spores are assumed to have a density slightly greater than that of water, 1.1 g/cm. Non-spherical or morphologically irregular particles, or those of different density are said to have an "aerodynamic equivalent diameter" (AED) equal to the diameter of a hypothetical unit-density spherical particle of equivalent TSV (Hinds 1999). Generally, bioaerosol particles with an aspect ratio (i.e. length : width) of less than 3 can be assumed to have an AED roughly equivalent to the diameter of a spheroid of equivalent volume (Hinds 2005). Particles with a larger aspect ratio than 3, and particularly greater than 10, exhibit variable TSV depending on their orientation, and require a more complex approach to predict their behaviour (Hinds 2005). The AED of a particle is also related to the efficiency with which that particle can be captured and retained by certain kinds of air sampling instruments, particularly those based on impaction.

Moisture

Most bioaerosol particles are hygroscopic. This, in combination with their small physical size and relatively large surface area-to-volume ratio, makes them increasingly susceptible to small changes in relative humidity, resulting in rapid changes in particle density and physical size. These changes ultimately cause variation in aerodynamic properties (Reponen *et al.* 1996). As such, the mechanical collection efficiency of a given bioaerosol sampler for the same aerospora will be greater when sampling is carried out under conditions of higher relative humidity.

Liquid impingement is a collection technique that circumvents the problem of desiccation-related cell death by drawing air through a liquid collection medium, often a nutrient broth, water or oil. However, the collection of viable cells in liquid media can result in premature cell germination and unintentional amplification, dilution of analytes used biochemical quantification or cell death. Culturable taxa may also continue to grow and amplify in the collection medium, resulting in an artifactual increase in reflected biomass and changes in cell morphology. Liquid impingement accepts this as a trade-off against the greater ability of liquid-based collection media to protect cell viability by preventing desiccation.

Electrostatic effects

Most bioaerosol particles acquire a small electrostatic charge through passive equilibration with normally occurring atmospheric positive and negative ions. Electrostatic charges can result in the deposition of particles on vertical surfaces, the agglomeration of particles of opposite charge, and other behaviors that are not predicted by gravitational effects alone. Electrostatic charges can also be an important source of sample loss in the collection head. The action of drawing air at a high flow rate through an orifice or slit can cause a static electric charge to develop on the sampler, typically in the region at and around the outlet of the sampling nozzle. This charge can be dispersed if the sampler is composed of conductive material; however, components of the sampling head composed of non-conductive materials, such as plastic and glass, can build up static electric charges to a sufficient degree that particles become diverted from the air stream during sampling (Cox *et al.* 1995: 18). The loss of collected particles by re-entrainment in the air stream during sampling is well-known (Hinds 1999).

Electromagnetic radiation

The strong influence of electromagnetic radiation on bioaerosol particles relates mainly to their size. A majority of indoor environmental bioaerosols are less than 10 µm in physical diameter, and therefore are close in magnitude to the wavelength of visible light. The energy imparted by light is readily propagated to aerosol particles. In incident light, opaque particles become heated on the aspect of the particle directly facing the light source and are propelled from the light source by convection. The opposite effect occurs with transparent particles, which function like lenses. Energy is concentrated on the far side of the particle, thereby causing the particle to be propelled under convective force towards the heat source. This is the principle underlying the tendency of incandescent light bulbs in kitchens to acquire a film of grease on their surfaces (the grease originates as an oil mist comprising transparent particles that are generated during cooking, depositing by thermal attraction on the surface of the bulb). Exposure to light in the visible range also increases Brownian motion, which can result in particle movement or deposition behaviors that are not predicted by gravity effects.

Convection effects predominate with longer wavelength, thermal radiation. Convective effects are mostly responsible for keeping fine particles suspended in the air for long periods in absence of persistent mechanical disruption. However, these effects may also result in particle deposition on vertical surfaces adjacent to heat sources, or horizontal surfaces above heat sources.

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Air sampling

Volumetric air sampling

A large range of sampling instruments is available for the volume-based measurement of biological particles in air. All air sampling instruments work by separating particles from air, and then collecting and concentrating the particles to permit their measurement and characterization. There are several methods of collection that are classified under the headings impaction, filtration and impingement. The devices most commonly used for bioaerosol detection are those relying on inertial properties, such as impaction samplers.

