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Sampling and Characterization of Bioaerosols

by William G. Lindsley, Brett J. Green, Francoise M. Blachere, Stephen B. Martin, Brandon F. Law, Paul A. Jensen and Millie P. Schafer, NIOSH

Centers for Disease Control and Prevention
National Institute for Occupational Safety and Health

1 Introduction

Bioaerosols are airborne particles that originate from biological sources including animals, plants, fungi, bacteria, protozoa, and viruses. Examples of bioaerosols encountered in occupational environments include plant pollen, algae, fungal spores, bacteria such as actinomycetes, droplets produced during coughing and sneezing that may contain bacteria and viruses, dust containing insect excreta, animal dander, and fragments derived from each of these sources. Bioaerosols are ubiquitous and can be isolated from indoor, outdoor, and occupational environments using a variety of methods that either enumerate viable or a collection of viable and non-viable bioaerosols. Photomicrographs of example viral, bacterial, fungal, and plant bioaerosols are presented in Figure 1.

Bioaerosol monitoring is a rapidly emerging area of industrial hygiene due to the improved analysis methods such as polymerase chain reaction (PCR) and the impact that occupational exposures may have on worker respiratory health, particularly in microbial contaminated environments [Eduard et al. 2012; Environment Agency 2009; Haig et al. 2016; Hung et al. 2005; Macher 1999; Morey 2007; Nazaroff 2016]. Some human diseases encountered in healthcare settings such as measles and tuberculosis can be spread by bioaerosols containing infectious microorganisms [Ijaz et al. 2016; Jones and Brosseau 2015]. Soil saprophytic fungi such as Coccidioides immitis can be aerosolized during occupational disturbance activities and, if inhaled, can result in an acute pulmonary infection [Das et al. 2012; Wilken et al. 2014; Wilken et al. 2015]. The measurement of these bioaerosols in industrial hygiene includes the measurement of viable (culturable and non-culturable) and nonviable bioaerosols in indoor settings (e.g., industrial, office, education, and residential buildings), industrial facilities (e.g., biotechnology, composting, waste disposal, manufacturing, textile, and food processing), and outdoor environments (e.g., farms, feed lots, and general air quality). Monitoring for bioaerosols in the occupational environment is one of the many tools the industrial hygienist uses in the assessment of indoor air quality, infectious disease outbreaks, agricultural exposures, and industrial health.

Bioaerosol monitoring may be appropriate during workplace health and exposure assessments, epidemiological investigations, research studies, or in situations deemed appropriate by an occupational physician or immunologist. Sampling can also be used to evaluate occupational environments before and after mitigation of microbial contaminants. When investigating bioaerosols as a possible source of workplace exposures and health issues, bioaerosol sampling should be part of an integrated assessment of work conditions. This should also include examining heating, ventilation and air conditioning (HVAC) systems; checking for water infiltration and moisture control; evaluating microbial contamination in evaporative cooling systems, metal working fluids, and waste water; evaluating possible

internal and external sources of bioaerosols; and other measures [Macher 1999]. In general, if visible growth or contamination (microbial growth on floors, walls, or ceilings, or in the HVAC system) is observed, this normally should be mitigated first before indoor bioaerosol sampling is conducted. If personnel remain symptomatic after remediation, air sampling may be appropriate, but the industrial hygienist should be aware that false negative results are possible and should be interpreted with caution.

The industrial hygienist has a variety of tools and methodologies available to conduct an environmental survey [ASTM 2014a; Flannigan et al. 2011; Hung et al. 2005]. However, many of these approaches have lacked standardization and this has made the interpretation and comparison between studies challenging [Flannigan et al. 2011]. In 2005, the American Industrial Hygiene Association (AIHA) published the second edition of the Field Guide for the Determination of Biological Contaminants in Environmental Samples [Hung et al. 2005]. This reference provides the industrial hygienist access to the most up to date methods to detect and quantify bioaerosols in the environment, and covers methods of how to conduct a survey, sample bioaerosols, and interpret the collected data [Hung et al. 2005]. Similarly, other reference sources have been published by Flannigan et al. [2011] and the American Conference of Governmental Industrial Hygienists (ACGIH) [Macher 1999] that extensively outline available methods to analyze collected bioaerosols as well as strategies to conduct an environmental survey. ASTM International has issued a wide range of standards on indoor air quality, including assessment of fungal growth and collection of bioaerosols and a guide to developing an air sampling strategy [ASTM 2009; ASTM 2014a; ASTM 2014b; ASTM 2014d]. The European Committee for Standardization has also published standards on sampling for bioaerosols and related topics [CEN 2000; CEN 2003; CEN 2004]. The sections presented below provide a very broad overview of the viable and non-viable methods available to detect bioaerosol sources that are described in the references listed above.

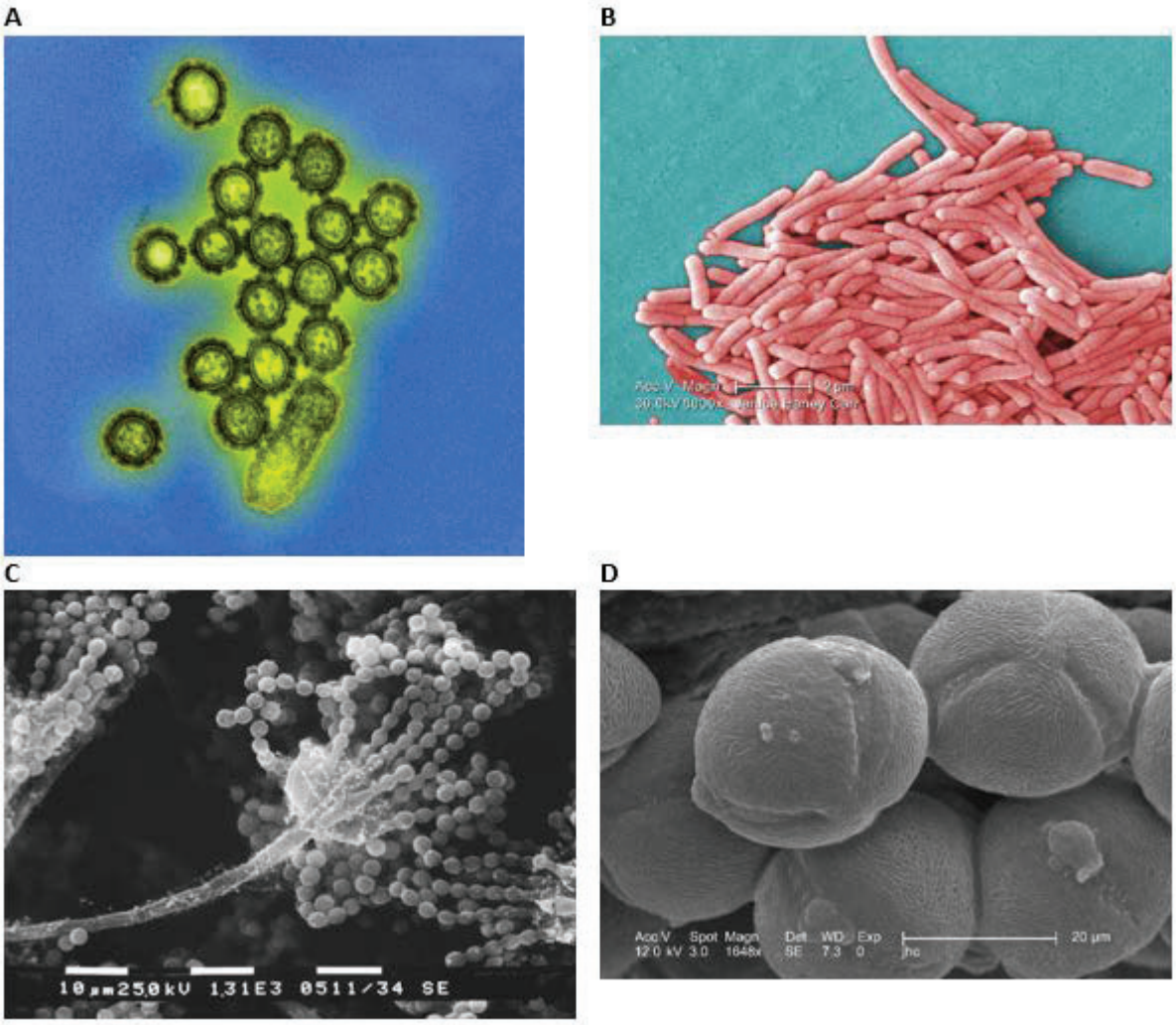

Figure 1: Photomicrographs of acellular, prokaryotic and eukaryotic microorganisms that can be encountered in occupational or industrial environments. (A) Transmission electron micrograph of Influenza/flu (H1N1) virus particles (Photo courtesy of National Institute of Allergy and Infectious Diseases; CDC Public Health Image Library (PHIL) ID#: 18156); (B) Scanning electron micrograph of bacilli derived from the Gram-negative bacteria, *Legionella pneumophila* (Photo courtesy of National Institute of Allergy and Infectious Diseases; CDC Public Health Image Library (PHIL) ID#: 11150); (C) Scanning electron micrograph of *Aspergillus* species reproductive structures including chains of asexual spores (Photo courtesy of CDC/ Robert Simmons; CDC Public Health Image Library (PHIL) ID#: 13367); and (D) Scanning electron micrograph of tricolpate pollen derived from the angiosperm plant species, *Oenothera fruticosa* (Photo courtesy of CDC/ Janice Carr, Betsy Crane; CDC Public Health Image Library (PHIL) ID#: 8729). The CDC Public Health Image Library at http://phil.cdc.gov/Phil/home.asp has thousands of health-related images available to the public free of charge.

2 Principles of bioaerosol collection

a. Aerodynamic diameter

The aerodynamic diameter of an airborne particle (usually written as "da" or "dae") is the single most important parameter that determines how the particle will behave in the air, including how long it will stay airborne and where it will deposit in the respiratory system if inhaled. If a particle is falling in still air, it will reach an equilibrium velocity where the gravitational force pulling it downward is balanced by the drag force on its surface. This velocity is called the terminal settling velocity, and it depends upon the size, shape and density of the particle. The aerodynamic diameter of a particle is defined as the diameter of a sphere with unit density (that is, a density of 1 g/cm^3) that has the same terminal settling velocity as the particle. Consider, for example, the irregularly-shaped fungal fragment shown in Figure 2. Suppose this particle has a terminal settling velocity of 0.05 cm/sec. This is the same settling velocity as that of a spherical particle with a unit density that has a diameter of 4 µm. Thus, the fungal fragment is said to have an aerodynamic diameter of 4 µm. Similarly, a different particle with a terminal settling velocity of 1.21 cm/sec has an aerodynamic diameter of 20 µm, since a 20 µm unit density sphere settles at that rate. It is important to note that the aerodynamic diameter may be very different from the physical size of a particle. A very dense and compact particle may have an aerodynamic diameter much larger than its actual dimensions, while a very light particle or one with fibrous branches may have an aerodynamic diameter that is much smaller than its physical size. It is possible for two particles to have very different shapes and physical sizes, but have the same aerodynamic diameter. Conversely, two particles may have similar physical sizes, but have very different aerodynamic diameters. A more detailed discussion of the aerodynamic diameter can be found in Hinds [1999] and Vincent [2007].

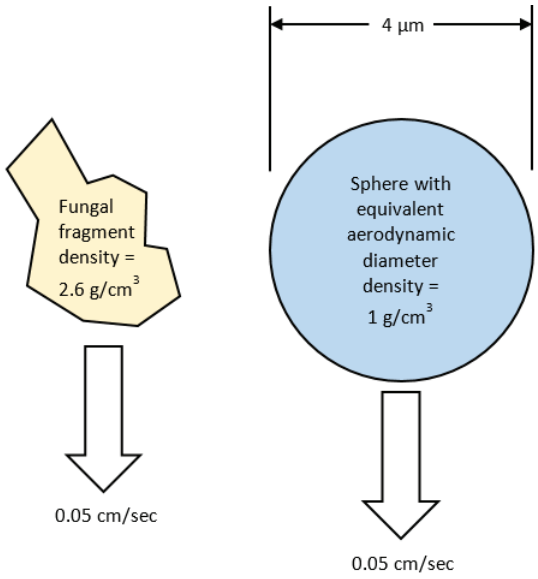

Figure 2: Aerodynamic diameter of an aerosol particle. In this case, the fungal fragment on the left is said to have an aerodynamic diameter of 4 µm, since it falls at the same terminal settling velocity as a 4 µm sphere with a unit density.

Aerodynamic diameter is used in aerosol science because particles with the same aerodynamic diameter tend to move and be collected in the same ways. For example, two particles with the same aerodynamic diameter will have the same likelihood of being collected by an impaction aerosol sampler even if they have different physical and morphological characteristics. For this reason, the performance of aerosol collection devices is usually described by giving the aerodynamic diameter of the particles that will be collected.

b. Collection efficiency and cut-off diameter

The collection efficiency of an aerosol sampler is the fraction of the aerosol particles of a particular aerodynamic diameter that will be collected by the sampler. For example, if 95% of the airborne particles with a 2 µm aerodynamic diameter that enter the sampler are deposited in the collection fluid or on the collection surface, then the sampler is said to have a 95% collection efficiency for 2 µm particles.

