This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.

Designation: D7391 – 17[ε1]

Standard Test Method for Categorization and Quantification of Airborne Fungal Structures in an Inertial Impaction Sample by Optical Microscopy[1]

This standard is issued under the fixed designation D7391; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

ε[1] NOTE—Research report information added editorially in May 2017.

1. Scope

1.1 This test method is a procedure that uses direct microscopy to analyze the deposit on an inertial impaction sample.

1.2 This test method describes procedures for categorizing and enumerating fungal structures by morphological type. Typically, categories may be as small as genus (for example, *Cladosporium*) or as large as phylum (for example, basidiospores).

1.3 This test method contains two procedures for enumerating fungal structures: one for slit impaction samples and one for circular impaction samples. This test method is applicable for impaction air samples, for which a known volume of air (at a rate as recommended by the manufacturer) has been drawn, and is also applicable for blank impaction samples.

1.4 Enumeration results are presented in fungal structures/sample (fs/sample) and fungal structures/m^3 (fs/m^3).

1.5 The range of enumeration results that can be determined with this test method depends on the size of the spores on the sample trace, the amount of particulate matter on the sample trace, the percentage of the sample trace counted, and the volume of air sampled.

1.6 This test method addresses only the analysis of samples. The sampling process and interpretation of results is outside the scope of this test method.

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

1.9 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

3. Terminology

3.1 *ASTM Definitions (see the ASTM Online Dictionary of Engineering Science and Technology*[3]):

3.1.1 *numerical aperture.*

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *circular impaction sample, n*—a sample of airborne particulate matter collected by means of a device that draws air through a round aperture at a specified rate, impacting the particles suspended in the air onto an adhesive medium, resulting in a circular area of deposition. A circular impaction sample may be collected by means of a cassette manufactured for that purpose, or by means of a sampling device that requires slides to be pre-coated with impaction medium.

3.2.2 *debris rating, n*—a distinct value assigned to an impactor sample based on the percentage of the sample area potentially obscured by particulate matter, and ranging from 0 to 5.

[1] This test method is under the jurisdiction of ASTM Committee D22 on Air Quality and is the direct responsibility of Subcommittee D22.08 on Sampling and Analysis of Mold.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] *ASTM Online Dictionary of Engineering Science and Technology* (Stock #: DEFONLINE) is available on the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org.

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3.2.3 *field blank, n*—a sample slide or cassette carried to the sampling site, exposed to sampling conditions (for example, seals opened), returned to the laboratory, treated as a sample, and carried through all steps of the analysis.

3.2.4 *fungal structure (sing.), n*—a collective term for fragments or groups of fragments from fungi, including but not limited to conidia, conidiophores, and hyphae and spores.

3.2.5 *fungus (s), fungi, (pl.), n*—eukaryotic, heterotrophic, absorptive organisms that usually develop a rather diffuse, branched, tubular body (for example, network of hyphae) and usually reproduce by means of spores. The terms 'mold' and 'mildew' are frequently used by laypersons when referring to various fungal colonization.

3.2.6 *hyaline, adj*—colorless.

3.2.7 *impaction medium, n*—a substance applied to a microscope slide used to collect (or capture) particulate matter during sampling.

3.2.8 *impaction sample, n*—a sample taken using impaction, for example, slit impaction sample, circular impaction sample.

3.2.9 *inertial impactor, n*—a device for collecting particles separated from an air stream by inertia to force an impact onto an adhesive surface. Inertial impactors are available in many designs, including those having a slit jet, yielding a rectangular sample trace, and a circular jet, yielding a circular sample trace.

3.2.10 *magnification/resolution combination 1, n*—~150–400× total magnification and a point to point resolution of 0.7 µm or better, as checked by a resolution check slide.

3.2.11 *magnification/resolution combination 2, n*— ~400× or greater total magnification and a point to point resolution of 0.5 µm or better, as checked by a resolution check slide.

3.2.12 *minimum reporting limit (fs/sample); minimum reporting limit (fs/m^3), n*—the lowest result to be reported for total spores or any spore category. Since both fs/sample and fs/m^3 are reported, there are two minimum reporting limits.

3.2.13 *morphology, n*—the form and structure of an organism or any of its parts; for fungi, the shape, form, ornamentation, or combination thereof.

3.2.14 *mounting medium, n*—a liquid, for example, lactic acid or prepared stain, used to immerse the sample particulate matter and to attach a cover slip to an impaction sample.

3.2.15 *sample trace, n*—the area of particle deposition, that is, the deposit on a slit impaction sample resembling a narrow rectangle, or the circular deposit on a circular impaction sample.

3.2.16 *septum (pl.: septa), n*—a cell wall or partition.

3.2.17 *slide adherent, n*—an adhesive or liquid used to affix an impaction sample substrate to a microscope slide.

3.2.18 *slit impaction sample, n*—a sample of airborne particulate matter collected by means of a device that draws air through a linear aperture at a specified rate, impacting the particles suspended in the air onto an adhesive medium, resulting in a rectangular area of deposition. A slit impaction sample may be collected by means of a cassette manufactured for that purpose, or by means of a sampling device that requires slides to be pre-coated with impaction medium.

3.2.19 *spore category, n*—a grouping used for identification and quantifation of fungal structures. A spore category may contain a specific genus (for example, *Stachybotrys*), or it may represent a combination of genera (for example, *Aspergillus/Penicillium*-like).

3.2.20 *traverse, n*—a portion of analysis of an impactor sample consisting of one scan under the microscope from a sample-less portion of the impaction medium across the deposit to a corresponding sample-less portion of the impaction medium on the other side.

3.3 *Symbols:*

3.3.1 *fs*—fungal structure

3.3.2 *fs/m^3*—fungal structures per cubic metre

3.3.3 *m^3*—cubic metre

3.3.4 *mm*—millimetre

3.3.5 *µm*—micrometre

4. Summary of Test Method

4.1 Samples have been previously collected utilizing an impaction device operating at the device manufacturer's recommended sample flow rate. Each sample consists of an optically clear substrate coated with an adhesive and optically transparent medium onto which particles have been deposited through inertial impaction.

4.2 A sample is mounted to a microscope slide and examined by bright field microscopy using at least two magnification/resolution combinations.

4.3 Spores are differentiated from each other, other fungal structures, and from non-fungal material by color, size, shape, presence of a septum or septa, attachment scars, surface texture, etc., by means of a taxonomic comparison with standard reference texts or known standard samples, or both (see Section A1.1 for suggested references). The number of spores that match each spore category are then calculated in units of fungal structures per sample (fs/sample) and also fungal structures per cubic meter of air (fs/m^3).

5. Significance and Use

5.1 This test method is used to estimate and categorize the number and type of fungal structures present on an inertial impactor sample.

5.2 Fungal structures are identified and quantified regardless of whether they would or would not grow in culture.

5.3 It must be emphasized that the detector in this test method is the analyst, and therefore results are subjective, depending on the experience, training, qualification, and mental and optical fatigue of the analyst.