Inertial samplers

Most inertial samplers are based on the principle of impaction. The most common impaction sampler is the jet-to-agar impactor. Air is drawn through a slit or round orifice, accelerating any suspended particles to the velocity of the air flow. The jet is directed against a surface producing a deflection in the air stream. Typically this surface is a glass or plastic impaction plate or a growth medium contained in a Petri plate. The trajectory of particles in the deflected air stream is determined by their momentum. Above a certain threshold momentum, particles become impacted on the plate, whereas particles with lower momentum remain in the air stream. In most impactors, the air sampling pump is situated downstream of the collection head and capture media, and the device operates under negative pressure.

Other samplers that utilize inertia as mechanism to separate particles from an air stream include centrifugal impactors and liquid impingers. Centrifugal impactors draw air through an impeller containing an annular collection plate, typically filled with an agar growth medium or coated with an adhesive. Particles are accelerated centrifugally and captured by impaction on the surface of the collection medium. The centrifugal impactors most commonly used in bioaerosol sampling are the RCS Standard and High-flow devices manufactured by BioTest (Dreieich, Germany). The RCS Standard has the strong disadvantage of exhausting air from the intake orifice, making it impossible to measure the flow rate of the device. This limitation was addressed in later versions of the sampler, such as the Plus and High Flow models, for which air flow rate can be calibrated.

Liquid impingers collect particles by passing air through a liquid medium. With these instruments, air is drawn through a series of orifices, producing a stream of bubbles in the liquid medium. The bubbles move through the medium much more

slowly than the speed of the air exiting the orifices, causing the inertial impaction of particles at the periphery of air bubbles. Liquid impingers are less prone to kill cells by desiccation than most other particle collection methods. However, this collection method is impractical for field use because of spillage or contamination during handling. Losses may arise from the re-entrainment of collected particles as bubbles burst at the medium surface. Foam accumulation during collection is common.

Mechanical collection efficiency

The effectiveness with which inertial samplers mechanically collect particles from the air is most commonly described by the d_{50} cut-point, which is the AED of particles that are collected with 50% efficiency. In general, particles of greater AED tend to be collected with high efficiency in keeping with the steep morphology of the collection efficiency curves (Hinds 1999: 125). It should be noted; however, that mechanical collection efficiency is contingent on the consistency of sampling flow rate, which determines the momentum of particles. A reduction in flow rate causes a simultaneous, non-proportional reduction in the d_{50} cut-point. Therefore, d_{50} cut-points are always given relative to a standard flow rate that must be maintained throughout the sampling period. The flow rate of the sampling device is usually directly controlled by the flow rate of the sampling pump used. The pressure drop contributed by the sampling head is low for most of the commonly used impaction samplers. Furthermore, sample loading during sample collection does not cause an increased pressure drop. Some air samplers are equipped with a "critical orifice", a precision narrowing of the air outlet that limits the maximum flow rate of air through the device. For these devices, the flow rate through the device will remain constant as long as the flow rate of the sampling pump does not fall below a certain minimum. Many older Andersen-style samplers are equipped with this flow restricting device; however, it is absent in most recent- model samplers. In these samplers, a careful calibration of the sampling pump is required.

Collection losses

Even with the foregoing factors optimized, several sampling phenomena remain that, can modify the capture characteristics of the sampler resulting in sample loss. The first of these relates to the modification or redistribution of particles by the air stream itself. At the flow rates used in most inertial impaction samplers, the process of entrainment and passage through the collection orifice creates sheer forces that can comminute aggregated particles, altering their deposition characteristics (Andersen *et al.* 1967). In our experience with Hirst-type slit samplers, cells that become comminuted during collection typically co-deposit in a spray pattern spanning

several cell diameters. Depending on the number of non-contiguous cells, the pattern of spread, and the overall loading of the sample, the microscopic interpretation of these cells as representing a single progenitor can be highly subjective.

In addition to modifying the deposition characteristics, the sheer forces produced during collection can also cause cell damage and reducing viability (Andersen *et al.* 1967). Sample loss can also arise from the redistribution of sampled particles by the air stream, depending on the ability of these particles to adhere to the collection plate. Poor adhesion to the capture medium can cause particles of AED greater than the d_{50} cut-point of the sampler to bounce off of the surface of the medium at the moment of impaction. Also, the persistent force of the air stream during sampling can cause captured particles to become re-entrained in the air stream and to exhaust from the sampler. With samplers that use direct microscopy to detect, quantify and characterize captured cells, redistribution of cells on the collection medium can artificially increase the number of apparent capture events.