Most commonly-used aerosol filters have a high collection efficiency for particles of all sizes [NIOSH 2016b]. However, impactors, cyclones and impingers use the inertia of airborne particles to separate them from the air stream, and thus they have a high collection efficiency for particles with larger aerodynamic diameters and a low collection efficiency for smaller ones (Figure 3) [Hering 2001; Hinds 1999; Marple and Olson 2011]. These devices are said to have a "cut-off diameter"; that is, particles with an aerodynamic

diameter larger than the cut-off diameter are collected while particles with an aerodynamic diameter less than the cut-off diameter are not collected and pass through the device. A perfect collection device would have a 100% collection efficiency for particles larger than the cut-off diameter and 0% for smaller particles. In practice, this is not the case: the collection efficiency curve for an inertia-based sampler looks like the example curve shown in Figure 3. The aerodynamic diameter at which the collection efficiency is 50% is defined as the cut-off diameter (usually written as d_{50}). A device with a more abrupt transition from 100% to 0% collection efficiency (that is, closer to the ideal device) is said to have a sharp cut-off.

For a given inertial collection device, the 50% cut-off diameter depends upon the air flowrate through the device. Increasing the flowrate will decrease the d_{50} and shift the collection efficiency curve to the left, while decreasing the flowrate will increase the d_{50} and shift the collection efficiency curve to the right. For example, the first stage of the NIOSH two-stage cyclone aerosol sampler has a d_{50} of 4.9 µm at 2 liters/minute of air flow, 4.1 µm at 3.5 liters/minute, and 2.1 µm at 10 liters/minute [Blachere et al. 2009]. For this reason, it is important to check the air flowrate before aerosol sampling and control it during sampling so that the particles are correctly segregated by size.

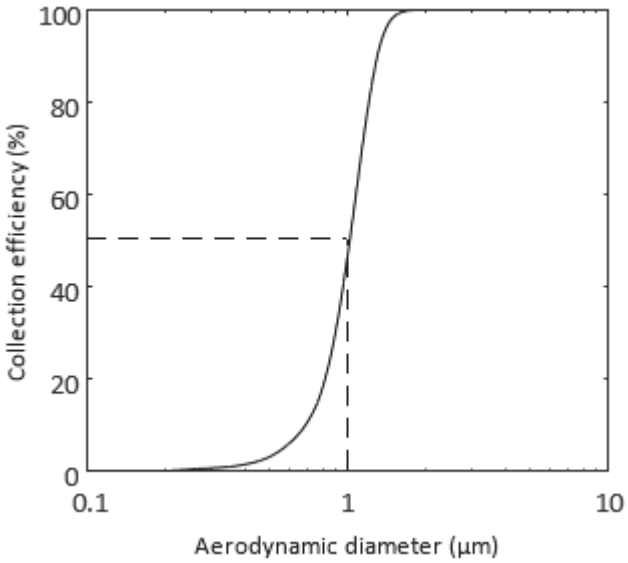

Figure 3: Example collection efficiency curve for an inertia-based aerosol sampler. Note that the collection efficiency is high for particles with large aerodynamic diameters and low for small particles. In this example, the 50% cut-off diameter (d_{50}) for this device is 1 µm.

c. Size-selective bioaerosol sampling in industrial hygiene

Size-selective bioaerosol sampling may be done for several reasons. Since the settling velocity of aerosol particles is determined by the aerodynamic diameter, knowing the size distribution of an aerosol helps in predicting how long the particles are likely to remain airborne and how far they can travel. In health care settings, for example, various medical procedures can produce a spray of droplets containing infectious microorganisms. Large droplets tend to fall onto surfaces fairly close to the source, while smaller droplets can remain airborne and carry pathogens many feet away from a patient [Davies et al. 2009; Jones and Brosseau 2015]. Another application of size-selection is to isolate different types of bioaerosol particles, such as separating fungal fragments from intact fungal spores [Adhikari et al. 2013; Seo et al. 2014].

Size-selective sampling is most commonly used to help understand the potential health effects of bioaerosol particles, which often depend upon where the particles are deposited in the respiratory tract. In general, larger bioaerosol particles tend to deposit higher in the respiratory tract (that is, in the nasal or oral cavities or larger airways), while smaller particles are able to travel deeper into the lungs to the smaller airways [Hinds 1999; Vincent 2005]. Some pathogens such as *Mycobacterium spp.*, *Bacillus spp.*, and *Aspergillus spp*. are thought to be more likely to cause a pulmonary infection if they reach the deeper airways, and the response to bioaerosols containing immunogenic material such as endotoxins or fungal antigens may also vary depending upon the site of deposition. For this reason, size-selective sampling is often used in industrial hygiene to better understand the potential risks that workplace bioaerosols present.

The American Conference of Governmental Industrial Hygienists (ACGIH), the International Organization for Standardization (ISO) and the European Standardization Committee (CEN) have defined three particle collection efficiency curves for aerosol samplers used to conduct size-selective aerosol sampling (Figure 4) [ACGIH 2001; ISO 2012; Vincent 2005]. The idea is that an aerosol sampler that conforms to one of the three criteria will collect aerosol particles in a way that approximates the fraction of particles that will reach different parts of the respiratory tract. These criteria are not specific to bioaerosols, but rather are applied to all types of aerosol particles.

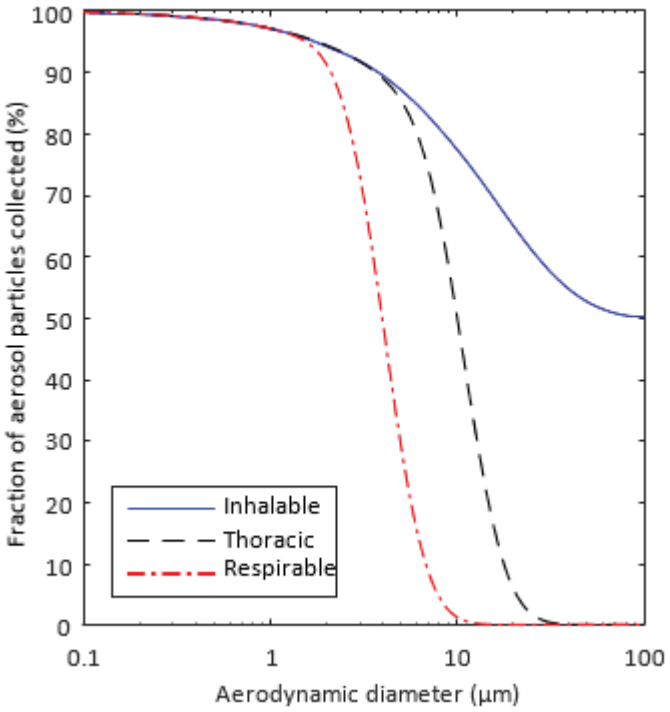

Figure 4: ACGIH/ISO sampling criteria for the inhalable, thoracic and respirable fractions of aerosol particles. The inhalable fraction contains all of the particles that are inhalable, which includes the particles in the thoracic and respirable fraction. Similarly, the thoracic fraction includes the particles in the respirable fraction. The 50% cut-off diameters are 100 µm for the inhalable fraction, 10 µm for the thoracic fraction, and 4 µm for the respirable fraction [ACGIH 2001; ISO 2012; Vincent 2005].

A sampler that collects the inhalable fraction accumulates the fraction of aerosol particles of each size that would be expected to be drawn into the nose or mouth during normal breathing. This includes larger particles that would be expected to be deposited in the nasal or oral cavities as well as smaller particles that can be conveyed to the lower airways. An aerosol sampler that conforms to the inhalable sampling criteria collects 50% of the 100 µm particles, 77% of the 10 µm particles, and 97% of the 1 µm particles in the ambient aerosol. The inhalable fraction is lower for larger particles because the greater inertia of these particles means they are less likely to be pulled into the body during inhalation. The thoracic fraction includes aerosol particles that are likely to travel into the trachea and bronchi. An aerosol sampler that conforms to the thoracic sampling criteria will collect 50% of the 10 µm particles and 97% of the 1 µm particles in the ambient aerosol. This fraction includes fewer large particles because these particles tend to be removed from the airstream by the head airways.

The respirable fraction includes aerosol particles that are able to reach the deepest airways, which are the respiratory bronchioles and the alveoli. An aerosol sampler that conforms to the respirable sampling criteria will collect 50% of the 4 µm particles, 97% of the 1 µm particles, and 99% of the 0.3 µm particles in the ambient aerosol. The respiratory bronchioles and the alveoli are of particular concern because these airways do not have cilia. Non-soluble particles that land in the nasopharyngeal region or upper airways tend to collect in the airway mucus and are removed from the respiratory tract by the cilia relatively quickly. However, particles that deposit in the alveoli and respiratory bronchioles can remain in the lungs for longer durations (in some cases, for life) unless they can be broken down or removed by migrating pulmonary macrophages. This fraction includes only the smallest particles because the larger particles are removed from the airstream by the head and thoracic airways.

It should be noted that, even though larger bioaerosol particles will tend to deposit in the upper airways and be cleared more quickly, they can still trigger an allergic/inflammatory response in susceptible individuals. Particles containing viable pathogens also commonly cause infections after being deposited in the upper airways.

When describing size-selective sampling, particles are often said to "penetrate" to a particular region of the respiratory tract. This does not mean penetrate in the sense of entering the tissue, but rather simply being present in the air stream flowing into that region, as compared to particles which were deposited before reaching a particular location. For example, an aerosol particle that is able to remain in the air stream and reach the lung alveoli is said to have penetrated to the alveolar region, even if it does not necessarily deposit there. This is the same context as with filtration, where a particle is said to penetrate a filter if it flows through the filter material and remains in the air stream. It also should be noted that the ACGIH/ISO criteria give an approximation of the fraction of aerosol particles that can penetrate to different regions of the respiratory tract. However, they do not indicate what fraction of the aerosol particles will actually deposit in the airways and what fraction will be exhaled. The lung deposition of aerosol particles is complex and depends upon many factors. More information about this topic can be found in Hinds [1999] and Vincent [2005; 2007].

3 Devices used for bioaerosol sampling

Most aerosol sampling devices involve techniques that separate particles from the air stream and collect them in or on a preselected medium. Impactors, filters, impingers and cyclones are four common sampling techniques used to separate and collect bioaerosols [Haig et al. 2016; Macher et al. 1995; Reponen et al. 2011b; Willeke and Macher 1999]. A few systems that use electrostatic precipitation or condensation-based collection are also available [Haig et al.

2016], and some real-time bioaerosol monitoring systems are available that do not require that the bioaerosol particles be isolated before analysis. Below are some specific types of bioaerosol sampling devices employed by industrial hygienists.

a. Filters

Aerosol filters are commonly used to collect bioaerosol particles because of their simplicity and low cost. Filter-based sampling is particularly useful for personal bioaerosol sampling because filter-based collectors are small and lightweight and work well with personal sampling pumps. Filters can be preceded by a size-selective inlet, such as a cyclone or impactor, to remove larger particles and provide size-classification of the bioaerosol particles. Most aerosol filter media can be classified as fibrous, membrane, or capillary pore (also called straight-through pore) [Raynor et al. 2011]. Fibrous filters are usually made of a deep mesh of glass fibers. Membrane filters are manufactured in a variety of pore sizes from polymers such as cellulose ester, polyvinyl chloride, or polytetrafluoroethylene (PTFE). Capillary pore filters are made of polycarbonate. The choice of a filter medium depends on the contaminant of interest and the requirements of the analytical technique. For gravimetric analysis, non-hygroscopic materials such as glass fibers, silver, or polyvinyl chloride membranes are selected because their masses are less affected by changes in humidity. For analysis by microscopy, cellulose ester or polycarbonate membranes are common choices because cellulose ester membranes can be rendered transparent for easier visualization, while polycarbonate filters have a smooth collection surface that works well with light or electron microscopy. Samples also can be eluted from cellulose ester and polycarbonate filters, but in some cases the recovery efficiency can be low [Eduard et al. 1990; Rule et al. 2007]. Samples to be cultured can be collected on gelatin filters, and the filters can then be dissolved in water and spread on culture plates, dissolved in growth media, or placed directly on culture plates and allowed to melt. Gelatin filters are fragile and can crack or melt in use. For analysis using immunological assays or polymerase chain reaction (PCR), PTFE filters are a common choice because they do not interfere with the assays and because samples can be readily eluted from them.

Filters are frequently described or specified using the term “pore size” or “equivalent pore diameter”. It is important to note that the filter pore size does NOT indicate the minimum particle size that will be collected by the filter; in fact, aerosol filters generally will collect particles much smaller than the nominal pore size. The mechanisms by which aerosol filters work and the role of pore size in selecting filters is described is more detail elsewhere [NIOSH 2016b].

Aerosol filters are usually supplied as disks of 25, 37 or 47-mm diameter. Because the flow resistance (often called the pressure drop) of a filter increases with the air velocity through the filter, the use of a larger filter results in a lower flow resistance for a given volumetric flow rate. On the other hand, the use of a smaller filter concentrates the deposit of the contaminant onto a smaller total area, thus increasing the density of particles per unit area of filter. This may be helpful for direct microscopic examination of low concentrations of organisms, and reduces the amount of elution media needed for immunological or PCR-based assays. In areas of high concentration, the microorganisms may have to be eluted, diluted, and then refiltered for microscopic analysis. Breuer [2012] reported on the flow resistance of common aerosol filters and its relationship to sampling pump selection. Soo et al. [2016] measured the filtration characteristics and flow resistance of a variety of commonly-used aerosol filters.

In the USA, the most common method of aerosol sampling with filters is to place the filters in disposable two-piece or three-piece plastic filter cassettes with a support pad to add rigidity. The three-piece cassette may be used either in open- or closed-face modes. Open-face sampling is performed by removing the end plug and the plastic cover from the three-piece cassette and is used when the particulate matter must be uniformly deposited (i.e., for microscopic analysis). If a three-piece cassette is used in the open-face arrangement, the plastic cover is retained to protect the filter after sampling is concluded. It should be noted that the aspiration efficiencies of open-face and closed-face filter cassettes are reported to be somewhat different [Beaulieu et al. 1980; Kenny et al. 1997]. In addition to collecting on the filter, aerosol particles (especially large particles) may collect on the internal walls of the filter cassette. Depending upon the purpose of the collection, wall-deposited material may need to be included in the analysis. This can be done by using a filter with an attached capsule or by washing or wiping the internal surfaces of the cassette [Ashley and Harper 2013].