6. Interferences

6.1 *Differentiation of Fungal Genera/Species*—Because of the similar size and morphology of some fungal spores of different genera and the absence of growth structures and mycelia in airborne samples, differentiation by microscopic

examination alone is difficult and spores must be grouped into categories based strictly on morphology. In many cases, identification at the genus level is presumptive. For example, differentiation between *Aspergillus* and *Penicillium* using this test method is not typical, so a combined *Aspergillus*/ *Penicillium*-like category is used. When differentiation between such genera is desired, a different test method must be used. Unequivocal identification of every spore in each category is not possible due to optical limitations, the atypical nature of some of the spores, overlapping morphology among different spore types, or combination thereof, and therefore, certain spores must be categorized as Miscellaneous/ Unidentifiable.

6.2 *Look-Alike Non-Fungal Particles*—Certain types of particles of non-fungal origin may resemble fungal spores. These particles and artifacts may include air or plant resin bubbles, starch, talc, cosmetic particles, or combustion products. Standards (mounted similarly to impactor samples) should be examined by laboratory analysts to know how to identify such particles. Examination of suspect particles using optical conditions other than bright field microscopy (for example, polarized light microscopy, phase contrast microscopy, differential interference contrast) may be helpful whenever significant concentrations of look-alike particles are present. In some cases dust and debris can mimic the morphology of particles of interest. When look-alike particles are present in high concentration, accurately counting spores with similar morphology is difficult. When these conditions exist, they should be reported in the analysis notes section of the report.

6.3 *Particle Overloading*—High levels of particulate matter on an impaction sample will bias the analysis in two ways:

(1) Particle capture efficiency decreases, and

(2) Debris obscures or covers spores.

Both of these factors produce a negative bias.

6.4 *Staining*—Staining, while optional, may help the analyst differentiate spores from debris. Without staining, clear spores (especially small ones) may exhibit negative bias because the analyst has insufficient contrast to notice them while scanning. Also, because spores of different fungal species absorb stains at different rates, under or over-staining makes identification difficult. The problem can be eliminated by careful control of stain concentrations.

6.5 *Impaction Medium Stability and Clarity*—Chemicals present in some mounting media may affect the physical stability or clarity of the impaction medium. For instance:

(1) Samples collected on silicone grease medium should first be warmed on a hot plate at approximately 40°C to "fix" the sample in place, when using lacto-phenol cotton blue stain, and

(2) Slides and cassettes using methyl cellulose ester + solvent adhesive medium, which is stable in lacto-phenol cotton blue stain, will "fog" with Calberla's stain due to the water and alcohol mixture; warming fogged slides may temporarily clear them.

The lab or analyst should develop through experimentation an impaction medium/mounting medium combination that will result in acceptable stability, clarity, and spore visibility.

6.6 *Uneven Impaction Medium Uniformity*—Uneven thickness may be present in greased slides, pre-coated slides and manufactured cassettes. The microscopist will compensate by adjusting the plane of focus. When grease is too thick, differentiating small spores from background artifacts (especially air bubbles) in the grease preparation becomes difficult. When grease is too thin, shrinkage and pooling may have occurred, causing particle loss during sampling.

7. Apparatus

7.1 *Marking pen,* for marking sample slides.

7.2 *Microscope or magnification system,* having a precision *x-y* mechanical stage. The microscope or magnification system used for analysis shall be capable of at least two magnification/ resolution combinations as follows: magnification/resolution combination 1 shall be ~150–400× total magnification and a point to point resolution of 0.7 µm or better; magnification/ resolution combination 2 shall be ~400× or greater total magnification and a point to point resolution of 0.5 µm or better. It is recommended that at least one microscope or magnification system in the lab be capable of magnification of ~1000× total magnification and a point to point resolution of 0.3 µm or better. That the resolution for combinations 1 and 2 is suitable is to be checked using a resolution check slide (see 13.2.3).

7.3 *Reference Slides*—a series of mounted field samples to be used as counting references. Analysts' results from these slides are expected to be within laboratory acceptance limits to prove competence.

7.4 *Reticule,* width defining, an optical device in the light path of the microscope capable of being reproducibly set to define a traverse width no larger than 0.75× the diameter of the ocular field of view, and having graduations of an appropriate dimension to allow measurement of spore size, for example, Walton-Beckett reticule (round) or 100 divisions in 10 mm (linear or square). If a non-round reticule is used, procedures must be in place to ensure that the reticule is correctly positioned for each analysis.

7.5 *Stage micrometer,* traceable to the National Institute of Standards and Technology (NIST) or equivalent international standard.

7.6 *Resolution check slide,* a microscope slide on which calibrated distances, shapes, and line widths provide reliable and simple image resolution and shape identification performance of the microscopic and analyst at magnification. Examples include: a slide onto which a variety of diatoms have been mounted, including examples of *Stauroneis phoenicenteron* and *Pleurosigma angulatum*, a brightfield resolution test slide, or equivalent.

7.7 *Syringe or dropper,* for dispensing liquid during sample preparation.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on

Analytical Reagents of the American Chemical Society where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type II of Specification D1193.

8.3 *Mounting medium (with or without stain),* for re-hydrating spores and for holding the cover slip to the impaction sample, for example, lactic acid, lacto-cotton blue stain, lacto-phenol-cotton blue stain, lacto-fuchsin stain (see Section X2.1 for stain preparation).

8.4 *Microscope cover slips,* large enough to cover the deposit (for example, 22 mm^2); for optimum performance, choose a cover slip thickness according to the recommendations of the microscope objective lens manufacturer.

8.5 *Microscope slides.*

8.6 *Slide adherent,* for affixing impaction cassette samples to microscope slides, for example, clear nail polish, immersion oil, tape.

9. Hazards

9.1 Components of re-hydrating liquids and stains, for example, lactic acid, phenol, are corrosive or hazardous. Consult the appropriate MSDS for any reagents used.

10. Preparation of Apparatus

10.1 *Microscope Alignment/Adjustments*—Follow the manufacturer's instructions.

11. Calibration and Standardization

11.1 *Diameter/Width and Graduation Spacing for Ocular Reticule*—see 13.2.2.

12. Procedure

12.1 *Sample Preparation:*

12.1.1 *Preparation of a Pre-Coated Slide* (the impaction medium is already on a microscope slide).

12.1.1.1 Mark each slide with a unique designation.

12.1.1.2 If necessary (for example, for grease medium), gently warm to no more than 40°C to "fix" impacted particles in place.

12.1.1.3 Place one drop of mounting medium near the deposition trace and cover with a clean cover slip. Gently lower the cover slip at a slight angle to minimize air bubble formation. If the liquid contains stain, allow the stain to fully penetrate the particles before enumeration.

12.1.2 *Preparation of a Cassette:*

12.1.2.1 Cut the sealer on the cassette and dismantle into two parts.

12.1.2.2 Mark each slide with a unique designation.

12.1.2.3 If the impaction substrate is not of suitable size/thickness to be examined on the microscope, it must be mounted on a microscope slide.