Filtration samplers

The use of filter membranes for the capture and collection of bioaerosols has a long history, but, nonetheless, the method is not widely used in routine air sampling. As a collection method, filtration has several advantages over inertial sampling. A primary benefit is that the mechanical collection efficiency of the sampler is governed by characteristics of the membrane. In relatively still air, this method is minimally dependent on face velocity (i.e. the speed of the air moving into the medium) above a certain minimum threshold dependent on the medium type; this velocity is usually about 100 cm/min for mixed cellulose ester membrane filters with a 0.45 µm-pore size (NIOSH 1994). Membranes are available in a wide variety of compositions and collection efficiencies. Membrane samples also have an advantage of being suitable for bioaerosol analysis in conjunction with microscopic or culture-based methods, or with other modes of characterization such as biochemical, immunochemical, genetic or spectral analyses.

Filtration samples are used widely in the characterization of integrated personal exposures to bioaerosols and other aerosols. This is because they can function with lower flow rates than inertial samplers, using relatively small pumps, and also because this sample format is minimally susceptible to overloading. These techniques have been underexploited outside of research-related assessments of bioaerosol exposure.

Passive air sampling

Settle plates

The use of gravity settle plates has a long history in environmental mycology and bacteriology, and these plates continue to be used in some specific applications. This method consists of exposing the agar medium in a Petri dish to the ambient air for a period of time, often 0.5-1 hr, incubating the plate and counting and identifying the resulting colonies. Because the effective volume of air sampled cannot be determined, the colony counts on settle plates cannot be expressed in volumetric units; hence, these data are semi-quantitative. Due to the different settling velocities of airborne particles according to their aerodynamic features, this method has long been known to under-represent taxa with small airborne propagules, such as *Aspergillus* and *Penicillium*, relative to those taxa with larger propagules (Sayer *et al.* 1969, Soloman 1975). This matter can be subjectively taken into consideration when results are evaluated (that is, the analyst can determine that the number of *Penicillium* colonies seen is probably a drastic under-representation), but there are no formulas available to allow formal transformation of the data to accommodate the differences in propagule size and shape. Despite these shortcomings, the persistent recovery of taxa commonly associated with indoor contamination is strongly suggestive of an indoor growth site. These taxa include: *Aspergillus niger*, *A. fumigatus*, *A. versicolor*, *Chaetomium* spp., *Cladosporium sphaerospermum*, *Paecilomyces variotii*, *Penicillium* subgen. *Penicillium*, and *Stachybotrys* spp. (Federal-Provincial Committee on Environmental and Occupational Health (Canada) 1995, ACGIH 2001, Flannigan *et al.* 2001, AIHA 2005).

Dusts

Studies comparing static area sampling with integrated personal sampling for aerosols have shown very poor agreement between the two methods (Lange *et al.* 2000, Niven *et al.* 1992). This inability to use area sampling to predict personal exposure is well recognized in industrial hygiene (AIHA 2005). Attempts to evaluate exposures in the non-industrial indoor environment face similar challenges. For example, analysis of fungal content in indoor air samples is of limited use in the evaluation of the contamination status of the affected environment. The fungal burden in indoor air varies episodically, related to factors including the total mass of settled dust present, the amount of activity, and the various features of the building that in some way moderate the ventilation (AIHA 2005, Ferro *et al.* 2004a). In most indoor environments, settled dust is the primary source of biological particulate contaminants (AIHA 2005: 95). Dust mass and disturbance measurements are

well correlated with measured levels of airborne fine particles (Afshari *et al.* 2005) including fine biological particles (Buttner and Stetzenbach 1993, Cole *et al.* 1996, Ferro *et al.* 2004b). Exposure to indoor biological contaminants mainly arises from activity-related disturbance of fine dusts on flooring and furnishings, contributing to a "personal cloud" of fine particulate matter (Burge 1995, Ferro *et al.* 2004a,b). The ability of indoor dust to act as a reservoir for seasonal outdoor aeroallergens, leading to perennial exposures in the indoor environment, has been well demonstrated (Arbes *et al.* 2005, Salo *et al.* 2006). Dust analysis provides a more robust proxy measure for personal exposure than short-term air sampling provides, and it is less influenced than air sampling by temporal and spatial variation. These measures are more reproducible than air sampling results, and provide an integrated picture of exposure, literally capturing transient bursts of indoor bioaerosols (Ren *et al.* 2001, Portnoy *et al.* 2004).