It is important to verify that the filter cassette and fittings are air-tight and have no bypass leakage around the filter. Cassettes should not be hand-assembled; they should be pressed together with a mechanical or hydraulic press. All plastic cassettes should be securely assembled and sealed with a cellulose shrink band or tape around the seams of the cassette to prevent external air leakage. The cassettes should be made of conductive or static dissipative materials to avoid losses due to electrostatic effects. More information on using filter cassettes for aerosol sampling can be found elsewhere [NIOSH 2003a; NIOSH 2016a].

b. Impactors

An impactor consists of a series of nozzles (circular- or slot-shaped) and an impaction surface [Hering 2001; Marple and Olson 2011; Marple and Willeke 1976]. Air is drawn into the impactor using a vacuum pump, and the air stream flows through the nozzles and toward the impaction surface, where particles are separated from the air stream by their inertia (Figure 5). Larger particles collect on the impaction surface, while small particles that do not impact follow the air stream. The impaction surface typically consists of a greased plate or tape, filter material, or growth media (agar) contained in Petri dishes. In some applications, impactors are not used as collection devices themselves, but rather to remove particles above a certain size before collection or characterization of the downstream aerosol.

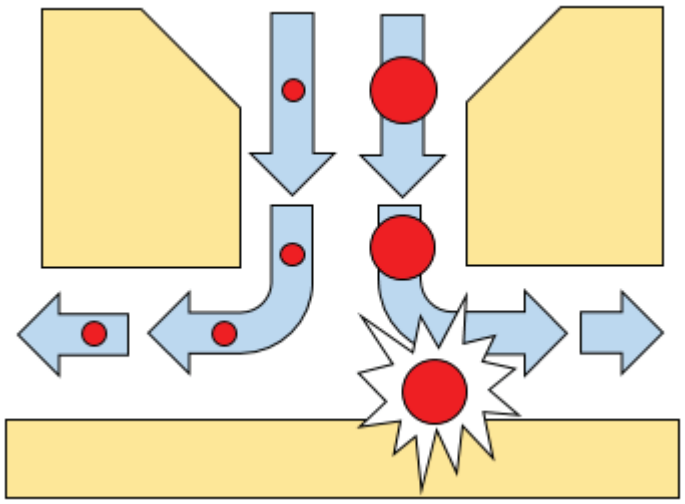

Figure 5: Impaction. As the air stream exits the impactor nozzle, it quickly changes direction as shown by the arrows. Smaller particles such as those on the left flow with the air stream and are not collected. Larger particles cannot change direction as quickly due to their higher inertia and collide with the collection surface, where they accumulate.

A cascade impactor consists of a stack of impaction stages: each stage consists of one or more nozzles and a target or substrate. The nozzles may take the form of holes or slots. Each succeeding stage has smaller nozzles and thus collects smaller particles (that is, each succeeding stage has a smaller cut-off diameter). A filter may be used after the final impaction stage to collect any particles smaller than the final cut-off diameter. If the substrate is a greased plate or filter media, it may be weighed to determine the collected mass, or it may be washed and the wash solution analyzed. If the substrate is growth media in culture plates, they may be incubated and examined for microbial growth.

The most commonly used impactor for sampling airborne culturable bacteria and fungi is the Andersen impactor, which uses from one to six impactor stages containing Petri plates as seen in Figure 6 [Andersen 1958]. Since the bioaerosol particles impact directly onto the growth media, the samplers can be directly transferred to an incubator and observed for microbial growth. However, this method depends upon collecting viable microorganisms that are capable of growth on the specific nutrient media.

Glass Petri plates are recommended for use with the Andersen impactor; plastic culture plates are often used, but this can result in loss of aerosol material due to electrostatic surface charges in the plastic [Andersen 1958; Kuo 2015]. NIOSH Method 0800 describes how to collect culturable airborne fungi and bacteria in buildings using an Andersen cascade impactor [NIOSH 2003b].

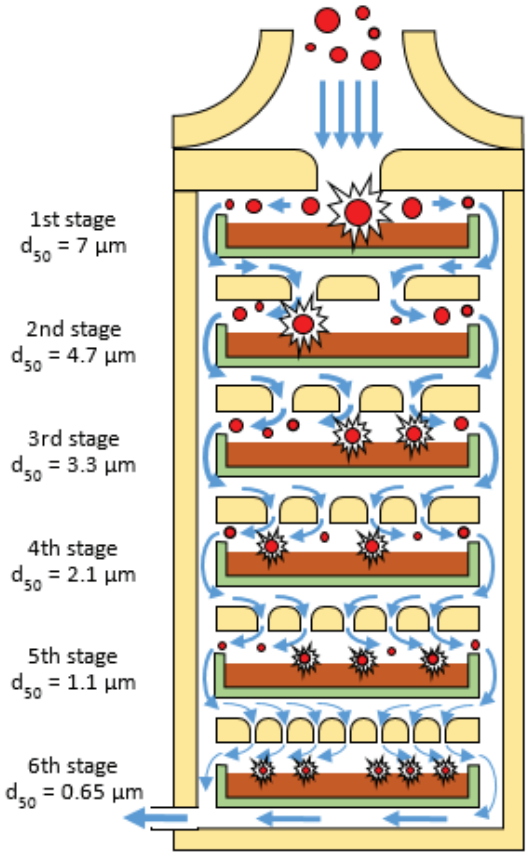

Figure 6: Schematic of a 6-stage Andersen cascade impactor [Andersen 1958]. Each stage contains a Petri plate (green) filled with nutrient agar (brown). The stages have progressively smaller nozzles, which create higher particle impaction velocities onto the agar. The aerosol particles (red) flow from the top into the first stage, where particles with aerodynamic diameters larger than 7 µm impact the agar. The remaining particles flow to the second stage, where particles with aerodynamic diameters between 7 µm and 4.7 µm are collected, and so on for the rest of the stages.

One significant advantage of the Andersen impactor is that samples can be collected directly onto culture plates and transferred to an incubator, which simplifies handling and eliminates some losses that can occur in processing. However, there are also several limitations. In low concentration environments, sampling time is limited to approximately 20 minutes to avoid drying the agar. The high flow rate (28.3 liters/minute) makes the sampler unsuitable for high concentration environments such as some agricultural sites (i.e. animal facilities) where a 1 minute sample may overwhelm the plates.

When using the Andersen impactor, it is also necessary to correct for "coincidence error" using a positive-hole correction factor. This occurs because it is possible for multiple particles, each containing one or more organisms, to pass through a particular hole during sampling and impact onto the growth medium, with one or more bacterial or fungal colonies forming at the same impaction sites. The colonies formed by the multiple particles can then be inaccurately counted as a single colony. As the number of organism-containing particles deposited onto the growth medium increases, the probability that the next organism-containing particle will impact an "occupied" hole increases. For example, if 75% of the holes have received at least one particle, the chance that the next particle will impact a "clean" hole is one in four (25%). To account for this, a probability-based coincidence correction factor needs to be applied to the results for each impactor stage. The basic formula for the coincidence correction is as follows [Andersen 1958; Macher 1989]:

$$P_r = N\left[\frac{1}{N} + \frac{1}{N-1} + \frac{1}{N-2} + \frac{1}{N-r+1}\right]$$

Where:

N = the total number of holes in the impactor stage

r = the number of colonies observed on the culture plate

P_r = the estimated culturable particle count

Andersen impactors have from one to 400 holes per stage. Macher [1989], Willeke and Macher [1999] and Andersen [1958] provide tables of positive-hole correction factors.

Investigators often employ stationary cascade impactors either as the primary collection mechanism, or as a preclassifier (for example, to remove nonrespirable particles from the sampled air stream). Marple and Willeke [1976] have reported that high velocity, inlet losses, interstage losses, and particle reentrainment affect the performance characteristics of an impactor. Particles larger than the cut-off diameter may bounce after impacting the collection surface and travel to subsequent impaction stages. This is particularly a problem with dry solid collection surfaces; for this reason, solid collection surfaces are usually greased or oiled [Hering 2001]. Fungal spores have been shown to be prone to de-aggregation and bounce when collected with an impactor, which can cause the spores to be collected on stages with smaller cut-off diameters. This can make the spore aggregates appear to have smaller aerodynamic diameters than is actually the case [Trunov et al.

2001]. Although personal cascade impactors are available, these devices are not as widely used in personal sampling for bioaerosols as are filters [Macher and Hansson 1987].
The slit-to-agar impactor is a type of impactor in which the aerosol particles are deposited on a Petri plate that slowly rotates. The rotation of the plate means that particles which are collected at different times deposit in different locations, and thus provides an indication of changes in the bioaerosol concentration over time [Ho et al. 2005; Jensen et al. 1992; Smid et al. 1989; USP 1997]. Examples of slit-to-agar samplers include the Dycor Slit Sampler from Dycor and the Air Trace Environmental Slit-to-Agar Sampler from Particle Measuring Systems.

The Hirst/Burkard spore trap has been widely used to collect outdoor aerospora. It was first described by Hirst [1952] and consists of a unit that houses a vacuum pump and rotating drum that is lined with polyester tape. The drum rotates at 2 mm per hour and is continuously run for seven days. Bioaerosols pass through an orifice on the sampler and particles impact on the tape. Following the seven-day sampling interval, the tape is removed and cut in 48 mm intervals that correspond to individual sampling days. Bioaerosols deposited on the tape are stained and then resolved, identified, and quantified using bright field microscopy.

[Tovey et al. 2016] developed a personal aerosol sampler with a rotating surface that allows time-resolved collection of aerosol particles onto an electret strip or an adhesive film. They used the sampler to study personal exposures to dust mite allergens over time.
A novel example of an impaction-based personal bioaerosol sampler is the intranasal air sampler fabricated by Graham et al. [2000], which fits within the intranasal cavity of the subject. Bioaerosols enter the nasal cavity following inhalation and pass through slits where particles are deposited by impaction on either an adhesive backed tape or collection cup lined with silicon grease. This impaction sampler has been utilized in a number of studies that have evaluated exposure to indoor and occupationally relevant aeroallergen sources [Gore et al. 2002; Mitakakis et al. 2000; Renstrom et al. 2002].

Other impaction-based approaches have also been used in the assessment of outdoor bioaerosols, including the Rotorod, Air-o-cell and Allergenco samplers [Frenz 1999; Lee et al. 2004a; Pityn and Anderson 2013; Portnoy et al. 2000]. ASTM Standards D7391 and D7788 discuss the collection and analysis of airborne fungal structures by inertial impaction [ASTM 2009; ASTM 2014d].

c. Cyclones

A cyclone sampler consists of a circular chamber with the aerosol stream entering through one or more tangential nozzles as shown in Figure 7 [Hering 2001]. Like an impactor, a cyclone sampler depends upon the inertia of the particle to cause it to deposit on the sampler wall as the air stream curves around inside the chamber. Also like an impactor, a cyclone sampler has a collection efficiency curve like the one shown in Figure 3, and the collection efficiency curve depends upon the flow rate. Cyclones are less prone to particle bounce than impactors and can collect larger quantities of material. They also may provide a more gentle collection than impactors, which can improve the recovery of viable microorganisms. However, cyclones tend to have collection efficiency curves that are less sharp than impactors, and it is simpler to design a compact cascade impactor compared to a cascade of cyclone samplers.

In industrial hygiene, cyclone aerosol samplers are frequently used in conjunction with a filter to conduct size-selective aerosol sampling [Hering 2001]. For example, in NIOSH Method 0600, a cyclone is used to remove the non-respirable fraction from the aerosol (following the ACGIH/ISO criteria described earlier), and a filter is then used to collect the respirable fraction [NIOSH 2003c]. A sampler developed at NIOSH uses two cyclones followed by a filter; the first cyclone collects the non-respirable fraction of the particles, the second cyclone collects the respirable particles > 1 μm, and the filter collects particles < 1 μm [Blachere et al. 2009]. The NIOSH cyclone aerosol samplers have been used in applications including measurements of airborne viruses in healthcare settings; airborne fungi and fungal fragments in residences; airborne dimorphic fungal pathogens such as Paracoccidioides brasiliensis in Brazil, and bioaerosols in agricultural operations [Arantes et al. 2013; Blachere et al. 2009; Blais Lecours et al. 2012; Kettleson et al. 2013; Lee and Liao 2014; Lindsley et al. 2010a; Lindsley et al. 2010b; Martin et al. 2015; Seo et al. 2014; Singh et al. 2011a; Singh et al. 2011b].