(1) Place a drop of slide adherent on a clean microscope slide.

(2) Carefully pull the glass or substrate that contains the adhesive film and the sample from the cassette, and place it, sample side upwards, on the slide adherent. Gently lower the glass slip at a slight angle to minimize air bubble formation if using liquid adherent.

12.1.2.4 Place one drop of mounting medium on the sample trace or cover slip. Gently lower the cover slip onto the sample trace at a slight angle to minimize air bubble formation. If the liquid contains stain, allow the stain to fully penetrate the particles before enumeration.

12.1.2.5 *(Optional)*—Mark the approximate maximum extent of scan (~3 mm larger than the visible deposit) on the underside of the slide using a marking pen. This is especially useful for lightly loaded samples, in which the area to be scanned may not be obvious when the slide is observed on the microscope.

12.2 *Preliminary Evaluation:*

12.2.1 The purpose of this examination is to note possible sample problems and to assign a debris rating. Use magnification/resolution combination 1.

12.2.2 Examine the entire sample trace. Note on the worksheet non-uniform deposition or other sample problems.

12.2.3 *Debris Rating Determination:*

12.2.3.1 From the amount of particulate matter present at that part of the sample trace having approximately the greatest particle load, assign the sample a debris rating on a scale from 0 to 5. (A description of the debris rating numbers is given below in Table 1.) Since the amount of debris in a field of view varies with the field of view chosen and its position in the sample trace, choose a rating that is most representative of a number of fields of view taken from the middle (not the more lightly loaded edges) of the trace.

12.3 *Counting Procedure for Slit Impactor:*

12.3.1 Categorize each observed spore based on color, morphology, size, etc.

12.3.2 Categorize, at a minimum, the spore categories:

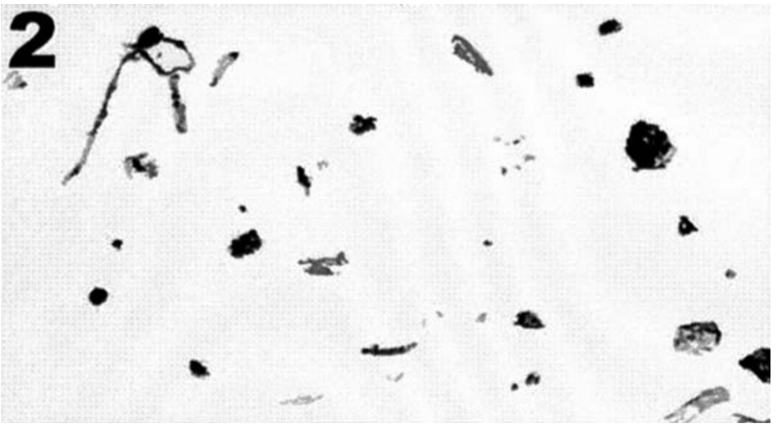
(1) Alternaria,
(2) ascospores (undifferentiated),
(3) Aspergillus/Penicillium-like
(4) basidiospores (undifferentiated),
(5) Chaetomium,
(6) Cladosporium,
(7) Curvularia,
(8) Drechslera/Bipolaris-like,
(9) smuts/Myxomycetes/*Periconia*,
(10) Stachybotrys/Memnoniella,
(11) Ulocladium, and
(12) hyphal fragments

For a fungal structure having characteristics inconsistent with all reported categories, enumerate it under the general category: Miscellaneous/Unidentified.

NOTE 1—Categories other than the above minimum may be used and reported, if desired. Fungal structures in the Miscellaneous/Unidentified category may be grouped by characteristics and reported separately, if desired, for example, Miscellaneous/Unidentified 1, Miscellaneous/Unidentified 2, etc.

12.3.3 Enumerate spore categories at an appropriate magnification/resolution. Enumerate the spore categories

TABLE 1 Debris Rating Table

Non-Microbial Particle Debris Rating (all photos taken at 600× magnification)	Description	Interpretation
0	No particulate matter detected in impaction area.	The absence of particulate matter in the impact area could indicate improper sampling or a blank sample, as most air samples typically contain some particles. Such absence of particulate matter should be noted on the report if the sample was not meant to be a blank.
	Minimal (>non detect to approx. 5 %) particulate matter present.	Reported values are minimally affected by particle load.
	Approx. 5 % toapprox. 25 % of the trace occluded with particulate matter.	Negative bias is expected. The degree of bias increases with the percent of the trace that is occluded.

Aspergillus/*Penicillium*-like and *Cladosporium* at magnification/resolution 2 and other spore categories at either magnification/resolution 1 or 2.

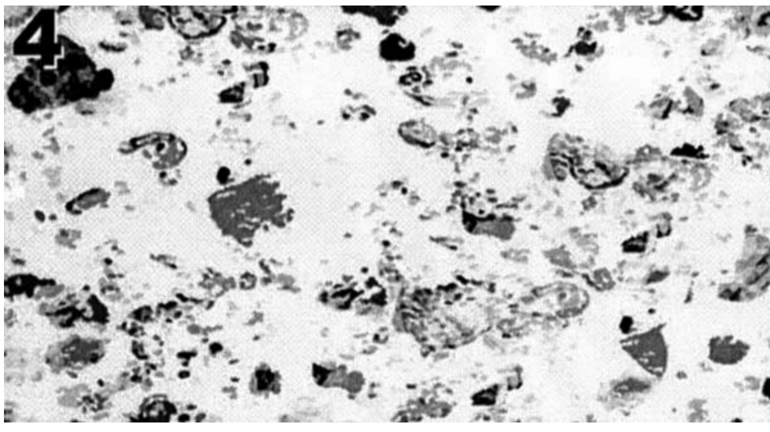
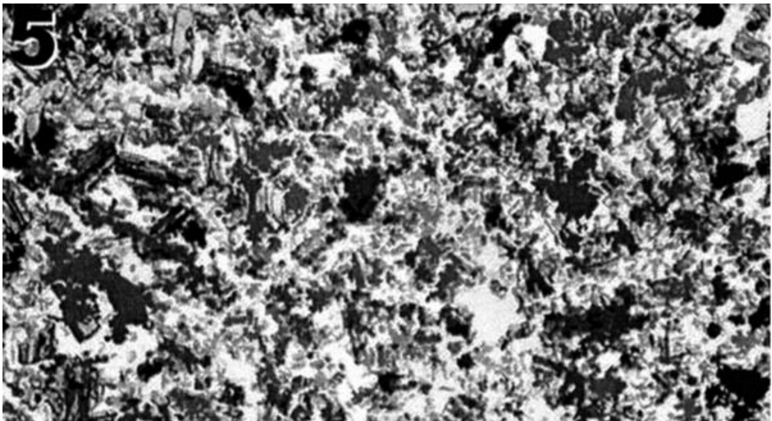
NOTE 2—Spores that are especially small or hyaline are best enumerated at magnification/resolution 2. The categories basidiospores (undifferentiated) and ascospores (undifferentiated) are so variable that some spores in these categories could be enumerated at magnification/resolution 1, but others should be enumerated at magnification/resolution 2. The spore categories *Alternaria*, *Chaetomium*, *Curvularia*, *Drechslera*/*Bipolaris*-like, smuts/Myxomycetes/*Periconia*-like, *Stachybotrys*/*Memnoniella*, *Ulocladium*, hyphal fragments, and Miscellaneous/Unidentified may be enumerated at either magnification/resolution 1 or 2.