While settled dust indoors may influence respiratory status independently of the exposure to any specific chemical constituent (Elliott *et al.* 2007), most commonly assayed contaminants such as allergens, endotoxins and glucans can be measured in a highly reproducible manner from dust (Antens *et al.* 2006, Heinrich *et al.* 2003). These analytes remain stable in dust during long-term storage (Morgenstern *et al.* 2006). The mass of sieved dust collected by standard methods from a pre-defined area (e.g. 2 m^2) provides an index of exposure both to total dust and to a series of health-relevant contaminants in the home (Elliott *et al.* 2007).

Analytical methods

The most commonly used bioaerosols samplers are grouped into two general categories, spore trap samplers and culturable samplers. Spore trap samplers are analysed by direct microscopy whereas samples collected on growth media must be cultured before identification and enumeration can be done.

Direct microscopic methods

The earliest methods of characterizing airborne fungi featured light microscopic analysis of liquid-impinged samples; this technique was devised by Louis Pasteur (Ariatti and Comtois 1993). The same approach continues to be a front-line technique for the identification and characterization of fungal bioaerosols. Direct microscopy is used in combination with culture-based techniques for the primary identification of taxa. As well, light microscopy is used increasingly in the visualization of fungal cellular material in methods excluding prior amplification via culture. These methods, while useful and rapid, are poorly able to distinguish many morphologically

similar common taxa such as *Acremonium*, *Aspergillus*, *Clonostachys*, *Paecilomyces*, *Penicillium*, *Phoma*, etc. Indeed, many common indoor contaminant genera of ascomycetes and basidiomycetes cannot be reliably identified via microscopic examination of their spores. Hyphal fragments from all sorts of fungi are similarly minimally distinguishable by light microscopy.

A range of methods and approaches have been applied to the analysis of spore trap samples. There are various types of counting procedures as well as conventions for identifying spores or other cells. Counting procedures vary according to the type of sampler used. Where the collected material is randomly deposited, as is the case on a filter membrane sample, the deposit may be enumerated by counting random microscopic fields. By contrast, sampling approaches that result in the non-random deposition of the catch must be analyzed using a systematic approach. For example, slit-type impaction samplers have long been used in aerobiological studies (Hirst 1953). These devices draw air through a slit-like orifice below which is situated an impaction plate, often a glass microscope slide, a coverslip or a piece of film coated with grease or adhesive. In sampling, the catch is deposited in a linear trace, shaped by the morphology of the orifice. Counts of captured cells are typically taken along microscopic transects perpendicular to the deposit. Transects must be counted completely in order to avoid bias based on the AEDs of the various particles. Nevertheless, incorrect counting methods have been applied in some large studies comparing sampler collection efficiencies under field conditions; the methodological correctness of published studies must be carefully evaluated (e.g. Lee *et al.* 2004).

It has been recommended that a minimum of 10-15% of the deposit be examined in order to obtain an adequate representation of the taxa collected (Foto *et al.* 2004). However, given the relatively short duration of sampling in all common methods, a number of authors have suggested that the quantitative data derived from bioaerosol sampling procedures are of little value, and that much more useful information is obtained from the qualitative composition of the sample. Thus, the expenditure of analytical time to establish a highly accurate count of spores or other cellular elements is often discouraged in favor of providing a thorough characterization of the taxonomic composition of the sample. The formal count is often followed by an exhaustive examination of the sample to ascertain the total number of distinguishable taxa that are present (Thorn *et al.* 2007).

Another important factor to consider in the microscope examination of spore-trap samples is microscopic magnification and resolving power. Many commonly occurring indoor fungal spores are small, often below 10 μm in diam. The discrimination of fine details of cell morphology and ornamentation necessary to differentiate cells,

such as the conidia of *Aspergillus* and *Penicillium*, from morphologically similar, hyaline basidiospores typical of many common outdoor *Polyporaceae*, can rarely be accomplished reliably at magnifications below 1000×, and cannot be done using a microscope with poor resolving power. Furthermore, the distinction of these and other small, hyaline fungal cells from background organic and inorganic debris can be exceedingly difficult at low or medium power. Therefore, the routine use of high-power magnification, often in addition to resolution-enhancing optical techniques such as Nomarski differential interference (DIC) or phase contrast microscopy is increasingly recommended for aerobiological analyses (AIHA 2005).