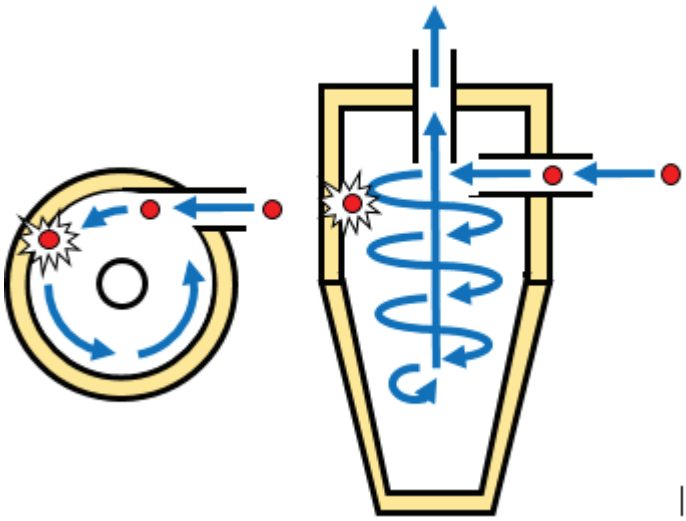

Figure 7: Cyclone aerosol collection. When the aerosol stream enters the body of the cyclone through the inlet, the air flow follows the curved interior wall and flows in a spiral pattern. If aerosol particles are larger than the cut-off diameter, then the inertia of the particles causes them to collide with the wall of the cyclone and accumulate. After spiraling downward, the air flow comes up through the center of the cyclone and exits through the outlet (called a vortex finder) at the top. The illustration shows a tangential inlet reversed-flow cyclone, which is the most common type of cyclone sampler.

d. Impingers

Many microorganisms can lose their viability if they are collected onto dry solid surfaces or filters because of impact damage and desiccation [Cox 1987; Jensen et al. 1992; Macher and First 1984; Verreault et al. 2008; Wang et al. 2001]. One way to avoid this is to collect culturable bioaerosols in liquids using an impinger [Henningson and Ahlberg 1994; Henningson et al. 1988; Lembke et al. 1981; Reponen et al. 2011b; Verreault et al. 2008]. A typical impinger is shown in Figure 8. The body of the impinger is filled with a collection liquid, and the aerosol stream flows down through a nozzle and enters the liquid at a high velocity. The aerosol particles are collected when they collide with the bottom of the collection vessel or disperse into the liquid. Impingers often have curved inlets to remove larger particles from the air stream before collection. Because impingers are essentially another type of inertial collection device, they have a collection efficiency curve and a cut-off diameter like impactors and cyclones. However, the collection efficiency curves tend to be less sharp. The high velocity air stream directed into the liquid also creates considerable agitation and can produce foaming if the collection liquid contains surfactants. Additives to the collection medium such as proteins, antifoam, or antifreeze aid in resuscitation of bacterial cells, prevent foaming and loss of the collection fluid, and minimize injury to the cells [Chang and Chou 2011; Cown et al. 1957; Dungan and Leytem 2015]. The presence

of proteins and other additives can also greatly influence the survival of airborne viruses during collection by impingers [Ijaz et al. 1985b; Schaffer et al. 1976; Verreault et al. 2008]. Water loss over time reduces the liquid level in the impinger and increases the concentration of the non-volatile components, which limits the available collection time [Lin et al. 1997]. Sample losses due to re-aerosolization and particle deposition inside the impinger can be significant [Grinshpun et al. 1997; Han and Mainelis 2012].

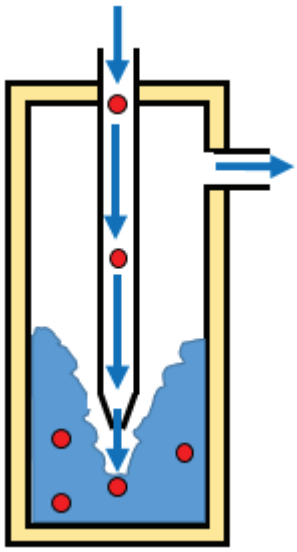

Figure 8: Impingement. Bioaerosol particles exit the nozzle of the impinger at high velocity and impact the liquid or the bottom surface of the collection vessel. Some types of impingers produce air bubbles in the collection media, which can enhance particle collection, but can damage some types of microorganisms.

Two common impingers used for bioaerosol sampling are the Greenburg-Smith impinger [Greenburg 1932] and the All-Glass Impinger with the nozzle 30 mm above the base of the collection vessel, called the AGI-30 [May and Harper 1957]. The Greenberg-Smith and AGI-30 samplers operate by drawing aerosols at nominal flow rates of 28.3 and 12.5 L/min, respectively, through an inlet tube [Macher et al. 1995]. The AGI-30 inlet tube is curved to simulate particle collection in the nasal passage [Cox 1987]. Investigators have reported problems with low sampling efficiencies and high losses due to particles in the collection being re-aerosolized and lost [Grinshpun et al. 1997; Kesavan et al. 2010; Lin et al. 1997].

When the AGI-30 is used to recover total airborne organisms from the environment, the curved inlet tube is washed with a known amount of collecting fluid after sampling because larger particles (i.e., over 15 μm) are collected on the tube wall by inertial force. After sampling for the appropriate amount of time, 10 mL of the full-strength collection

fluid is filtered through a 0.45-µm pore size membrane filter. Serial dilutions of the remaining collection fluid are handled similarly [Greenberg et al. 1992]. The membrane filters are placed in sterile plastic petri plates filled with the appropriate medium and incubated for later identification and enumeration.

e. Wetted-surface bioaerosol samplers

Several types of bioaerosol sampling devices have been developed in which the aerosol stream impacts onto a wetted surface or onto the wall of a cyclone wetted with collection media [Kesavan and Sagripanti 2015; Kesavan et al. 2011]. These systems largely avoid the bubbling and agitation associated with conventional impingers, which may be detrimental to some microorganisms [Lin et al. 2000], and can provide sharper collection efficiency curves. One of the simplest examples of a wetted-surface sampler is the SKC BioSampler [Lin et al. 2000; Willeke et al. 1998]. It is similar to an AGI-30, except that it has three nozzles that curve so that the aerosol stream is tangential to the wall of the collection vessel. This causes the collection liquid to swirl and greatly reduces the agitation, bubbling and consequent reentrainment seen with the AGI-30. The BioSampler collects particles with aerodynamic diameters of approximately 0.3 µm to 8 µm into the collection media, although the upper cut-off diameter is not sharp [Hogan et al. 2005; Kesavan et al. 2010; Willeke et al. 1998]. The BioSampler reportedly can be used with non-evaporating fluids such as mineral oil to eliminate the collection time limits imposed by water evaporation, provided that the microorganism can survive collection and processing [Lin et al. 2000]. Alternatively, fluid can be exchanged or added to the sampler as needed [Rule et al. 2005; Rule et al. 2007].

The CIP10-M, a modified version of the CIP10 aerosol sampler, collects airborne microorganisms in a liquid layer on the interior surface of a rapidly-rotating cup. As with the BioSampler, the CIP10-M can be used with mineral oil as the collection fluid to avoid fluid evaporation. It is reported to have collection efficiencies of >80% for particles >2.8 µm, 50% for 2.1 µm particles, and <10% for particles of <1 µm [Görner et al. 2006; Simon et al. 2016].

May [1966] designed a three-stage sampler in which aerosol particles are collected by impaction onto a wetted fritted surface in the first two stages and the third stage is a swirling aerosol collector similar to the BioSampler. Both glass and stainless steel versions are available. In his original report, May [1966] used particles with a density of 1.5 g/cm^3 and reported cut-off sizes of 6 µm, 3.3 µm and 0.7 µm, which correspond to aerodynamic diameters of about 7.3 µm, 4 µm, and 0.86 µm. The May sampler is reported to give comparable results to the Andersen impactor [Zimmerman et al. 1987].

Several wetted-surface bioaerosol samplers recirculate the collection fluid and add additional fluid as needed to replace evaporative losses. This extends the collection time available and allows the concentration of the aerosol from a large volume of air at a high flow rate into a relatively small volume of liquid, which is of great advantage when searching for pathogens that may be present in very low concentrations. For this reason, such systems are often used for bioterrorism and homeland security applications. The Coriolis sampler [Carvalho et al. 2008], the OMNI-3000 [Zhao et al. 2014], the SASS 2000 [Ravva et al. 2012], and the SpinCon [Yooseph et al. 2013] use a wetted wall cyclone for bioaerosol collection, while the BioCapture 650 [Ryan et al. 2009] collects particles onto a wetted rotating impactor. Kesavan and Sagripanti [2015] reported the results of performance tests for several of these types of bioaerosol samplers.

When conducting long-term bioaerosol collection into liquid media, it is important to note that if the collected bioaerosol particles remain in the collection media for an extended time and if steps are not taken to inhibit growth, spore germination and cell amplification of some fungi and bacteria can occur. This can result in the appearance of much higher bioaerosol concentrations than are actually present in the environment.

f. Condensation-based bioaerosol samplers

Some bioaerosol particles are too small to be readily collected by impactors or impingers. These particles can be collected using filters, but filter collection can reduce the viability of microorganisms. One solution is to humidify the aerosol stream and then cool it, which causes water vapor to condense on the aerosol particles and create a droplet surrounding the particle. This larger particle can then be collected by impaction or impingement, as shown in Figure 9. This is similar in principle to condensation-based particle counters, which are used to measure the concentration of small airborne particles. Some researchers showed that adding water vapor to an aerosol stream enhanced the recovery of airborne viruses and bacteriophages, which may work by this method (although this is unclear) [Hatch and Warren 1969; Trouwborst and Kuyper 1974; Warren et al. 1969]. More recently, Milton developed a condensation-based system to collect fine particles containing influenza virus from the exhaled breath of human subjects [McDevitt et al. 2013; Milton et al. 2013]. A condensation-based bioaerosol sampler called a growth-tube collector has been used to collect MS2 bacteriophage and influenza virus in the laboratory, and is reported to be especially effective at recovering viable virus in sub-micrometer particles [Lednicky et al. 2016; Pan et al. 2016; Walls et al. 2016]. A version of this system called the Spot Sampler (Aerosol Devices, Inc.) is commercially available.

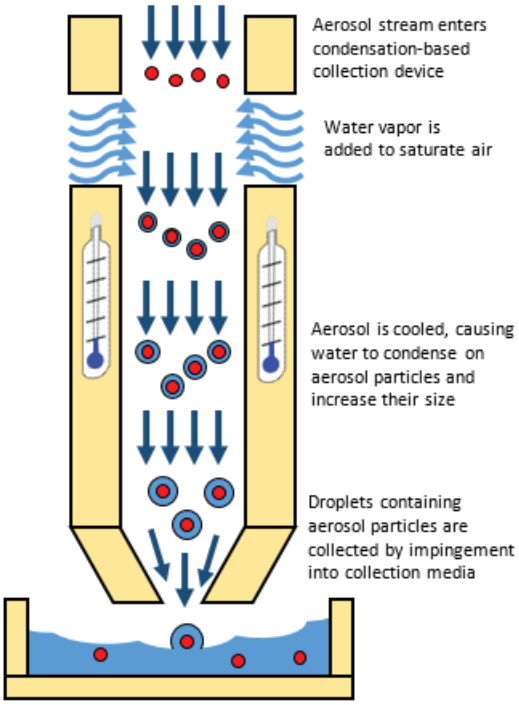

Figure 9: Condensation-based aerosol particle collector.

g. Electrostatic samplers

Electrostatic precipitation works by using a strong electric field to create a high concentration of unipolar ions. The rapid motion of these ions causes them to collide with and charge airborne particles, and the resulting charge on the particles causes them to be attracted to the collection surface [Hinds 1999]. Electrostatic precipitation systems have been used to collect bioaerosol particles such as allergens, bacteria and viruses [Artenstein et al. 1968; Artenstein et al. 1967; Custis et al. 2003; Donaldson et al. 1982; Heitkamp et al. 2006; Lee et al. 2004b; Parvaneh et al. 2000; Roux et al. 2013]. Such devices offer simplicity of design with few moving parts, and are generally effective at collecting small particles. One electrostatic bioaerosol sampling device is available commercially from Inspirotec [Gordon et al. 2015].

Some electrostatic bioaerosol samplers collect particles into liquid to concentrate the particles and help preserve the viability of microorganisms. The Large Volume Air Sampler (LVS) developed by Litton in the 1960's washed the collection surface with recirculating fluid; this sampler was successfully used to collect pathogenic respiratory bacteria and viruses in a variety of settings [Artenstein et al. 1968; Artenstein et al. 1967; Donaldson et al. 1982]. Pardon et al. [2015] developed a system that collects particles directly on a microfluidic chip. The electrostatic aerosol collector devised by Han et al. [2015] collects the deposited aerosol into rolling water droplets, which greatly

concentrates the particles. The Aerosol-to-Liquid Particle Extraction System (ALPES) uses an electrostatic system to collect aerosol particles into recirculating liquid, which helps preserve the viability of microorganisms [Heitkamp et al. 2006].

Electrostatically-charged cloths are used to collect airborne particles that settle onto them, and also to wipe settled dust from surfaces. These are discussed in the next two sections.

h. Passive bioaerosol samplers

Passive bioaerosol sampling refers to the collection of bioaerosols by allowing them to gravitationally settle onto a collection device, such as a culture plate, foil sheet, electret-based filter or electrostatically-charged cloth. Compared to active sampling, passive bioaerosol sampling has several advantages, including simplicity, low cost, lack of disturbance of the surrounding air, and the ability to collect for extended time periods [Haig et al. 2016; Pasquarella et al. 2000; Vincent 2007].

Passive bioaerosol sampling can be limited by several variables including the air currents around the device and airborne particle size. As discussed earlier, large particles settle much more quickly than small particles. Thus, large particles are much more likely to be collected by passive samplers [Haig et al. 2016; Reponen et al. 2011b]. As a result of these limiting variables, results from passive bioaerosol sampling cannot be directly related to the concentration of airborne particles and may not correlate well with results from active sampling [Reponen et al. 2011b]. However, some authors have proposed that passive sampling may be useful in evaluating the likelihood that bioaerosol particles will contaminate surfaces such as open wounds in operating rooms, since they mimic the contamination event more closely than does an active sampler [Friberg et al. 1999; Haig et al. 2016; Pasquarella et al. 2000].

Passive bioaerosol collectors are often placed 1.5 to 2 meters above the ground to avoid collection of large dust particles from sources other than airborne particles, such shoes, clothing, skin and animals [Frankel et al. 2012; Lioy et al. 2002; Noss et al. 2008; Rintala et al. 2012]. Grills, screens or shields may also be placed around or over the collection device to screen out large debris [Brown et al. 1996; Wagner and Macher 2003; Whitehead and Leith 2008; Wurtz et al. 2005].