12.3.4 Enumerate a minimum of 20 % sample trace.

NOTE 3—An analyst/lab could decide to enumerate the minimum of 20 % for all spore categories, or decide to enumerate 100 % for all spore categories, or decide to enumerate for each spore category a percentage between 20 and 100 based on experience, on quality objectives or on how many spores in that category appeared to be present during the initial screening.

12.3.5 Enumerate during traverses across the sample trace. A traverse is one scan across the sample trace in a direction perpendicular to the longest dimension of the sample trace.

TABLE 1 *Continued*

	Approx. 25 % to approx. 75 % of the trace occluded with particulate matter.	Negative bias is expected. The degree of bias increases with the percent of the trace that is occluded.
	Approx. 75 % to approx. 90 % of the trace occluded with particulate matter.	Negative bias is expected. The degree of bias increases with the percent of the trace that is occluded.
	Greater than approx. 90 % of the trace occluded with particulate matter.	Quantification is not possible due to large negative bias. A new sample should be collected at shorter time interval, or other measures taken to reduce the particle load.

12.3.5.1 Start above or below the visible apparent deposit and scan across the deposit until well off the visible deposit. This process constitutes one traverse.

12.3.5.2 As a traverse is made, the outer edges of the ocular reticule will be used to describe the boundaries of a rectangular counting area.

12.3.5.3 During a traverse, identify and categorize, the fungal structures that appear to fall within the outer edges of the ocular reticule If a fungal structure appears to lie on the left boundary line of the ocular reticule, count this structure. If a fungal structure appears to lie on the right boundary line of the ocular reticule, do not count it.

12.3.5.4 An analysis consists of a number of complete traverses. Do not use partial traverses.

12.3.5.5 If enumerating varying percentages for each spore category, pre-determine the % of the sample trace to be enumerated, so that traverses may be chosen to cover the sample trace more or less evenly. That is, do not start counting 100 % of the sample trace for all spore categories (using adjacent traverses), and then stop counting certain spore

categories part of the way through the analysis, since such a count would be biased low due to the lightly loaded first few traverses.

12.3.5.6 If enumerating 100 % of the sample trace, start the traverses slightly outside the visible end of the sample trace, to ensure that all spores are within the scanned area. If enumerating less than 100 % of the sample trace, start the traverses slightly inside the visible end of the sample trace, where the deposit appears to become uniform in order to obtain a representative traverse. Avoid the extreme end of the sample trace where the deposit appears to become less dense.

12.3.5.7 If enumerating 100 % of the sample trace, choose each subsequent counting area so that it abuts the previous counting area to provide full coverage, as shown in Fig. 1. If enumerating less than 100 % of the sample trace, separate traverses to prevent overlap. In this case, it is recommended that the total number of traces more or less evenly cover the trace, as shown in Fig. 2.

12.3.6 *Stopping Rule:*

12.3.6.1 Start an analysis for that spore category as if less than 100 % of the sample trace is to be enumerated, that is, not at the extreme end and separating the traverses.

12.3.6.2 Discontinue counting after the completion of the traverse during which either: Option (*1*) 100 fungal structures are reached for that category (and continue counting for the remaining categories), or Option (*2*) 300 total fungal structures are reached.

NOTE 4—Option 1 may be useful when one or two fungal categories predominate – counting may be discontinued on predominant categories while still producing data on other categories. Option 2 is useful if category counts are adequately characterized by 300 total counts.

12.3.6.3 If <3 traverses are to be counted for a given spore category, take care to choose traverses that appear to be representative for that spore category.

12.3.6.4 For extremely high fungal structure counts (>100 per traverse), enumerate by grouping fungal structures into approximately even groups (for example, 10 or 50) and count the groups.

NOTE 5—The justification for using 100 fungal structures as a stopping rule in this test method is that it results in a 10 % relative standard deviation for a Poisson distribution.

12.3.7 Record the fungal structure count and either the number of traverses performed or the percentage of trace counted for each spore category.

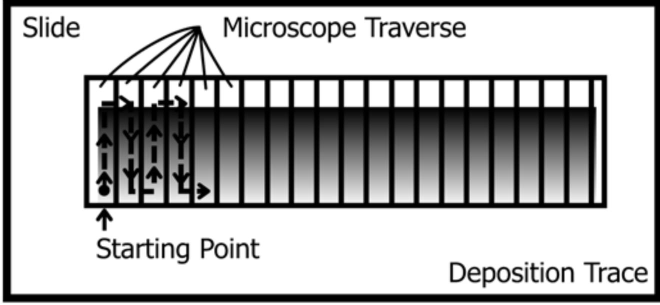


FIG. 1 Slit Impactor Location of Traverses for Counting 100 % of the Sample Trace

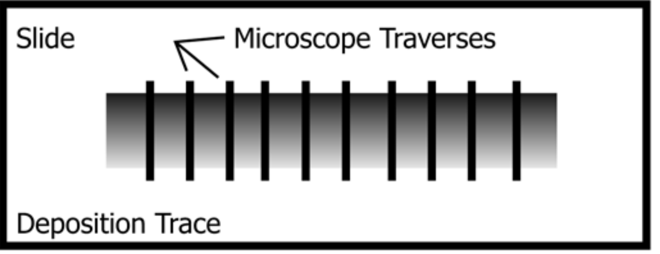


FIG. 2 Slit Impactor Location of Traverses for Counting <100 % of the Sample Trace

12.3.8 *Calculations:*

12.3.8.1 For each spore category:

$$\text{percentage scanned} = 100 * \frac{(\text{width of ocular reticle in } \mu\text{m}) * (0.001 \text{ mm}/\mu\text{m}) * (\text{number of traverses})}{(\text{length of deposit in mm})}$$

$$\text{fungal structures/sample} = 100 * \frac{\text{fungal structure count during traverses}}{\text{percentage scanned}}$$

$$\text{fungal structures/m}^3 = \frac{\text{fungal structures/sample}}{\text{volume } (\text{m}^3)}$$

12.3.8.2 Example: cassette (deposit length = 14.4 mm), 75 L volume, 30 traverses, ocular reticle width = 100 µm, one fungal structure observed.

$$\text{percentage scanned} = (100 * (100 \ \mu\text{m}) * (0.001 \ \text{mm}/\mu\text{m}) * 30)/14.4 \text{ mm} = 20.8 \%$$

$$\text{fs/sample} = (100 * 1 \ \text{spore})/20.8 \% = 4.8 \text{ fs/sample}$$

$$\text{fs/m}^3 = (4.8 \ \text{fs/sample})/0.075 \text{ m}^3 = 64 \text{ fs/m}^3$$

NOTE 6—Because the example is based on one fungal structure, the calculation is the same as the minimum reporting limit for this spore category (see 15.3).