The enumeration of spore clumps or chains is another area where methodology varies among researchers. Many aerobiological studies have taken the approach of counting (or estimating the count) of individual cells or spores in an aggregated particle. This practice, strongly influenced by early pollen aerobiologists investigating outdoor exposures and allergic sensitization, is rooted in the assumption that numerical counts of individual particles, even when aggregated in clumps, may correlate with increased inhalation exposure. While there is some validity to this assumption for long-term, integrative pollen sampling (Van Leeuwen 1924), the relevance of this approach to commonly-used, short-duration fungal spore sampling is unclear. First of all, the purpose for using these methods often relates to the evaluation of whether or not building interiors harbor fungal growth sites. In this type of evaluation, the "dose" is irrelevant; instead, the community composition is of primary importance, since indoor fungal growth generally features distinctive assemblages of species. Secondly, the individual counting of aggregated cells treats the process of collection for each of these cells as if it were a statistically independent event. Actually, the clumps or chains often arise as masses from individual conidiophores or microcolonies, and the individual cells thus collectively represent statistically *dependent* "capture events". This is an important distinction in air-sampling data in practice and research. For example, a coherent chain of 30 *Cladosporium* conidia, if interpreted as adding 30 units to the count per cubic meter of air, artificially implies a higher airborne prevalence of this fungus than is statistically realistic. The potential for surface contamination by spore deposition appears to be 30-fold greater than it actually is. If the goal of air sampling is to express the frequency of occurrence of airborne cells or propagules, counts are much more informative when they document "collection events" rather than the total number of cells collected. Depending on the planned applications of air-sampling data, it may be useful to document details of the morphology of bioaerosol particles as they occur in the air, particularly in regard to whether they are unitary or composite.

Culture-based methods

Most of the commonly used air sampling methods rely on cultivation of fungi on agar as a means of detection. Bioaerosol sampling methods that rely on culture are subject to recovery losses that arise from mechanical as well as biological effects:

- *Jet-to-plate distance.* The stability of the d_{50} cut-point from sample to sample relies on a consistent distance between the terminus of the sample jet and the surface of the collection plate. In culturable samplers, like the Andersen samplers and other jet-to-agar impactors, this distance is governed by the medium volume of the Petri plates used for collection. In theory, the air stream should not diffuse appreciably between the nozzle exit and a location not more than one jet diameter away from the surface of the collection plate (Hinds 1999: 125). However, the use of a consistent jet-to-plate distance through adherence to the recommended medium volume is important in the maintenance of collection efficiency (Macher *et al.* 2001). Although user guides that accompany sampling devices typically provide these requirements, in practice they are often ignored and it is worthwhile here to review several common standards.

  The most commonly used jet-to-agar impactor is the single stage Andersen samplers, also known as the N6 because it derives from the sixth stage of larger, size-partitioning instrument, the 6-stage Anderson cascade impactor. The N6 has an empirically determined d_{50} cut-point of 0.65 μm (Andersen 1958) using a sampling flow rate of 28.3 l/min with standard 100 mm Petri dishes filled with 41 ml of medium (Thermo Electron Corporation 2003). Actually, plate volumes ranging from 40-50 ml are suitable (Macher *et al.* 2001: 687). In contrast, the 2-stage Andersen uses 20 ml per plate, and the 6-stage uses 27 ml per plate (Thermo Electron Corporation 2003). Substitution of 90 mm Petri plates for 100 mm plates is acceptable for all devices. This may create a problem if no adjustment is made for the difference in interior volume. For example, the critical height of the medium surface in a 100 ml diam Petri dish with straight sides containing 41 ml of medium is 5.2 mm. However, many researchers now commonly use 90 mm polystyrene Petri plates with tapered sides. As a case in point, our laboratory ordinarily uses "100×15 mm" Petri plates manufactured by Fischer Scientific Inc., Canada, catalogue no. 08-757-13, which have an inner diameter at the bottom of the plate of 84 mm, and an inner diameter at the top of the lower plate of 87 mm. In order to achieve the predicted d_{50} cut-point with an Andersen N6 sampler using these Petri plates, a 30 ml volume of medium is required. It should be noted that the critical medium height of 5.2 mm is a minimum required to achieve the predicted d_{50} cut-point of the device, and that further increasing the medium height (i.e. increasing the plate volume) would reduce the d_{50} cut-point.