1.) Settle plates

Settle plates (also called settling plates or sedimentation plates) are culture plates containing nutrient agar that are opened and placed collection-side up in a location of interest. Airborne particles are allowed to settle onto the plates for a specified time, and the plates are then closed, incubated and inspected for growth. Settle plates are

commonly used to assess airborne microbial contamination and are listed in methods and standards from the ISO, the American Public Health Association (APHA) and the United States Pharmacopeia (USP) [Dyer et al. 2004; ISO 2003; USP 1997]. However, because the results from settle plates cannot be directly compared to the amount of airborne microbes, they should only be used for qualitative, not quantitative, evaluations. The CDC recommends the use of high-volume air samplers rather than settle plates when investigating airborne fungal spore contamination in health care facilities [CDC 2003].

Settle plate methods suffer from a lack of standardization of methodology, which makes results difficult to compare. Pasquarella et al. [2000] reviewed the use of settle plates and proposed an Index of Microbial Contamination (IMA) to standardize the use of settling plates. To measure the IMA, 90 mm culture plates are placed 1 meter above the floor and 1 meter from any walls, and collect settled particles for 1 hour (called the 1/1/1 scheme). The number of colony-forming units (CFUs) detected on each plate is then used to calculate the IMA in CFUs/dm2/hour [Pasquarella et al. 2000].

2.) **Electrostatic dust collectors**

Noss et al. [2008] developed a method called the electrostatic dustfall collector (EDC) that collects settling airborne particles onto four electrostatically-charged cloths. EDC's have been used in studies of culturable bacteria and fungi, endotoxin, glucan and inflammatory mediators in airborne particles [Adams et al. 2015; Frankel et al. 2012; Huttunen et al. 2016; Kilburg-Basnyat et al. 2016; Kilburg-Basnyat et al. 2015; Noss et al. 2010; Noss et al. 2008]. Noss et al. [2008] and Frankel et al. [2012] reported good correlations between the EDC and active aerosol samplers. Adams et al. [2015] compared EDC's to Petri dishes and other passive collection materials and found that the results correlated reasonably well, but that a rigorous extraction protocol was required to get consistent results from the EDC's. Brown et al. [1996] developed a passive electrostatic-based personal aerosol sampler and reported that it gave a reasonable correlation with inhalable dust measurements at farms and a rubber plant.

3.) **Other passive bioaerosol samplers**

The UNC Passive Aerosol Sampler consists of a 6.8 mm diameter collection substrate mounted on a scanning-electron microscope stub and shielded by a protective screen [Wagner and Macher 2003; Whitehead and Leith 2008]. Airborne particles settle or diffuse onto the substrate and can be analyzed by optical or electron microscopy. Other investigators have used aluminum sheets in boxes, Petri dishes, and sheets of various plastic materials as passive bioaerosol collectors [Adams et al. 2015; Meadow et al. 2015; Wurtz et al. 2005].

i. Settled dust collection devices

The collection and analysis of dust that has settled onto floors, carpets, and other surfaces is widely used as a means of identifying bioaerosols in buildings, especially allergens, endotoxin and molds [Hung et al. 2005; Lioy et al. 2002; Martyny et al. 1999; Morey 2007; Rintala et al. 2012]. Settled dust sampling allows for the collection of large quantities of material, provides a long-term sample, and does not require a dedicated sampling device for each location. Dust assays allow quantitative data to be generated per weight and surface area of dust. Some investigators find it useful to compare different sites in a building or to sample before and after remediation efforts to see if the source of a bioaerosol has been eliminated.

Settled dust will vary within a building depending upon the location and collection surface [Lioy et al. 2002; Rintala et al. 2012]. In addition to settling from the air, dust can be produced by a variety of other mechanisms, making it difficult to distinguish the source. Floor and carpet dust, for example, will include outside material brought in by shoes, skin flakes, clothing fibers and animal dander. Sampling locations well above floor level are often chosen to minimize the amount of dust that is not from settled airborne particles [Frankel et al. 2012; Rintala et al. 2012].

1.) Vacuums

The US Department of Housing and Urban Development has developed a protocol for the vacuum collection of home dust samples to test for allergens [HUD 2008]. Vacuum collection of settled dust from floors and carpets has been used to determine the Environmental Relative Moldiness Index (ERMI), which is a measure of mold contamination in homes [Kettleson et al. 2015; Reponen et al. 2012; Reponen et al. 2011a; Taubel et al. 2016; Vesper et al. 2013; Vesper et al. 2007]. ERMI is discussed in more detail later in this chapter. Note that vacuuming can increase the levels of bioaerosols in a location. Thus, air sampling should be completed before collecting surface samples by vacuuming [Hung et al. 2005; Hunter et al. 1988].

2.) Swabs

Swabs are widely used to collect airborne material that has settled onto surfaces. Swabs are also used to identify microbial contaminants that may be colonizing building materials within the indoor environment. However, obtaining consistent and reliable results from swab sampling is far more difficult than is often appreciated, and careful attention is needed to the choice of swab material, elution media, and method of swabbing. If swab samples are to be cultured, aseptic technique is needed to avoid contamination. ASTM International has a standard for collecting fungal material by swab [ASTM 2012]. The APHA has published a standard method for swab sampling of

food-contact surfaces [Dyer et al. 2004], while the USP and ISO have standards that include swab sampling for microbiological contamination in clean rooms [ISO 2003; USP 1997].

An example of a validated protocol for swab sampling is that provided by NIOSH for surface sampling for Bacillus anthracis spores [Hodges et al. 2010; Hodges et al. 2006; NIOSH 2012b]. In this procedure, a defined area is first outlined using a template or a ruler and masking tape. A sterile macrofoam swab is then moistened using a buffer solution that neutralizes disinfectants. The surface is swabbed using horizontal strokes, followed by vertical strokes, and finally diagonal strokes, and the swab is then placed in a sterile tube for transport and analysis. Aseptic technique is used throughout the procedure.

The choice of swab material can have a significant impact on the collection of microorganisms from a surface. Moore and Griffith [2007] studied the recovery of Escherichia coli and Staphylococcus aureus from stainless steel squares using nylon-flocked swabs and spatulas, cotton swabs and rayon swabs. They reported that nylon-flocked and cotton swabs were equally effective at removing bacteria from dry surfaces, but that cotton swabs removed bacteria more effectively from wet surfaces than rayon or nylon-flocked swabs. However, nylon-flocked swabs and spatulas released the bacteria into the elution media more readily than rayon swabs, which in turn released more bacteria than cotton swabs. For viruses, polyester-tipped swabs were found to be more effective than cotton swabs or antistatic wipes at recovering MS2 bacteriophage from stainless steel and plastic [Julian et al. 2011], while macrofoam swabs performed best when recovering wet or dried norovirus from stainless steel surfaces, followed by cotton, rayon and polyester swabs [Park et al. 2015].

The elution media used to wet the swabs and recover the bacteria from the swabs also can have a substantial effect on sampling. Moore and Griffith [2007] tested eleven different swab wetting solutions containing various combinations of salts, surfactants and nutrients. They found that the recovery efficiency varied widely depending upon the species of bacteria, type of swab, and whether the surface was wet or dry. For MS2 bacteriophage, saline or Ringer's solution (an isotonic salt solution) worked better than viral transport media or pure water [Julian et al. 2011]. It is important to note that the elution media must both remove the biological material from the surface and subsequently elute it from the swab in order to be effective.

Although they may be overlooked, storage conditions play an important role in swab sampling. After sample collection, room temperature storage of moist swabs may lead

to microbial growth if the elution media or swab contain nutrients, while the presence of chemicals such as Tween 80 may reduce viability over time. These problems can be alleviated by placing the swabs in cold storage as quickly as possible [Moore and Griffith 2007].

3.) **Wipes**

All of the considerations and limitations of swab sampling also apply to wipe sampling. Swabs are typically more useful for small surfaces and hard-to-reach locations, while wipes are more effective at collecting dust from large non-porous surfaces [NIOSH 2012b]. Electrostatic wipes have been used to collect settled dust for studies of mold and endotoxin [Bolaños-Rosero et al. 2013; Thorne et al. 2005]. However, Thorne et al. [2005] found that wipes and gloves themselves were frequently contaminated with endotoxin and needed to be tested before use.

4.) **Adhesive tape**

Adhesive tape can be used to collect dust samples from surfaces for microscopic examination (this is called tape lift or cellotape sampling) [ASTM 2014c; Martyny et al. 1999; Morey 2007]. Typically, a section of adhesive tape is gently pressed onto a surface of interest, removed with a slow steady force, and then attached to a glass slide or placed in a vial. The samples are relatively simple to collect, but the results depend upon the ability of the examiner to identify microorganisms and their fragments, and do not provide a quantitative assessment of exposure.

5.) **Contact plates**

Contact plates are typically round culture plates in which the agar is poured so that the top of the agar forms a meniscus slightly above the top rim of the plate. A surface sample is collected by inverting the plate and pressing the agar directly onto a flat surface of interest. The plate is then removed, incubated and inspected for microbial growth. This sampling method is often called the replicate organism direct agar contact (RODAC) procedure, and it is commonly used for biocontamination monitoring in the pharmaceutical and food industries [Dyer et al. 2004; ISO 2003; USP 1997]. Because many of the surfaces of interest in these industries are routinely disinfected, contact plates are available with agars that contain neutralizers for disinfectants. One report indicated that nitrocellulose membranes were slightly more effective than RODAC plates at surface sampling, and are easier to use on curved surfaces [Poletti et al. 1999].

j. Heating, ventilation and air conditioning (HVAC) filters

Building HVAC systems filter large quantities of outside and recirculated inside air as they maintain environmental conditions inside buildings. Researchers have taken advantage of these existing filtration systems as a way to study bioaerosols in a variety of structures [Goyal et al. 2011; Haaland and Siegel 2016; Noris et al. 2011]. Testing the collected particulate material on HVAC filters provides an inexpensive way of studying bioaerosols collected from large volumes of air over long time periods. However, some limitations must be kept in mind. Extracting bioaerosols from these filters can be difficult and the methods require validation [Farnsworth et al. 2006]. Many microorganisms lose viability after collection, so although PCR-based methods may be effective, culture-based methods likely will not work except for very hardy microbes [Farnsworth et al. 2006]. Finally, commonly-used HVAC filters can have relatively low collection efficiencies, especially for small particles [ASHRAE 2009]. Haaland and Siegel [2016] reviewed 60 studies in which HVAC filter analyses were used to study bioaerosols in buildings.

k. Real-time bioaerosol monitoring

Many biological molecules have an intrinsic autofluorescence, and this phenomenon has been used as the basis for continuous real-time bioaerosol detection systems [Pöhlker et al. 2012]. This technique is most commonly employed for studies of atmospheric bioaerosol particles and for biodefense and biosecurity applications. These systems can distinguish biological from non-biological particles, and can usually provide information about the particle size and some characteristics of the bioaerosols. One device, the TSI Ultraviolet Aerodynamic Particle Sizer (UV-APS), was used in several studies [Bhangar et al. 2016; Hairston et al. 1997; Kanaani et al. 2008]; it has been replaced by an updated version called the Fluorescence Aerosol Particle Sensor (FLAPS) III. Other real-time bioaerosol detectors include the BioScout [Saari et al. 2014], the Wideband Integrated Bioaerosol Sensor (WIBS-4) [Toprak and Schnaiter 2013], and the Fido B2 (formerly called the Instantaneous Bioaerosol Analysis and Collection, IBAC) [Santarpia et al. 2013].

4 Considerations for bioaerosol sampling

a. Development of a bioaerosol sampling strategy

The first step in designing a sampling strategy for bioaerosol sampling is to determine the purpose of the sampling [ASTM 2014a]. For example, bioaerosol sampling may be conducted to estimate worker exposure to bioaerosols, or to select or evaluate engineering controls to reduce exposures, or to identify the source of a bioaerosol. A sampling strategy then should begin with an overview of the site of interest and development of initial hypotheses regarding the types, sources and distributions of bioaerosols. After this, the sampling methods, times, durations, and the analytical methods can be selected. Note that

bioaerosol sampling is almost always done in conjunction with the collection of other types of data, such as worker health information, visual observations, air flow measurements, surface sampling, and information about possible sources.

b. Sampling locations

The sampling locations should be selected to assist in evaluation of the working hypotheses about possible exposures [ASTM 2014a]. If worker exposures are being evaluated, then the samplers should be placed in areas occupied by the workers. If contamination of a ventilation system is being examined, then sampling in the system and at the ventilation louvers would be appropriate. Care must be exercised to ensure that people do not tamper with the samplers and that microorganisms on surfaces or in duct work are not inadvertently aerosolized.

Bioaerosol samples should be drawn directly into the sampler rather than being transported to the sampler by tubing. If transport tubing must be used, it should be as short and straight as possible. Abrupt flow constrictions and bends in the tubing should be especially avoided, as considerable sample deposition can occur at these locations. The tubing diameter should be large enough that the flow is not turbulent and that the d50 of any bends is well above the size of the bioaerosol particles [Pui et al. 1987; Tsai and Pui 1990]. The tubing should be made of a material that does not lead to losses through electrostatic deposition [Liu et al. 1985]. A review of the many issues surrounding the transporting of aerosols through sampling lines is provided by Brockmann [2011].

Personal aerosol sampling provides a much better representation of worker and resident exposure to aerosol particles than area (static) sampling [Cherrie et al. 2011; Kissell and Sacks 2002; Rodes and Thornburg 2005]. However, most samplers for viable bioaerosols do not lend themselves to personal sampling. Thus, a combination of personal and area sampling may be necessary to fully characterize the exposure [Toivola et al. 2002].

c. Concentrations of indoor and outdoor bioaerosols

Indoor bioaerosol sampling is conducted in occupational (industrial, education, and office environments) and non-occupational (residential and buildings) settings. Outdoor bioaerosol sampling is often performed to provide comparative data for indoor sampling and to help determine possible sources of contaminants. Outdoor bioaerosol sampling also is conducted in occupational environments such as agricultural settings, composting sites and sewage treatment plants [Environment Agency 2009; Lee and Liao 2014; Masclaux et al. 2014]. In addition, outdoor sampling may be performed for pollen and fungi to assist allergists in their treatment of patients by identifying taxa distribution and concentrations in air over time.