12.4 *Counting Procedure for Circular Impactor*—Impactors having a circular deposit have been observed to have a toroidal-shaped area of heavier deposit.[4] For this reason, the following rules have been designed to either count the entire deposit, or to eliminate bias by traverses across the middle of the toroid.

12.4.1 Categorize each observed spore based on color, morphology, size, etc.

12.4.2 The minimum categories to be reported are:

(*1*) *Alternaria*,
(*2*) ascospores (undifferentiated),
(*3*) *Aspergillus*/*Penicillium*-like
(*4*) basidiospores (undifferentiated),
(*5*) *Chaetomium*,
(*6*) *Cladosporium*,
(*7*) *Curvularia*,
(*8*) *Drechslera*/*Bipolaris*-like,
(*9*) smuts/Myxomycetes/*Periconia*,
(*10*) *Stachybotrys*/*Memnoniella*,
(*11*) *Ulocladium*, and
(*12*) hyphal fragments.

[4] Grinshpun, S., et al. "Collection of Airborne Spores by Circular Single-Stage Impactors with Small Jet-to-Plate Distance," *Journal of Aerosol Science*, Vol 36, No. 5, 2005, pp. 575–591.

For a fungal structure having characteristics inconsistent with all reported categories, enumerate it under the general category: Miscellaneous/Unidentified.

NOTE 7—Categories other than the above minimum may be used and reported, if desired. Fungal structures in the Miscellaneous/Unidentified category may be grouped by characteristics and reported separately, if desired, for example, Miscellaneous/Unidentified 1, Miscellaneous/Unidentified 2, etc.

12.4.3 Enumerate fungal structures at an appropriate magnification/resolution. Enumerate the spore categories *Aspergillus*/*Penicillium*-like and *Cladosporium* at magnification/resolution 2 and other spore categories at either magnification/resolution 1 or 2.

NOTE 8—Spores that are especially small or hyaline are best enumerated at magnification/resolution 2. The categories basidiospores (undifferentiated) and ascospores (undifferentiated) are so variable that some spores in these categories could be enumerated at magnification/resolution 1, but others should be enumerated at magnification/resolution 2. The spore categories *Alternaria*, *Chaetomium*, *Curvularia*, *Drechslera*/*Bipolaris*-like, smuts/Myxomycetes/*Periconia*-like, *Stachybotrys*/*Memnoniella*, *Ulocladium*, hyphal fragments, and Miscellaneous/Unidentified may be enumerated at either magnification/resolution 1 or 2.

12.4.4 Enumerate during traverses across the sample trace. A traverse is one scan across the sample trace. Traverses are parallel to one another.

12.4.4.1 Start left or right (or above or below) the visible apparent deposit and scan across the deposit until well off the visible deposit. This process constitutes one traverse.

12.4.4.2 An analysis consists of a number of complete traverses. Do not use partial traverses.

12.4.4.3 During a traverse, identify and categorize, the fungal structures that appear to fall within the outer edges of the ocular reticule If a fungal structure appears to lie on the left boundary line of the ocular reticule (or upper if traversing horizontally), count this structure. If a fungal structure appears to lie on the right boundary line of the ocular reticule (or lower if traversing horizontally), do not count it.

12.4.5 Enumerate a minimum of 20 % sample trace.

NOTE 9—An analyst/lab could decide to enumerate the minimum of 20 % for all spore categories, or decide to enumerate 100 % for all spore categories, or decide to enumerate for each spore category a percentage between 20 and 100 based on experience, on quality objectives or on how many fungal structures in that category appeared to be present during the initial screening.

12.4.5.1 If enumerating 100 % of the sample trace, start the traverses slightly outside the visible end of the sample trace, to ensure that all spores are within the scanned area. Choose each subsequent counting area so that it abuts the previous counting area to provide full coverage. Continue as shown in Fig. 3.

12.4.5.2 If enumerating less than 100 % of the sample trace, start the traverses at the apparent middle of the sample trace. Choose each subsequent counting area so that it is adjacent to the previous counting area. Continue towards one end as in Fig. 4.

12.4.5.3 If it is desired to count 50–100 % of the deposit, first count an entire half, then count a partial half as above.

12.4.6 *Stopping Rule:*

12.4.6.1 Start an analysis for that spore category as if less than 100 % of the sample trace is to be enumerated, that is, Fig. 4.

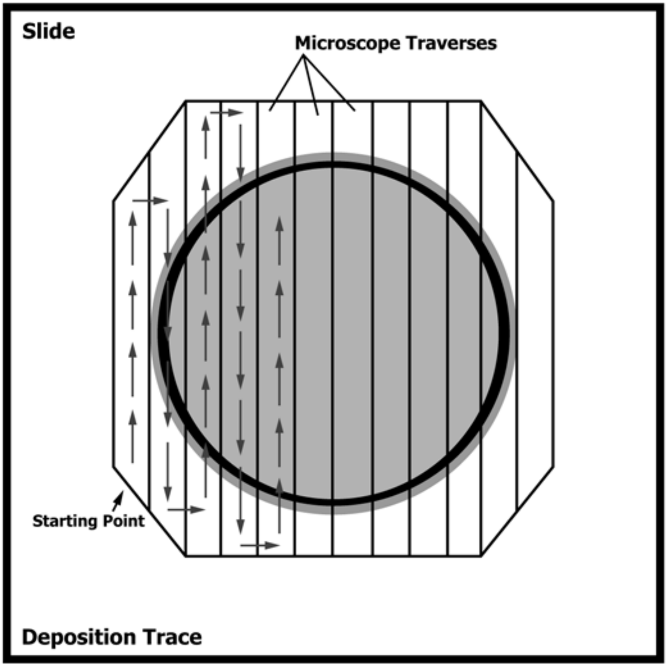


FIG. 3 Circular Impactor Location of Traverses for Counting 100 % of Sample Trace

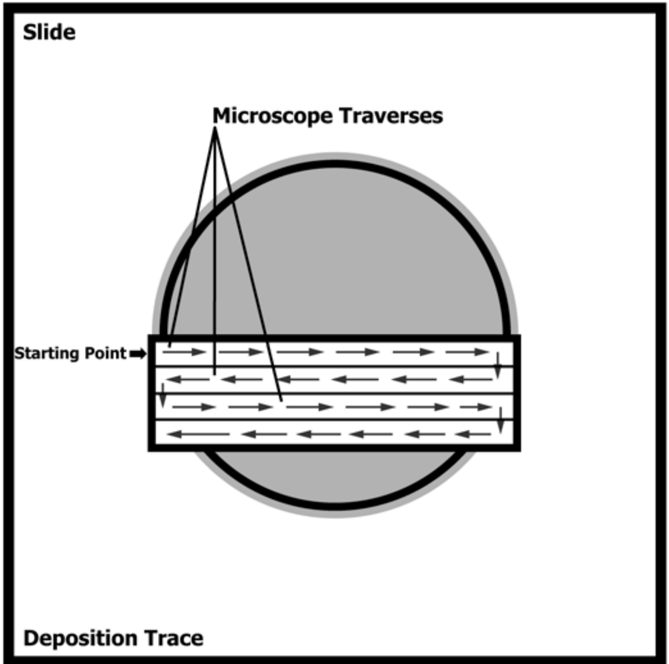


FIG. 4 Circular Impactor Location of Traverses for Counting <100 % of Sample Trace

12.4.6.2 Discontinue counting after the completion of the traverse during which either: Option (*1*) 100 fungal structures are reached for that category (and continue counting for the remaining categories), or Option (*2*) 300 total fungal structures are reached.