- *Biological recovery efficiency.* The recovery of propagules or "colony-forming units" requires that the captured cells be amplified by culture. However, the process of sampling itself can dramatically modify cell culturability or viability. These effects become pronounced where solute-rich, semi-solid growth media are used for collection. In the case of a jet-to-agar impactor, for example, air passing through an orifice strikes a highly localized area on the medium surface. Over the course of sampling, this may result in localized water loss accompanied by transient hyperconcentration of solutes on the upper surface of the agar (Andersen 1958, Blomquist *et al.* 1984, Burge 1995, Morris 1995). As the spot impacted by the jet is the locus of cell deposition, this region becomes osmotically ever less hospitable to incident cells as sampling continues. At the conclusion of the sampling period, there is some rebound rehydration of the impaction loci from water provided by the underlying medium; however, the osmotic damage that arises in the deposited propagules may be irreversible. This is particularly true for propagules impacted early in the course of sampling or those of taxa that exhibit low osmotolerance. Depending on the prevailing relative humidity, the duration of sampling, and the degree of osmotic tension that develops while the medium is exposed to the sampling air stream, varying degrees of irreversible cell damage may result. Similar desiccation-related loss effects have been reported with air sampling and vacuum-collection of surface dust on filter membranes (Collett *et al.* 1999, Hinds 1999).

  Another important source of biological loss arises from intraspecies and interspecies competition at sites of co-impaction (Cox and Wathes 1995). This phenomenon is predominantly associated with jet-to-agar impactors like the Andersen N6, where deposition on the surface of the collection medium is non-random, mirroring the arrangement of the jets. The likelihood of co-impaction increases logarithmically with the number of propagules collected, expressed as the number of "positive holes". A statistical correction procedure known as "positive hole correction" has been applied to resolve this problem. This method, however, is widely misapplied and its utility is poorly understood. To be statistically valid, the application of a positive hole correction factor requires the user to determine the number of jets through which at least one particle has impacted. This can only be determined by examination of each jet impaction site by direct microscopy immediately following sampling. The enumeration of colonies following incubation cannot be used as a proxy measure for several reasons. Firstly, the deleterious effects of competition between co-impacted propagules may result in failure to detect any of the co-impacted propagules by culture. Secondly, the turbulence resulting from the deflection of air streams immediately beneath jets can lead to tangential impaction of free particles in the air stream, and can deposit particles arising from secondary redistribution or from comminution of previously collected particles. Thus, only particles located immediately below jets can be counted and all other particles

present on the plate must be ignored. After incubation, however, it is impossible to distinguish colonies coinciding with jets from those of interstitial origin. Given these limitations, the proper use of the positive hole correction method for multi-orifice jet-to-agar samplers is impractical for routine bioaerosol sampling.

- *Additional confounders*. The collection of culturable samples necessitates careful handling of sample media to avoid unintended contamination during handling. Carry-over from location to location on the exterior of the sampler, pump or ancillary equipment must also be avoided. Handling-related contaminants are often recognizable by their patterns of distribution, typically concentrated at the margins of agar plates or strips, and also by their consistent taxonomic composition from sample to sample. The use of so-called "field blanks", sample media that are taken to the field, loaded in the sampler and repackaged without sampling, handled always in the same manner as actual samples. The presence of contaminants on field blanks is confirmatory of handing-related contamination, and warrants cautious interpretation of data from the sample set.

Another potential confounder in culturable sampling on agar media arises from the presence of free water in the form of condensation in the Petri dish or sample container. It is widely recommended to cool agar plate or strip samples between field collection sites and the laboratory. This is usually accomplished by placing samples in an insulated box cooled with ice packs. However, rapid temperature changes may cause condensation to form in the sample containers. Free water is problematic on semi-solid growth media because collected cells tend to be redistributed on the medium surface. For yeasts, this redistribution can result in the formation of satellite colonies. Conversely, free water can wash the propagules of filamentous taxa from the surface of the medium and deposit them on the plastic walls of the container. Semi-solid growth media used for culturable sampling should be free of standing water at the time of sampling.

The risk of condensation forming on the interior of growth medium containers is greatly reduced when sample plates or strips are over-packed in strongly insulative materials, such as expanded polystyrene foam or paper, prior to transport. The use of cooling packs during transport should be carefully considered as this practice risks the formation of interior condensation which may lead to spurious results. During incubation, sampling media should be kept inverted to prevent the flow of any free water that may form to the medium surface.

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Biochemical detection of fungal extrolites

Quantification of fungal bioaerosols using analytical biochemical techniques has recently complemented traditional viable and non-viable sampling methodologies. These bioanalytical approaches encompass various separations, analytical, immunological and biophysical methods that have enabled the isolation, detection, structural identification and quantitative determination of biologically inactive and active compounds. For fungi, the analysis of fungal antigens, allergens, secondary metabolites, macromolecules, ergosterol and microbiological volatile organic compounds (mVOCs) has been made possible using various immunological and biophysical techniques.