The concentrations of bioaerosol particles vary widely depending upon the meteorological parameters, the location of sources, the time of year and the amount of ventilation. Shelton et al. [2002] studied 1,717 buildings in the United States. They found that outdoor levels of airborne fungi are usually higher than indoor levels, and that fungal levels were highest in the fall and summer and lowest in the winter and spring. Outdoor levels varied from 1 to more than 8,200 colony-forming units (CFU)/m^3 of air, with a median of 540 CFU/m^3. Indoor levels ranged from 1 to over 10,000 CFU/m^3, with a median of 82 CFU/m^3. An examination of fungi in flood-damaged homes found fungal concentrations of 1,100 to 8,400 spores/m^3 outside and 500 to 101,100 spores/m^3 inside [Reponen et al. 2007]. An investigation of 100 large office buildings by Tsai and Macher [2005] found that airborne bacterial concentrations tend to be higher outdoors than indoor (except for Gram-positive cocci). Outdoor concentrations tended to be higher in the winter (194 vs. 165 CFU/m^3), while indoor concentrations were higher in the summer (116 vs. 87 CFU/m^3). Forty-one percent of the bioaerosol samples were below the detection limit, and >95% of the culturable bacteria were mesophilic (grow at moderate temperatures). In a report on agricultural workers working in animal confinements, Lee et al. [2006] found breathing zone culturable bioaerosol exposures of 300 to 36,000 CFU/m^3 for fungi, 3000 to 3.3 x 108 CFU/m^3 for bacteria, and up to 2,800 CFU/m^3 for actinomycetes. During grain harvesting, workers were exposed to culturable bioaerosol levels of 82,000 to 7.4 × 106 CFU/m^3 for fungal spores, 40,000 to 1.4 × 106 CFU/m^3 for bacteria, and up to 2.6 × 104 CFU/m^3 for actinomycetes.

If one or more genera of fungi or bacteria are found indoors in concentrations greater than outdoor concentrations, then the source of amplification may need to be found and remediated. When conducting indoor bioaerosol sampling, it is advisable to sample before, during, and after the sampling area is occupied, including times when the heating, ventilating, and air conditioning system is activated and inactivated.

d. Viable and nonviable bioaerosols

Viable microorganisms are metabolically active (living) organisms with the potential to reproduce, grow and colonize. Viruses are not metabolically active but are considered viable if they are capable of reproducing in an appropriate cellular host. Viable microorganisms may be culturable or non-culturable. Culturable organisms reproduce under controlled laboratory conditions. Non-culturable organisms do not reproduce in the laboratory because of intracellular stress or because the conditions (e.g., culture medium or incubation temperature) are not conducive to growth. Some bacteria can be very difficult or impossible to culture from bioaerosols. For example, although human *Mycobacterium tuberculosis* is readily transmitted among people and from people to Guinea pigs, it has never been successfully cultured from an environmental aerosol

sample, probably because of its extremely low airborne concentrations and slow growth rate [Nardell 2016]. Other bioaerosols such as *Histoplasma capsulatum* or *Pneumocystis carinii* may take weeks to grow or may not even grow in culture at all [Dennis 1990; Ibach et al. 1954]. As the name implies, viable bioaerosol sampling involves collecting a bioaerosol and culturing the collected particles. Only culturable microorganisms are enumerated and identified, thus leading to an underestimation of bioaerosol concentration. Non-viable and viable but non-culturable microorganisms are often studied by collecting them with a dry aerosol sampler or a membrane filter. The microorganisms are then enumerated and identified using microscopy, classical microbiology, molecular biology, or immunochemical techniques [Hung et al. 2005; Macher 1999; Reponen et al. 2011b; Tortora et al. 2013].

Assessment of viable bacteria is also dependent on a number of variables including nutrient media, temperature and culture conditions. In indoor environments the collection of viable bacteria may be confounded by endogenous bacterial microflora such as *Staphylococcus epidermis* that sheds with skin flakes [Hung et al. 2005]. Concentrations of viable bacteria have been reported to be as high as 105 CFU/m^3 in indoor environments; however, like fungi, the proportion of the total bacterial burden may be higher if non-viable bacteria are also included [Hung et al. 2005]. In addition, viable assessment of several bacterial species of clinical significance may not be the best approach as these bacteria do not remain viable in the air. Alternative methods such as immunoassays or molecular-based methods may provide suitable approaches for quantifying bacterial pathogens.

e. Bioaerosol particle sizes

As noted earlier, the aerodynamic diameter (d_{ae}) of an airborne particle is the most important factor determining how long it will remain in the air, how likely it is to be inhaled, and where it will deposit in the respiratory tract. The sizes of bioaerosol particles can range from tens of nanometers for small fragments to hundreds of micrometers for pollen, fungi or large agglomerations. However, most of the bioaerosol particles of interest in the indoor environment fall between about 100 nm and 10 µm [Nazaroff 2016]. For bacteria, vegetative cells typically have physical diameters of about 0.2 to 2 µm and are 2 to 8 µm in length, while bacterial spores are somewhat smaller [Tortora et al. 2013]. Airborne particles containing bacteria were found to have aerodynamic diameters of about 1 to 3 µm in indoor environments [Gorny et al. 1999; Kujundzic et al. 2006; Meklin et al. 2002]. *Mycobacterium tuberculosis* is a rod-shaped bacteria with a length of about 6.6 µm [Schafer et al. 1999]. When aerosolized from a liquid culture, *M. tuberculosis* DNA was found in particles with aerodynamic diameters of 0.6 to 1.8 µm [Schafer et al. 1999]. Aerosolized *Mycobacterium bovis* BCG (a commonly-used surrogate for *M. tuberculosis*) was found in

particles with aerodynamic diameters of 0.5 to 9.9 µm [Schafer et al. 1998]. Air sampling around indoor whirlpools in a public facility found airborne mycobacteria DNA in particles with aerodynamic diameters of 0.5 to 9.9 µm [Schafer et al. 2003]. *Actinomycete* spores tend to be smaller, with aerodynamic diameters of cultured spores ranging from 0.6 to 1.5 µm [Madelin and Johnson 1992; Reponen et al. 1998]. Fungal spores have physical diameters of about 0.5 to 30 µm or larger, while the aerodynamic diameters of airborne fungal spores and spore clusters are reported to be from 0.9 to 5 µm [Eduard 2009; Hussein et al. 2013; Reponen et al. 2011b].

Airborne microorganisms are often present as parts of aggregations, droplets or agglomerations that can be much larger than the size of the native microorganism. In indoor environments with large amounts of other aerosol particles like cigarette smoke, bacteria have been found on particles with aerodynamic diameters up to 10 µm, which was larger than airborne bacterial particles in cleaner environments. This was thought to occur because the aerosol particles were forming agglomerates [Gorny et al. 1999]. In a farm study, airborne *Actinomycetes* and fungal spores were more likely to be found in aggregates in environments with higher spore concentrations [Karlsson and Malmberg 1989]. In two studies of airborne influenza virus in health care facilities, about half of the airborne virus was found in particles with aerodynamic diameters of 4 µm or greater, even though the virus itself is only about 100 nm in diameter, because the virus was contained in aerosolized droplets of respiratory fluids [Blachere et al. 2009; Lindsley et al. 2010a]. Agglomerates of fungal spores can break apart upon impaction inside an impactor and be collected on subsequent stages with smaller cut-off diameters [Trunov et al. 2001]. Bioaerosols may also be present as cellular fragments that are much smaller than the source microorganisms. Endotoxins are fragments of the cellular walls of Gram-negative bacteria that have been implicated in a variety of illnesses [Eduard et al. 2012; Jacobs 1989; Olenchock 2002]. Fragments of fungal cell walls also are thought to be associated with several types of adverse respiratory health effects [Green et al. 2011; Green et al. 2006b; Olenchock 2002]. Very high levels of fungal fragments have been measured in flood-damaged homes contaminated with mold [Reponen et al. 2007]. Fungal fragments also contain a variety of secondary metabolites, mycotoxins, beta-glucan, antigens and allergens [Green et al. 2011; Green et al. 2006b]. In one study of indoor air in homes, the majority of the endotoxin and fungal wall material was found in particles with aerodynamic diameters of less than 1 µm [Adhikari et al. 2013]. Another study found considerable amounts of endotoxin in aerosol particles from metalworking fluids that were between 0.16 and 0.39 µm [Wang et al. 2007]. Indoor and outdoor measurements of endotoxin levels found that the largest proportion was detected in particles with aerodynamic diameters of less than 1 µm [Kujundzic et al. 2006].

It is common to use an aerosol spectrometer in conjunction with bioaerosol sampling to better understand the size distribution of the airborne particles. One consideration when interpreting the data is, of course, that the large majority of these devices do not distinguish between biological and non-biological aerosols. Another less-obvious factor is that while a few aerosol spectrometers such as the TSI Aerodynamic Particle Sizer measure the aerodynamic diameter of the airborne particles, many aerosol spectrometers measure particles using light scattering and thus provide an approximate physical diameter instead [Hinds 1999; Sorensen et al. 2011]. The difference between the aerodynamic and optical diameters may be significant depending upon the shape and density of the particles.

f. Temperature and humidity

The temperature and humidity of the environment can affect the size of bioaerosol particles, the viability of airborne microorganisms, the growth of microorganisms on surfaces, and the amount of electrostatic charges on aerosols and surfaces. Because of these effects, the environmental temperature and humidity should be recorded during bioaerosol sampling.

Water evaporates rapidly from wet aerosol particles [Hinds 1999]. If an airborne particle is initially an aqueous solution containing non-volatile substances such as salts and organic material, and if the relative humidity is above the crystallization relative humidity (CRH, also called the efflorescence relative humidity), then some of the water will evaporate and the solution will become more concentrated, but the particle will remain liquid. If the relative humidity is below the CRH, then all of the water will evaporate (that is, the particle will desiccate) [Nicas et al. 2005]. Similarly, if an airborne particle is initially a dry combination of salts and organic material, and if the relative humidity is below the deliquescence relative humidity (DRH), then the particle will remain desiccated. However, if the relative humidity is above the DRH, then the particle will absorb water until it liquefies and becomes an aqueous solution. The DRH is always greater than the CRH [Nicas et al. 2005]. A particle in an environment above its CRH (or DRH if it was initially dry) will be larger and heavier and will settle faster than the same particle when the humidity is below the CRH, which can affect the size and amount of bioaerosol particles that are collected during sampling [Mikhailov et al. 2004]. This phenomenon was seen in a study of particles in human exhaled breath, where the particles detected in low humidity air were substantially smaller than those detected when the air was more humid [Holmgren et al. 2011].

Bioaerosol particles may also undergo an increase in size when the humidity increases due to water absorption and swelling of hygroscopic components. An increase in relative humidity has been shown to increase the aerodynamic diameter of fungal spores [Madelin

and Johnson 1992; Reponen et al. 1996]. Similar results have been reported for *Actinomycetes* spores [Madelin and Johnson 1992].

For airborne viruses, survival decreases as air temperature increases [Ijaz et al. 2016; Tang 2009]. Exposing most viruses to temperatures of 60°C or higher for 60 minutes will inactivate them, although the viruses can be somewhat protected if they are encased in organic material [Tang 2009]. For example, in one set of experiments, airborne particles containing vaccinia virus, influenza virus, and Venezuelan equine encephalomyelitis virus all showed higher survival rates at 7-12°C than at 21-24°C, and still lower survival at 32-34°C [Harper 1961]. Aerosol transmission of influenza virus among Guinea pigs is blocked at air temperatures of 30°C [Lowen et al. 2008]. The effect of humidity on virus survival depends upon the virus; in general, viruses with lipid envelopes tend to survive better at low humidity, while non-enveloped viruses survive better at high humidity [Ijaz et al. 2016; Tang 2009]. For example, influenza viruses and coronaviruses have enveloped capsids, and both survive better at low humidities compared to high [Ijaz et al. 1985a; Ijaz et al. 2016; Noti et al. 2013; Schaffer et al. 1976]. On the other hand, rotaviruses and rhinoviruses have non-enveloped capsids and survive better at high humidities compared to low [Ijaz et al. 1985b; Ijaz et al. 2016; Karim et al. 1985].

The survival of airborne bacteria also decreases as air temperature increases; the survival of virtually all airborne bacteria declines when temperatures are above 24°C [Ijaz et al. 2016; Tang 2009]. However, as with viruses, the effects of humidity on bacterial survival are much more complex, and depend not only upon species but also upon the methods of culture and aerosolization [Cox 1989; Tang 2009]. In field experiments in a greenhouse, survival of certain bacteria was 35- to 65-fold higher at 80% RH than at 40% [Walter et al. 1990]. In laboratory experiments, survival of certain bacteria was virtually complete at low RH but was reduced at RH values above 80% [Cox 1968]. Higher humidities can also significantly decrease the efficacy of ultraviolet germicidal irradiation (UVGI) for reducing levels of viable airborne bacteria [Peccia et al. 2001]. Cox [1987] believes the potential for the movement of the solvent water is an important environmental criterion in assessing survivability of bacteria, viruses, and phages.