NOTE 10—Option 1 may be useful when one or two fungal categories predominate – counting may be discontinued on predominant categories while still producing data on other categories. Option 2 is useful if

category counts are adequately characterized by 300 total counts.

12.4.6.3 For extremely high fungal structure counts (>100 per traverse), enumerate by grouping spores into approximately even groups (for example, 10 or 50) and count the groups.

NOTE 11—The justification for using 100 fungal structures as a stopping rule in this test method is that it results in a 10 % relative standard deviation for a Poisson distribution.

12.4.7 Record the spore count and either the number of traverses performed or the percentage of trace counted for each spore category.

12.4.8 *Calculations:*

12.4.8.1 *For Each Spore Category*—The area scanned for a <100 % count is the portion of the circle that includes the areas marked 1, 2 and 3 in Fig. 5.

$$R = \text{radius of the deposit circle (mm)}$$

$$d = \text{width of counting (mm)}$$

$$= \text{number of traverses} * \text{width } (\mu m) \text{ of the ocular reticle} * 0.001 \text{ mm}/\mu m$$

$$\theta = \text{angle subtended by the last traverse} = 2 * \cos^{-1} (d/R)$$

$$\text{percentage scanned} = 100 * \frac{\text{areas of pie pieces 1 and 2} + \text{area of the triangle 3 in Figure 5}}{\text{area of entire deposit}}$$

$$= 100 * \frac{2 * \left(\pi R^2 * \left(\frac{0.5 * (180 - \theta)}{360} \right) \right) + d * R * \sin (0.5 * \theta)}{\pi R^2}$$

$$\text{fungal structure/sample} = 100 * \frac{\text{fungal structure count during traverses}}{\text{percentage scanned}}$$

$$\text{fungal structures/m}^3 = \frac{\text{fungal structures/sample}}{\text{volume (m}^3)}$$

12.4.8.2 Example: cassette having deposit diameter = 2.3 mm, 50 L volume, four traverses, ocular reticle width = 100 µm, one fungal structure observed.

R = 1.15 mm
d = 4 * 100 µm * 0.001 mm/µm = 0.4 mm
θ = 2 * $\cos^{-1}$(d/R)(0.4 mm/1.15 mm) = 139.3°

$$\text{percentage scanned} = 100 * \frac{2 * \left(\pi R^2 * \left(\frac{0.5 * (180 - \theta)}{360} \right) \right) + d * R * \sin (0.5 * \theta)}{\pi R^2}$$

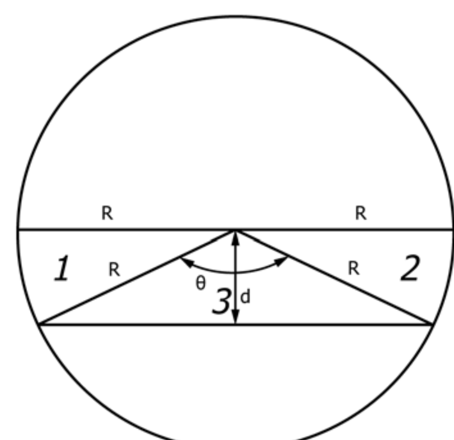


FIG. 5 Circular Impactor Calculation Diagram for Counting <100 % of Sample Trace

$$= 100 * \frac{2 * (4.155 \text{ mm}^2 * 0.0565) + (0.4 \text{ mm} * 1.15 \text{ mm} * 0.9375)}{4.155 \text{ mm}^2}$$

$$= 100 * \frac{0.4695 \text{ mm}^2 + 0.4312 \text{ mm}^2}{4.155 \text{ mm}^2} = 21.7 \%$$

$$\text{fungal structures per sample} = 100 * 1 \text{ fungal structure}/21.7$$

$$= 4.6 \text{ fungal structures per sample}$$

$$\text{fs/m}^3 = 4.6 \text{ fungal structures per sample}/0.050 \text{ m}^3 = 92 \text{ fs/m}^3$$

NOTE 12—Because the example is based on one fungal structure, the calculation is the same as the minimum reporting limit for this spore category (see 15.3).

13. Quality Assurance/Quality Control

13.1 Establish and maintain a quality assurance/quality control system for this analysis, to include, at least, the following (Accreditation bodies, such as the American Industrial Hygiene Association, may require specific frequencies for the following, or may require other QA/QC tasks.):

13.2 *Calibration:*

13.2.1 *Width of Reticule (width of traverse)*—Measure and calculate at least once per year, and after any major service or repair to the microscope, to be performed using a stage micrometer, and at the magnification(s) where a reticule width is used for counting.

13.2.2 *Spacing of Measuring Gradations on the Ocular Reticule*—Calibrate the µm per graduation, using a stage micrometer, at the magnification(s) used for counting at least once per year, and after any service or repair to the microscope. The graduations are used to measure the size of spores as an aid to identification.

13.2.3 *Resolution Check*—Check the resolution of magnification/resolution combinations 1 and 2 at least annually for each analyst, as in accordance with manufacturer's instructions for the resolution check slide used.

13.3 *Contamination Control:*

13.3.1 *Housekeeping*—Keep preparation and analysis areas clean, for example, routinely wet-wipe to minimize transfer of lab dust to samples.

13.3.2 *Process/Medium Blank*—At a defined frequency, place in the sample preparation area during sample preparation a slide containing blank impaction medium, for example, a disassembled cassette, or a clean, greased slide. When the sample batch has been prepared, place a drop of mounting medium on the impaction medium followed by a cover slip, to create a process blank that includes all glass and liquid components of a typical sample. Analyze in the same manner as a sample. Establish acceptance criteria for such blanks.

13.4 *Precision and Accuracy:*

13.4.1 *Analyst Training and Qualification*—Qualify an analyst to be competent to perform this test method by a combination of background and education, aerobiological and mycological training, experience, and performance on impactor samples of known/reference content (for example, reference slides). Analyst qualification should be continuing, through routine comparison with other analysts. For single-person organizations, such comparison would necessarily be inter-laboratory exchange.