Antigens

The development of monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) has been a significant analytical biochemical advancement. Antibodies are specialized immune proteins that are produced by B lymphocytes following immunization with native or reduced antigens. Specifically, pAbs are mixtures of immunoglobulin G (IgG) molecules secreted by different B cell lines against multiple epitopes of a specific antigen. In contrast to pAbs, mAbs are very specific Abs produced by fusing single antibody-forming B cells to tumor cells grown in culture. The resulting cell is called a hybridoma; it produces relatively large quantities of identical antibody molecules. Antibodies have been extensively used in combination with enzyme-linked immunosorbent assays (ELISA) for the qualitative or quantitative analysis of fungal allergens, antigens and mycotoxins in indoor, outdoor and occupational environments.

Important considerations when utilizing mAbs or pAbs in fungal exposure assessment studies include the extent of antibody cross reactivity (Trout *et al.* 2004, Schmechel *et al.* 2006) and metabolic spore activity (Green *et al.* 2003, Schmechel *et al.* 2008). This concern was recently addressed following studies that used an enzyme immunoassay-based assay for *A. alternata* (Barnes *et al.* 2006, Salo *et al.* 2005). In the National Survey of Lead and Allergens in Housing study, a pAb-based inhibition ELISA was used to measure the prevalence of *A. alternata* antigen in house dust samples from different locations throughout the United States (Salo *et al.* 2005). Salo *et al.* (2005) concluded that *A. alternata* antigens were detectable in 95-99% of American homes. However, the extent to which this pAb cross reacted with other fungi remained unclear. Recently, Schmechel *et al.* (2008) demonstrated that over 50% of all tested fungi (n=24) inhibited the binding of the same *A. alternata* pAb when tested by ELISA. These findings were confirmed by other immunoassays including

inhibition immunoblotting and Halogen Immunoassay (HIA) (Schmechel *et al.* 2008). The strongest inhibition was observed in pleosporalean fungi, including *Alternaria*, *Drechslera*, *Exserohilum*, *Stemphylium* and *Ulocladium* species. Several of these fungi may be common in indoor environments (Verhoeff *et al.* 1994). They have been shown to extensively cross react with *A. alternata* and some medically important fungi (Hong *et al.* 2005, Bowyer *et al.* 2006, Saˊenz-de-Santamaria *et al.* 2006).

The interpretation of antigen quantification data obtained with mAbs or pAbs should include consideration of the extent of spore and hyphal metabolic activity. This has recently been demonstrated by Schmechel *et al.* (2008) using the HIA. In this study, no pAb reactivity to *Curvularia lunata* ungerminated spores was identified; however, following spore germination elevated concentrations of antibody binding was recorded. Similar increases in antigen concentrations have been reported following spore germination for Asp f 1 (Sporik *et al.* 1993), Alt a 1 (Mitakakis *et al.* 2001) and other fungal aeroallergens (Green *et al.* 2003). Although the rate of germination varies according to the fungus involved as well as the incubation time, buffer type and presence of other fungi (Green *et al.* 2006), it is recommended to limit ELISA incubation times to avoid analytical variability associated with spore germination.

Continuing development of species specific antibodies for an extending range of common indoor fungi will allow comprehensive assessment of fungal exposure in the future. Until then, investigators undertaking fungal exposure assessment studies should thoroughly characterize the specificity of the antibody used for the quantification of airborne fungi, and they should limit incubation times. These actions will eliminate analytical bias and ambiguities in the interpretation of epidemiological results.

Ergosterol

Ergosterol is a sterol contained within fungal cell membranes. The presence of ergosterol in fungi and some protists has made this constituent a useful biomarker of fungal biomass in environmental assessments. The determination of ergosterol content in environmental samples is biochemically complex and involves a series of extractions and hydrolysis steps. Ergosterol is quantified using either high-performance liquid chromatography (HPLC) or gas chromatography-tandem mass spectrometry (GC-MS). Recent studies have demonstrated the utility of detecting this biomarker to provide a proxy measure of fungal biomass in contaminated indoor environments (Park *et al.* 2008). However, these methods are generally expensive and require a large amount of infrastructure and time to process and analyze samples.