Fungi and fungal spores generally are better able to withstand environmental stresses compared to vegetative bacteria and viruses [Ijaz et al. 2016; Tang 2009]. Warm temperatures, wet substrates and humid air conditions favor the growth of fungi on surfaces [Eduard 2009; Tang et al. 2015]. Temperature can induce morphological changes in dimorphic fungi such as the pathogen *Histoplasma capsulatum* [Salvin 1949]. It is not clear, however, how air temperature and humidity affect the viability of airborne fungi and fungal spores [Tang 2009].

g. Electrostatic effects

Aerosol particles in the workplace can be highly charged, and the electrostatic charge can vary considerably depending upon the aerosol generation mechanism and the particle characteristics [Johnston et al. 1985]. Aerosol particles are especially prone to develop electrostatic charges in low humidity environments [Baron and Deye 1990]. Like most particles, freshly generated microbial aerosols are nearly always electrostatically charged unless steps are taken to neutralize them. Lee et al. [2004b] found that airborne fungi and bacteria carried a net negative charge in most of the laboratory and field environments that they studied. Mainelis et al. [2002] found that a strong positive electrostatic charge reduced the viability of *Pseudomonas fluorescens* bacteria but did not affect *Bacillus subtilis* spores.

The effect of electrostatic charge on aerosol collection is often overlooked, resulting in the possible bias of sampling results [NIOSH 2016a; Vincent 2007]. Aerosol samplers made of non-conductive plastics can develop substantial electrostatic charges, which can degrade their performance significantly [NIOSH 2016a; Baron and Deye 1990]. The use of polyethylene or polytetrafluoroethylene (PTFE) tubing to transport air streams to a sampler can remove a sizeable amount of aerosol particles by electrostatic deposition [Liu et al. 1985]. As noted above, the use of plastic Petri dishes in an Andersen impactor can result in bioaerosol particle losses [Andersen 1958; Kuo 2015]. Whenever possible, it is better to use aerosol samplers made of conductive materials such as metals or specially-treated plastics [NIOSH 2016a].

h. Flow calibration

Accurate airflow rates are very important in calculating the concentration of microorganisms in the air. All samplers should be calibrated before and after sampling to ensure that the flow rate is within the manufacturer's specifications and does not change from the initial calibration. Calibration may be performed using a primary standard such as a spirometer or bubble calibrator. Where it is not possible to calibrate using a primary standard, a calibrated secondary standard such as a dry gas meter may be used. The calibration of such a secondary standard should be traceable to a primary standard. A detailed explanation of the calibration of airflow rates is given by McCammon Jr. and Woebkenberg [NIOSH 2016c].

i. Blanks

Laboratory media blanks are unexposed, fresh samples of media, such as agar plates, filters and impinger fluids. These samples are generally not taken into the field. Before using any batch of media, incubate at least three culture plates under the same conditions as planned

for the field samples, in order to check for sterility of the media. Approximately five media blanks should be included with each sample set. If the samples are to be analyzed by an outside laboratory, consult the specific laboratory procedure for the number of blanks to be submitted. Similarly, blank filters should be processed in the same manner as planned for field samples in order to check for contamination.

Field blanks are simply unopened, fresh media samples that are handled in the same way as field samples, including labeling, except that no air is drawn through the sampler. The generally recommended practice for the number of field blanks is to provide at least two field blanks for every 10 samples with a maximum of 10 field blanks for each sample set.

5 Selection of bioaerosol samplers

The first step in selecting a bioaerosol sampling device is to establish the purpose of the sampling. Once the goal of the bioaerosol sampling is determined, the appropriate sampling methods may be chosen. The selected bioaerosol sampler must be capable of high efficiency particle collection within the physical and biological conditions required by the microorganisms to be sampled. The most appropriate sampling methods will be dictated in part by the techniques that will be used to analyze the sample. Methods for bioaerosol sample analysis are discussed in the next section. A list of some manufacturers and suppliers of bioaerosol sampling equipment and supplies is shown in Appendix I. The characteristics of several commonly used bioaerosol samplers are shown in Appendix II.

a. Sampling for airborne bacteria and fungi

Choosing a bioaerosol sampler for bacteria and fungi begins by deciding how the bioaerosol will be analyzed, and in particular whether the viability of the bacteria or fungi will be evaluated. Culturable bioaerosol sampling instruments must minimize injury during the collection process and maintain the culturability of the collected microorganisms. If the sample will not be cultured, then the samples usually can be collected dry using a membrane filter, cyclone, impactor, or a combination of these. Dry collection is typically simpler and less expensive to perform, and filters and cyclones can handle a wide range of particle concentrations. Organisms that are difficult or impossible to grow in culture are often collected using dry techniques and assessed using polymerase chain reaction (PCR) based methods, which have the advantage of speed and specificity. PCR has been used for rapid detection of *Histoplasma capsulatum* and mycobacteria [Reid and Schafer 1999; Schafer et al. 1999; Schafer et al. 2003]. A DNA-based mold specific quantitative PCR (msQPCR) method is widely used to evaluate indoor fungal bioaerosols in the academic, government and commercial sectors, and is the basis for the Environmental Relative Moldiness Index (ERMI) used to quantify mold contamination in

homes [Kettleson et al. 2015; Vesper et al. 2013]. The ERMI and other PCR-based assays are discussed in greater detail later in this chapter.

If viability is to be studied, then the samples usually will need to be collected with an impinger or in an Andersen impactor loaded with agar plates, because many microorganisms will lose viability due to damage or desiccation if collected dry [Cox 1987; Hung et al. 2005]. For example, a membrane filter sampler is not appropriate for sampling culturable *Escherichia coli* because the cells desiccate and become either nonviable or viable but not culturable under these conditions [Jensen et al. 1992]. Similar results have been reported for other bacteria and fungi [Macher and First 1984; Wang et al. 2001]. Depending upon the target microorganism, impingers may be filled with distilled water or a buffered isotonic solution, sometimes with antifoaming agents to reduce foaming and proteins to enhance survival. Mineral oil has also been used in impingers instead of aqueous solutions to avoid evaporation [Lin et al. 2000]. Impactors are loaded with agar plates; the choice of agar depends upon the microorganisms of interest and the desired selectivity (discussed in the next section).

As noted previously, depending upon the investigation that is being conducted, the particle size distribution of the bioaerosol may be very important in the evaluation of the data obtained. If particle size information is needed to, for example, determine how much of the bioaerosol is in the respirable size fraction, then a size-selective sampler should be used for at least some of the collections if possible. For example, if an SAS-Compact sampler was the selected sampler for collection of culturable Escherichia coli, an Andersen 6-Stage sampler could be used to determine the particle size distribution at each location sampled. The expected size of the bioaerosol particles is also an important factor in choosing a sampler. For example, an impactor with a d50 of 4 µm should not be used to collect *Aspergillus niger* spores (dae 1-3 µm) because most spores would remain entrained in the air and pass through the instrument.

NIOSH Method 0800 discusses sampling for culturable airborne bacteria and fungi with an Andersen cascade impactor [NIOSH 2003b]. Standard methods for the collection of airborne fungi by inertial impaction are presented in ASTM Standards D7788 [ASTM 2009; ASTM 2014d]. ASTM Standard D7391 also discusses the aspects related to the laboratory analysis.

b. Sampling for airborne viruses

Airborne viruses are more difficult to study in bioaerosols than bacteria and fungi for a variety of reasons [Prussin et al. 2014; Verreault et al. 2008]. Viruses are more difficult to culture because they are obligatory intracellular parasites that require a host cell for

reproduction [Tortora et al. 2013]. Bioaerosols of pathogenic viruses have been found in many settings to be present in low concentrations that can be difficult to detect [Blachere et al. 2009; Bonifait et al. 2015; Lindsley et al. 2010a; Tseng et al. 2010; Yang et al. 2011]. Viruses also are generally more susceptible to damage during aerosol collection than are bacteria or fungi, although their sensitivity varies widely with the collection method and species [Appert et al. 2012; Turgeon et al. 2014; Zuo et al. 2013]. Aerosol sampling methods for viruses have been reviewed by Verreault et al. [2008].

Bacteriophages are viruses that infect bacteria rather than multicellular organisms. They are used in laboratory aerosol studies as tracers for aerosol particles and as surrogates for airborne viruses that infect humans [Fisher et al. 2012; Tseng and Li 2005; Turgeon et al. 2014]. Bacteriophages are not known to be hazardous to humans but are of interest to industries that rely on bacteria such as cheese manufacturers [Verreault et al. 2011]. Polymerase chain reaction (PCR) based methods are often used to study viral bioaerosols. PCR has the advantages of being very sensitive and very specific, and considerably easier to perform than viral cultural assays. For this reason, most recent field studies of airborne viruses have used PCR as the detection method. Examples include studies of viruses in healthcare facilities [Blachere et al. 2009; Booth et al. 2005; Lindsley et al. 2010a; Thompson et al. 2013; Tseng et al. 2010], influenza at poultry and pig farms [Corzo et al. 2013; Jonges et al. 2015], airborne viruses in a sewage treatment plant [Masclaux et al. 2014], and respiratory viruses in human coughs and exhaled breath [Gralton et al. 2013; Lindsley et al. 2010b; Milton et al. 2013].

PCR has both the advantage and disadvantage of not requiring that the virus be viable in order to be detected. This eliminates the need to preserve viability during and after collection and allows the use of dry collection methods such as cyclone samplers, dry impactors and filters, which are simpler and easier to carry out. On the other hand, this also means that it is unclear whether the airborne virus is infectious or not, which makes interpretation of data more difficult. This is a common criticism of PCR-based bioaerosol studies.

If the virus in a bioaerosol sample is to be cultured, in most cases the sample will need to be collected into an aqueous media using an impinger or wetted surface aerosol collector. Fabian et al. [2009] showed that collecting airborne influenza virus in aqueous media using an SKC BioSampler preserved infectivity much better than dry collection using filters or an impactor. A less-common method is to collect viable viruses using an Andersen impactor. Gustin et al. [2011] collected airborne influenza virus using an Andersen impactor by placing a filter and a thin layer of gelatin on top of the agar in the culture plates. After collection, the gelatin was removed and melted at 37°C to allow

subsequent culture of the virus. Note that the collection media must be compatible with the cell culture system used to host the virus.

6 Sample preparation for culturable bioaerosols

Collecting and culturing viable airborne microorganisms is the most common technique used by industrial hygienists to assess bioaerosols [Macher 1999]. However, the appropriate sample preparation method is highly dependent upon the microorganism(s) of interest, sample source, and down-stream analysis. These sampling approaches are further confounded as viable bioaerosols have been estimated to account for approximately 1% of the total bioaerosol load, and non-viable bioaerosols are often overlooked [Hung et al. 2005]. In contrast, non-culturable bacteria and fungi cannot be grown in conventional lab-based conditions, but their presence is still important from a health perspective [Green et al. 2011; Mitakakis et al. 2003]. Non-viable bioaerosols can be determined through other detection methodologies such as microscopy, proteomic, immunological, and molecular analysis methods, and some of these approaches are discussed in section 9. A list of the common bioaerosols encountered in indoor and outdoor environments, as well as the fungi that are common contaminants of indoor building materials, can be found in Flannigan et al. [2011].

a. Sample preparation for bacteria and fungi

Viable bacterial and fungal bioaerosol identification is made through the collection, deposition, and growth of a viable propagule or intact cell on a selected nutrient agar medium contained in a sterile petri dish or liquid culture suspension [Macher 1999]. These methods are similar for both fungal and bacterial bioaerosols [Flannigan et al. 2011; Hung et al. 2005]. Selection of the nutrient media, incubation conditions (time and temperature), and potential damage to the culturable bioaerosol during sampling are among several critical variables to review before the collection, growth and proliferation of a viable propagule [Eduard et al. 2012; Hung et al. 2005; Macher 1999]. These parameters have been reviewed elsewhere, but should be taken into consideration when planning an environmental survey [Hung et al. 2005; Macher 1999].

Growth media can be defined as either broad or selective [Macher 1999]. As the term implies, broad nutrient media supports the growth of a diverse number of microorganisms. In contrast, a selective growth medium, with appropriate energy sources, nutrients, and pH, is used to enrich growth of the specific microorganism in question and inhibit the growth of competitive organisms [Macher 1999]. A variety of broad and selective nutrient media for bacteria and fungi are available to the industrial hygienist and can be found in Hung et al. [2005] and Macher [1999].

Following sample collection, liquid or agar cultures are incubated at a suitable temperature and atmosphere (facultative versus aerobic) for an appropriate time. Fast-growing bacteria may develop microcolonies in hours, while fungi may take days to develop into a visible colony and perhaps sporulate. Organisms such as *Mycobacterium tuberculosis* or the dimorphic fungal pathogens, *Histoplasma capsulatum* or *Blastomyces dermatitidis* may require weeks of incubation to produce visible colonies [ATS 1990; Babady et al. 2011]. For fungi, plates are typically incubated at room temperature (18°C-25°C) or, if it is a clinically relevant isolate, at 35°C [ACGIH 1989; Baron and Finegold 1990; Hung et al. 2005; Macher 1999]. In contrast, environmental bacteria are grown between 18°C and 28°C, while thermophilic bacteria are grown between 50°C and 58°C [Hung et al. 2005; Macher 1999].

After allowing for vegetative growth of all viable propagules on the selected nutrient medium, the number of colonies is identified, quantified and presented as colony forming units (CFUs) [Eduard et al. 2012]. Media blanks (laboratory and field) should be processed using the same methods as samples to control for environmental or laboratory contaminants. A collection of bioaerosol identification manuals is presented in both the AIHA and ACGIH manuals [Hung et al. 2005; Macher 1999]. Color micrographs of common fungal contaminants are also presented in Flannigan et al. [2011]. Along with the quantification of viable microorganisms, taxonomic data and an interpretation of the datasets are generally reported [Hung et al. 2005].