13.4.2 *Re-Analysis*—Recount a minimum of 10 % of client samples (determined randomly or arbitrarily). Set statistically-based acceptance limits (for example, maximum relative percent difference).

14. Records

14.1 Record at least the following data for each sample:

(1) analyst (for example, initials on the worksheet)

(2) date of analysis

(3) reference to the microscope used (if multiple scopes are present)

(4) cassette brand or other designation of sample type, including whether it is slit or circular design

(5) laboratory number or unique number for each sample

(6) debris rating

(7) raw fungal structure counts for each spore category

(8) magnification used for each spore category

(9) number of traverses counted or % of deposit counted for each spore category, if the entire sample was not counted for that category, and whether estimation has been used

(10) identities and counts for any additional spore categories used above the minimum categories

(11) notes on sample condition, broken cassette or substrate, missing cap, loading, out-dated cassettes, analytical problems, conidiophores seen, etc.

15. Report

15.1 The test report shall include at least the following:

(1) Reference to this test method, and which counting rules were used

(2) Laboratory identification, address, telephone number

(3) Client identification and address

(4) Client sample identification

(5) Laboratory unique identification/laboratory number

(6) Date and time of sampling, if known

(7) Date and time of sample receipt

(8) Condition of sample (that is, any problems with condition)

(9) Date of analysis

(10) Date of report

(11) Analyst name

(12) Signature and printed name of person taking responsibility for the data in the report

(13) Significant modifications to this procedure, if any

(14) Page number and total number of pages in the report on each page or other mechanism for identifying each page as part of the report and for indicating the end of the report

(15) Statement that the analysis relates only to the items tested

(16) Debris rating for each sample

(17) fs/sample for each spore category reported and total fs/sample

(18) An indication of the proportion of the trace that was analyzed for each spore category (for example, % of trace read, # traverses, minimum reporting limit, multiplication factor).

(19) fs/m^3 for each spore category and for total spores reported for samples having non-zero volumes

(20) Minimum reporting limit (fs/m^3) for each spore category reported

(21) Summary of any out of control situations connected to the analysis

(22) Notes on sample condition, broken cassette or substrate, missing cap, loading, out-dated cassettes, analytical problems, conidiophores seen, apparent sampling problems, etc.

15.2 Report results for, at a minimum, the following spore categories:

(1) Alternaria

(2) ascospores (undifferentiated)

(3) Aspergillus/Penicillium-like

(4) basidiospores (undifferentiated)

(5) Chaetomium

(6) Cladosporium

(7) Curvularia

(8) Drechslera/Bipolaris-like

(9) smuts/Myxomycetes/*Periconia*

(10) Stachybotrys/Memnoniella

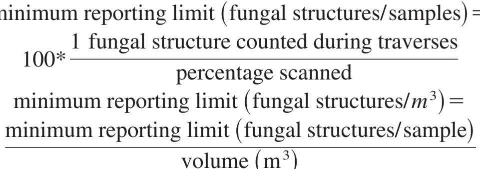
(11) Ulocladium

(12) hyphal fragments, and

(13) at least one category of Miscellaneous/Unidentifiable spores.

Additional categories may provide valuable information.

15.3 *Minimum Reporting Limit*—For each spore category, report results no lower than the minimum reporting limit for that category (see below). For spore categories in which no spores were counted, report as "n.d." (not detected) or as "<" and minimum reporting limit.



$$\text{minimum reporting limit (fungal structures/samples)} = 100 * \frac{1 \text{ fungal structure counted during traverses}}{\text{percentage scanned}}$$

$$\text{minimum reporting limit (fungal structures}/m^3) = \frac{\text{minimum reporting limit (fungal structures/sample)}}{\text{volume (m}^3)}$$

NOTE 13—It is suggested that a statistically based method detection limit or method quantification limit be calculated and reported as well as the minimum reporting limit.

15.4 *Significant Figures*—Report numbers that are actual counts as whole numbers; report numbers that are calculated counts to no greater than two significant figures.

15.5 *Bias*—For a sample having a debris rating of 2–4, report as being possibly negatively biased.

15.6 *Overloading*—For a debris rating of 5, do not report quantitative results using this counting method. Report the sample as overloaded. Note on the report the presence or identification, or both, of fungal material.

16. Precision and Bias

16.1 *Bias:*

16.1.1 Bias cannot be determined because certified reference materials are unavailable.

16.1.2 Sources of negative bias include: spores being overlain or otherwise obscured by other particles, analyst missing spores during scanning, analyst mistaking spores for non-fungal particles, spores being transported off the impaction medium and out of the analytical area by the mounting medium.

16.1.3 Sources of positive bias include: analyst mistaking bubbles or other non-fungal particles for spores; recounting of the same spore or spores in adjacent traverses.

16.2 *Interlaboratory Precision Assessment Study:*

16.2.1 The precision of this test method is based on interlaboratory study conducted in 2011. Seventeen analysts from eight laboratories participated in the study, analyzing six impaction samples for 13 different types of fungal structures. The interlaboratory study consisted only of samples having a debris rating of 2. Every analyst reported a single test result for each of the samples in the study. Except for the absence of replicate analyses, Practice E691 was followed for the study design; the results of this interlaboratory study have been filed at ASTM Headquarters.[5]

16.3 *Precision Data:*

16.3.1 The 13 types of fungal structures in this study included:

(1) Alternaria;
(2) ascospore (undifferentiated);
(3) Aspergillus/Penicillium-like;
(4) basidiospore (undifferentiated);
(5) Bipolaris/Drechslera-like;
(6) Chaetomium;
(7) Cladosporium;
(8) Curvularia;
(9) hyphal fragments;
(10) miscellaneous;
(11) smuts/Myxomycete/Periconia;
(12) Stachybotrys/Memnoniella; and
(13) Ulocladium.

16.3.2 These 13 types of fungal structures were partitioned into 2 size categories that included (*1*) Small (that is, <8 µm in size and either require or may require use of magnification/resolution 2 for enumeration), and (*2*) Large (that is, ≥8 µm in size and magnification/resolution 1 or 2 could be used for enumeration). For purposes of this study, small spores included ascospore, *Aspergillus/Penicillium*-like, basidiospore, *Cladosporiun*, and miscellaneous. Large spores included *Alternaria*, ascospore, *Bipolaris/Drechslera*-like, *Chaetomium*, *Cladosporium*, *Curvularia*, hyphal fragments, smuts/myxomycete/Periconia, *Stachybotrys/Memnoniella*, *Ulocladium*, and miscellaneous. Ascospores, *Cladosporium*, and miscellaneous types of fungal structures were included in both size categories because they can be small and large.

16.3.3 For each sample and analyst, enumerations for each type of fungal structure within a size category were added. Hence, each analyst had 1 enumeration for small and large spores for each sample within the round robin. Small and large spore enumerations were placed into one of two spore loading categories that included (*1*) >100 spores/slide, and (*2*) ≤100 spores/slide. Two fungal structure size categories and loading categories were defined because it was hypothesized that both smaller spores and lightly loaded slides would elicit less precision since smaller spores are harder to see by means of compound microscopy and lightly loaded samples would have greater probability of spores not being detected especially if only a portion of the deposit area was assessed.