Accordingly, these associated limitations have prevented the widespread use of this biomarker to quantify fungal biomass.

Beta-(1,3)-D-glucan

Glucan has been shown to be a useful measurement. An excellent review of the biochemical measurement of this airborne burden is given by Miller (2011).

Molecular genetic methods

The identification of indoor fungi by means of diagnostic sequences has been a well established and commonly used technique since the late 1990s (Haugland *et al.* 2004). Though initially developed for identifying *in vitro* cultures, the techniques can readily be modified to analyse the fungal contents of filtered air samples, dust samples or contaminated indoor materials. In an early example, Haugland and Heckman (1998) introduced specific primers for the important indoor fungus *Stachybotrys chartarum*. This study was shortly followed by the first of a series of species-specific quantitative PCR (qPCR) studies for indoor fungi, based on use of the TaqManä fluorogenic probe system combined with the ABI Prismâ Model 7700 Sequence Detector (Haugland *et al.* 1999). In this study, *S. chartarum* was again the object of study. qPCR counts of *S. chartarum* conidia were found to be highly comparable to counts obtained with a haemocytometer. The method was further developed by Roe *et al.* (2001) for direct quantitative analysis of *S. chartarum* in household dust samples.

This Taqman-based qPCR methodology was extended over subsequent years into a broad ranging methodology encompassing many major indoor air fungal groups (Haugland *et al.* 2004) and a variety of applications. In conjunction, key studies considered how best to extract DNA for qPCR and related PCR-based analyses of indoor fungi (Williams *et al.* 2001; Haugland *et al.* 2002; Kabir *et al.* 2003). Meklin *et al.* (2004) employed qPCR to evaluate indoor dust from the presence of 82 mold species or species complexes, including members of the genera *Aspergillus, Cladosporium, Penicillium, Trichoderma* and *Ulocladium*, in addition to *Stachybotrys* and the closely related *Memnoniella*. Comparisons among techniques showed that culturing underestimated numbers of key *Aspergillus* species by 2 to 3 orders of magnitude. "Moldy homes" could be distinguished from putatively uncontaminated "reference homes" using qPCR-based quantification. More medically oriented environmental studies developed Taqman qPCR for *Candida* yeasts in water samples (Brinkman *et al.* 2003) and *Aspergillus fumigatus* conidia in filtered air samples (McDevitt *et al.* 2004). More recently, the accuracy of qPCR for *A. fumigatus* detection in hospitals

has been very acutely tested using a comparison with green fluorescent protein (GFP)-expressing conidia of this species (McDevitt *et al.* 2005).

qPCR was used in conjunction with other modern techniques such as a quantitative protein translation assay for trichothecene toxicity in an evaluation of *Stachybotrys* from a house where a case of idiopathic pulmonary hemosiderosis had occurred (Vesper *et al.* 2000). However, haemocytometer counts of the relatively large and conspicuous *Stachybotrys* conidia were heavily relied on for quantitation in the data used in the subsequent analysis. A much more detailed, later qPCR analysis of homes where pulmonary hemosiderosis had occurred showed that *S. chartarum* was part of a group of species, also including *A. fumigatus* and several other *Aspergillus* species, that was significantly elevated in quantity in dust samples in affected homes (Vesper *et al.* 2004). Species abundant in affected homes tended to be hemolytic in *in vitro* testing, whereas the common species associated with reference homes were generally not hemolytic. Another significant application of the qPCR technique was to sensitively monitor *Aspergillus* contamination during hospital renovation (Morrison *et al.* 2004) and related infection control applications.

Relatively recent developments have included detailed studies of the fungal contents of dust from various sources, including studies optimizing qPCR to overcome chemical PCR inhibitors in dust (Keswani *et al.* 2005, Vesper *et al.* 2005). Multi-species qPCR has been applied to compare outdoor and indoor air (Meklin *et al.* 2007) and to analyse both fungi and bacteria in building materials such as chipboard, paper materials and insulation (Pietarinen *et al.* 2008). An elegant recent study has used propidium monoazide to inactivate DNA from dead cells prior to using qPCR to quantify viable conidia (Vesper *et al.* 2008). An online information page is available about the now widely used technology developed by R.A. Haugland, S.J. Vesper and other members of the US Environmental Protection Agency group (http://www.epa.gov/nerlcwww/moldtech.htm). Primers are given for 130 target species. For a more detailed account on molecular methods for bioaerosols characterization see Summerbell *et al.* (2011).

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Strategies – remediation