16.3.4 Coefficients of variation for fungal structure size and loading categories within each round robin sample is displayed in Table 2.

16.3.5 CV values from Table 2 were pooled for each fungal size and loading category and displayed in Table 3.

NOTE 14—Pooled CV for small spores with:

$$> 100\ \text{spores/side} = \sqrt{[((0.731)2 + (0.585)2 + (0.363)2 + (0.779)2)/4]}$$
$$= 0.636$$

All other pooled CVs within Table 3 were calculated similarly.

16.3.6 Pooled CV values ranged from 0.636 to 1.847. The small spore category showed a higher CV value than the large spore category when spore loading was less than or equal to 100 spores per slide. At higher spore loadings greater than 100 spores per slide, large and small spores showed similar CV values.

16.3.7 Pooled CV value for total fungal structure enumerations across all 17 analysts and 6 round robin samples regardless of fungal size or loading category is 0.933.

17. Keywords

17.1 bioaerosol; fungal; fungal structures; fungi; fungus; impaction; impactor; indoor air quality; mold; mould; spore; spores

TABLE 2 CV for Each Fungal Size and Loading Category Within Each Round Robin Sample

Sample Number & Size Category	>100 Spores/Slide	≤100 Spores/Slide
1 small[A]	0.731	
1 large[A]		0.599
2 small		0.628
2 large		0.497
3 small	0.585	
3 large	0.450	
4 small[A]	0.363	
4 large[A]	0.384	
5 small		2.536
5 large		1.047
6 small	0.779	
6 large	1.016	

[A] Samples 1 and 4 are based on 15 assessments instead of 17.

[5] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D22-1037. Contact ASTM Customer Service at service@astm.org.

TABLE 3 Pooled CV for Each Fungal Size and Loading Category

Fungal Size Category	>100 Spores/Slide	≤100 Spores/Slide
small	0.636	1.847
large	0.679	0.753

ANNEX

(Mandatory Information)

A1. REFERENCES FOR FUNGAL SPORE IDENTIFICATION

A1.1 Fungal spores and other structures should be identified by a combination of reference texts (for example, those suggested for the Environmental Microbiological Laboratory Accreditation Program (EMLAP) of the American Industrial Hygiene Association (AIHA),[6] those suggested by the Pan-American Aerobiology Certification Board (PAACB),[7] or those suggested by the American Phytopathological Society (APS)[8]) and microscopical mounts from known sources (for example, national culture collections). All analyses should be performed by a trained and qualified analyst (see 13.4.1) utilizing appropriate mycology reference materials.

[6] Available from American Industrial Hygiene Association (AIHA), 3141 Fairview Park Drive, Suite 777, Falls Church, VA 22042, http://www.aiha.org.

[7] Available from Pan-American Aerobiology Certification Board (PAACB), P.O. Box 45, Amherst, MA 01004, http://www.paacb.org/PAACBguidancedocs.pdf.

[8] Available from American Phytopathological Society (APS), 3340 Pilot Knob Road, St. Paul, MN 55121, http://www.apsnet.org.

APPENDIXES

(Nonmandatory Information)

X1. DIATOM RESOLUTION CHECK SLIDE

X1.1 A test slide is available (Diatomaceae Test Plate 8 Forms) that can be used to document the resolution of an optical system.

X2. DIRECTIONS FOR PREPARATION OF COMMON STAINS

X2.1 Lacto-Cotton Blue Stain

X2.1.1

glycerol	250 mL
85 % lactic acid	100 mL
cotton blue stock	3 mL
de-ionized water	50 mL

LCB Mounting Medium:

(1) Mix the water, lactic acid, and glycerin (in that order) for one hour on a stir plate.

(2) Once the solution is homogenous, add 3 mL of Cotton Blue Stock solution (recipe given below) to the above solution.

(3) Stir the entire mixture for an additional hour.

(4) Cover the flask while the mixture is stirring to ensure against airborne contamination.

Cotton Blue Stock Solution:

85 % lactic acid	99 mL
aniline (cotton) blue crystals	0.1 g

(1) Add cotton blue crystals to lactic acid while stirring vigorously on a stir plate.

(2) Stir until cotton blue crystals are dissolved.

(3) Filter the solution (for example, through a #50 Whatmann 90-mm filter disc).

(4) After filtration has occurred, check the clarity of the stock dye solution by placing one drop of the dye on a clean microslide and examine at 400×. Dye should be dissolved, not suspended.

NOTE X2.1—It may be desirable to prepare to a lighter color by decreasing the amount of Cotton Blue Stock added to the stain.

X2.2 Lacto-Phenol-Cotton Blue Stain

X2.2.1

glycerol	30 mL
lactophenol cotton blue stain (prepared solution, VWR catalog # VW3427 or equivalent)	0.5 gm
de-ionized water	30 mL
phenol	1 gm

(1) Heat 30 mL de-ionized water to near boiling
(2) Add 30 mL of glycerol; stir until mixed and solution is clear
(3) Remove from heat
(4) Add 1.0 gm phenol; stir until dissolved
(5) Add 0.5 mL of lactophenol cotton blue stain

X2.3 Lacto-Fuchsin Stain

X2.3.1

85 % (or higher) lactic acid	40 mL
acid fuchsin	0.04 gm

(1) Heat 40 mL of 85 % lactic acid to near boiling.
(2) Add 0.04 g of acid fuchsin and stir on hotplate until completely dissolved.
(3) Remove solution from heat.
(4) Aliquot into 4-mL screw cap vials with septated caps.
(5) Label with "Acid Fuchsin" and date.

X2.4 Levetin 89 – L-PVA Permanent Slide Mount[9]

X2.4.1

polyvinyl alcohol powder (PVA)	7.5 gm
distilled water	50 mL
85 % (or higher) lactic acid	22 mL
phenol	22 gm

(1) Heat the distilled water to 80°C in a water bath.
(2) Add the polyvinyl alcohol (PVA) powder to the distilled water while stirring.
(3) Continue stirring until the solution attains the viscosity of thick molasses. If there are a few lumps that do not dissolve, remove them.
(4) Add the lactic acid to the PVA solution and mix well.
(5) In a separate beaker, melt the phenol using a water bath.
(6) Add the phenol to the PVA solution and stir until homogeneous.
(7) Store in tightly closed small vials. The shelf life is months. Over time, the phenol will cause the medium to darken, but the clarity of mounts will be unaffected.
(8) Mounts may be examined immediately, but harden gradually over a few days.

NOTE X2.2—This medium is compatible with double sided tape, acid fuchsin, and phenosafranin. This medium is not compatible with cotton blue stain.

[9] Levetin, E., "Basidiospore Identification," *Annals of Allergy*, Vol 62, 1989, pp. 306–310.

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