The interest in detecting and quantifying fungi has increased following consensus documents that reported associations between fungi in damp indoor environments and adverse respiratory health effects [IOM 2004; Mendell et al. 2011; WHO 2009]. Compared to bacteria, additional variables need to be taken into consideration by the industrial hygienist when evaluating viable fungal bioaerosols including water activity, colony competition, and carbohydrate nutrient sources [Hung et al. 2005; Macher 1999]. Broad viable culture approaches favor species belonging to the phylum Ascomycota, as well as species that outcompete slower-growing species. Several different types of media and physiological conditions (e.g. temperature) may also need to be employed to assess complete fungal diversity using this approach. For fungi, selection of the nutrient media may potentially bias the growth of specific viable fungal bioaerosols. Common nutrient media include malt extract agar (MEA) supplemented with chloramphenicol or rose bengal agar to suppress bacterial growth [Hung et al. 2005; Macher 1999]. Cellulose agar can also be used for the selection of indoor fungal contaminants such as *Stachybotrys chartarum* [Hung et al. 2005]. Dichloran glycerol (DG18) can be used to select for those fungi that are xerotolerant [Flannigan et al. 2011; Hocking and Pitt 1980; Macher 1999]. Temperature and incubation time can also be used to select for specific fungal bioaerosols such as *Aspergillus fumigatus* which are capable of growth within human hosts [Flannigan

et al. 2011; Hung et al. 2005]. A selection of nutrient media and growth conditions that can be used for viable fungal culture can be found in Hung et al. [2005], Flannigan et al. [2011] and Macher [1999].

b. Sample preparation for viruses

Because viruses are obligate intracellular parasites, special precautions must be taken in an effort to minimize damage to the collected virus-laden aerosol. Environmental factors such as humidity, temperature and gas composition of the air can significantly impact the infectiousness of a virion and should be monitored closely [Ijaz et al. 2016]. Several studies have shown that the inactivation of an airborne virus is directly related to the relative humidity and temperature [Weber and Stilianakis 2008]. In one study, high humidity levels caused a loss of infectious influenza virus from simulated coughs [Noti et al. 2013]. Similarly, using a ferret animal model, Lowen et al. [2007] demonstrated a correlation between airborne transmission of influenza and the relative humidity and temperature. Through the use of an ozone-oxygen delivery system, researchers were able to show that ozone-mediated reactive oxygen species (ROS) caused lipid peroxidation and subsequent damage to the lipid envelope and viral capsid [Murray et al. 2008]. Such studies highlight the importance of collecting viral aerosols under optimal environmental conditions and, when possible, minimizing the detrimental effects of environmental factors on collected samples.

Air sampling techniques also may cause damage to the virus and compromise analysis. Before collecting viral aerosols, the hardiness of the target virus must be taken into account. Currently, there are over 200 known respiratory viruses that fall under one family of DNA viruses (Adenoviridae) and four families of RNA viruses (Orthomyxoviridae, Paramyxoviridae, Picornaviridae and Coronaviridae) [Abed and Boivin 2006]. While all viruses package their genome in a protective protein coat known as the capsid, some viruses also possess a lipid bilayer envelope that, as the name implies, surrounds the viral capsid. Once outside the host, the viral envelope is highly sensitive to desiccation, temperature fluctuations and readily undergoes degradation. Variations in temperature can greatly affect viral enzymatic activity and nucleic acid stability [Tang 2009]. As noted earlier, viruses with lipid envelopes tend to survive better at low humidity, while non-enveloped viruses survive better at high humidity [Tang 2009]. Also, RNA viruses are inherently more unstable than DNA viruses due to the presence of the 2'-hydroxyl group on the ribose sugar molecule of RNA that is susceptible to base-catalyzed hydrolysis and degradation. Therefore it is critical that collection methods do not disrupt the lipid membrane and/or compromise the integrity of nucleic acids. Impairment of either the lipid envelope or nucleic acids can significantly impact detection of the viral aerosol and lead to false negatives.

To optimize collection efficiency while maintaining infectiousness of the viral aerosol, researchers must be discriminating when deciding on what type of aerosol sampler to use and how long the sampling collection period should be. As discussed in Section 3, some commercially available bioaerosol sampling devices exist, each of which possesses unique collection properties. In a review by Verreault et al. [2008], liquid impingers were found to be the most effective sampling devices for capturing small viral particles while maintaining virion integrity and infectiousness [Verreault et al. 2008].

While liquid impingement preserves and maintains viral integrity (in comparison to dry impaction), the type of collection medium must also be considered. With culture-based identification methods, it is of utmost importance to maintain the stability of the collected viruses while using cell-culture compatible media. The type of liquid medium, as well as the volume used, are important variables to consider. Virus collection and transport media are typically isotonic solutions with a buffer to control the pH, protein to protect the virus, and antibiotics to prevent microbial growth. If used in an impinger, the viral collection media may also include an antifoaming agent. Specimen handling, storage time and storage temperature can significantly impact the integrity of sample analysis. Collection and storage under suboptimal conditions can result in viral inactivation and degradation of nucleic acids. Bioaerosol samples containing viruses should be processed as soon as possible after collection. They should be refrigerated or frozen and transported as quickly as possible. The stability and retention of viability depend upon the virus [Johnson 1990].

7 Identification of culturable bioaerosols

Identification of the microbial taxa is a critical element in the determination of the viable bioaerosol load in an industrial or occupational environment. The science of classification, especially the classification of living forms, is called taxonomy. The objective of taxonomy is to classify living organisms to establish the relationship between one group of organisms and another, and to differentiate between them based on phenotypic and genotypic characteristics. The identification of viable fungal bioaerosols has been challenging due to the confusion of current nomenclature [Flannigan et al. 2011]. As a result, investigators often use synonymous names that over time have been placed in another group. Familiarity with taxonomy and nomenclature is critical when undertaking assessments of the viable microbial burden in an environment [Flannigan et al. 2011]. Several criteria and methods for the classification of culturable microorganisms are briefly discussed in the following subsections. Besides using these methods, the nonviable and non-culturable methods of identification discussed in Section 9 may also be used in combination with these viable methods.

Classical microbiology includes general methods for classifying or identifying microorganisms. The least specific of these is the observation of growth characteristics.

Growth characteristics include the appearance of the microorganisms in a liquid medium, colony morphology on solid medium, pigmentation, and arrangement of reproductive structures such as fungal sexual or asexual spores.

a. Bacteria

Bacteria are prokaryotes with distinguishing morphological characteristics that include the cell shape, cell size, arrangement of cells, and the presence or absence of flagella, capsules, or endospores. Simple and differential staining may be performed on bacteria to enhance visualization and to aid in grouping and identification [Tortora et al. 2013]. In simple staining, a single basic dye is used that highlights the cellular morphology. Stains such as methylene blue, carbolfuchsin, crystal violet, or safranin may be used for bacteria.

A differential stain distinguishes among structures or microorganisms based on varying reactions to the staining procedure. Two examples of differential stains are the Gram stain and the acid-fast stain. In Gram staining, bacteria are stained and then washed with alcohol. Gram-positive bacteria possess a cell wall composed of a relatively thick peptidoglycan layer and teichoic acids, which retains the dye complex. Gram-negative bacteria possess a cell wall composed of a thin peptidoglycan layer and an outer membrane which consists of lipoproteins, lipopolysaccharides, and phospholipids, and do not retain the dye complex when washed [Tortora et al. 2013]. A few of the commercially available identification kits require a Gram-stain prescreening to assure that the correct reagents are used. Acid-fast stains are used for some species of bacteria, particularly those of the genus Mycobacterium, which do not stain readily. In the acid-fast staining process, the application of heat facilitates the staining of the microorganism [Tortora et al. 2013].

b. Legionella

Bacteria that are placed in the genus *Legionella*, are the etiological agents of pulmonary infections called Legionellosis [Fields 2002]. *Legionella pneumophila* (Figure 1B) is the most widely known species that has been implicated in Legionnaires' disease, which can result in pneumonia. Milder illness with fever and body aches is referred to as Pontiac fever. Bacteria placed in this genus consist of Gram negative rods and are associated with freshwater in the environment [ASTM 2015; Macher 1999]. Exposure to warm temperatures (25-42°C) can result in the growth and proliferation of the bacteria, a problem that has emerged in water and air handling systems within the indoor built environment [Hung et al. 2005]. Growth and persistence within Protista have also been reported [Hung et al. 2005]. For health care facilities, ASHRAE Guideline 12–2000 recommends storing and distributing cold water at <20°C (68°F), whereas hot water should be stored at >60°C (140°F) and circulated with a minimum return temperature of 51°C (124°F). In other settings, hot water should be stored at ≥40°C (≥120°F) [ASHRAE

2000]. Building air conditioning cooling towers, humidifiers, or structures used for bathing such as hot tubs are particularly susceptible to Legionella amplification. Legionella can be aerosolized within water droplets via abiotic disturbance mechanisms and disseminated into the breathing zone of the subject. Airborne levels lower than 10 CFU/mL have been associated with Legionnaires' disease [ASTM 2015; Demirjian et al. 2015; Hung et al. 2005].

Since the identification of *L. pneumophila* and association with Legionnaires' disease in 1976, there have been many studies that have focused on a variety of approaches to detect and mitigate this bacterial species from the built environment. These approaches are broadly reviewed in Hung et al. [2005]. Along with industrial hygiene practices and building maintenance, environmental monitoring and surveillance programs are critical to ensure effectiveness of employed engineering controls and maintenance/disinfection programs [ASTM 2015]. ASTM International has published a standard for inspecting water systems and investigating outbreaks [ASTM 2015]. The CDC has also published a sampling procedure [CDC 2015].

In 2015, ASHRAE published a consensus standard for the primary prevention of Legionnaires' disease in building water systems [ASHRAE 2015]. Similar environmental assessment methods are utilized in maintenance programs and outbreak cases. In this approach, bulk water samples are typically collected (250 mL to 1 L for non-potable and 1000 mL for potable water), concentrated via filtration, resuspended, and then plated on a growth medium (such as buffered charcoal yeast extract media) to enable the propagation and identification of *Legionella spp*. [CDC 2015]. Samples may also be direct plated, acid treated, or heat treated to enhance the recovery of the bacterium. Colonies represent the viable fraction of *Legionella* in a water sample and these colonies are quantified and reported as CFU/mL

In addition to viable culture-based approaches, molecular-based methods such as PCR as well as antibody-based methods have been developed to enable the detection of *Legionella*. Air sampling is not considered a reliable method for *Legionella* surveillance in the built environment [Hung et al. 2005].

Using an environmental microbiology laboratory with expertise in propagating *Legionella* spp. is important when evaluating Legionella contamination of water systems within the built environment. A number of laboratories participate in the CDC's Environmental *Legionella* Isolation Techniques Evaluation (ELITE) Program. Participation in the program is voluntary and enables laboratories to test their proficiency in *Legionella* isolation and identification techniques against standardized samples. A list of ELITE member laboratories can be accessed at https://wwwn.cdc.gov/elite/Public/MemberList.aspx.

c. Fungi

In general, culturable fungi are classified by colony features including the septation of hyphae and colony morphological phenotypes, including pigmentation and the presentation of asexual and sexual spores on hyphae. Stains such as Calberla's solution, lactophenol cotton blue, periodic acid-Schiff stain, Grocott's methenamine silver stain, and calcofluor white may be used in combination with potassium hydroxide (10% KOH) to resolve these colony structures using microscopic-based approaches [Hung et al. 2005]. The identification and classification of fungal colonies should be performed by an examiner that is skilled in microbiology and mycology. A number of commercial labs that employ examiners skilled in the identification of microorganisms encountered in indoor and occupational environments are accredited by the AIHA Environmental Microbiology Laboratory Accreditation Program (EMLAP).

In addition to viable culture-based approaches, biochemical, physiological, and nutritional tests for bacteria and fungi can be used [Flannigan et al. 2011]. These testing strategies offer identification based on numerous variables including cell wall constituents, pigment biochemicals, storage inclusions, antigens, optimum temperature and temperature range, the effect of oxygen on growth, pH tolerance, osmotic tolerance, salt requirement and tolerance, antibiotic sensitivity, energy sources, carbon sources, nitrogen sources, fermentation products, and modes of metabolism (autotrophic, heterotrophic, fermentative, respiratory). As a rule, batteries of such tests, rather than any one individual test, are used to identify or classify microorganisms. A few commercially available test batteries are discussed briefly in the section on biochemical approaches.

8 Enumeration of culturable bioaerosols

a. Enumeration of bacteria and fungi

The total concentration of culturable airborne microorganisms in a sample is determined by collecting the bioaerosol sample on a culture plate or plates (or inoculating culture plates with a bioaerosol sample), incubating the plates, and dividing the number of colonies observed on the culture plates by the volume of air sampled. Note that, as discussed in section 2, the number of colonies counted on an agar plate from a bioaerosol impactor must be adjusted using a positive-hole correction factor to correct for multiple microorganisms depositing beneath an impactor hole [Andersen 1958; Leopold 1988; Macher 1989]. A colony is defined as a macroscopically visible growth of microorganisms on a solid nutrient medium. Concentrations of culturable bioaerosols collected during air sampling are normally reported as colony forming units (CFU) per unit volume of air [Eduard et al. 2012]. CFUs also can be determined from samples collected in a swab or dust sample collected from the floor or area of contamination [Hung et al. 2005].
Often, it is difficult to identify multiple colonies at one location on a plate because of the lack of differential colony morphology [Burge et al. 1977]. In addition, some organisms produce large, spreading colonies while others produce microcolonies. Analysis of plates containing multiple types of microorganisms can be difficult because the chemicals secreted by one microorganism might inhibit the growth of other microorganisms at that same location [Burge et al. 1977]. The morphology of the colony of one microorganism also may completely obscure that of another, and a fast-grower might obscure a slow-grower.

b. Enumeration of viruses

Before the advent and mainstreaming of molecular-based detection methodologies, cell culture-based methods and serological assays were considered the gold standard for the detection of viral pathogens. Typically, through the use of commercially available immortal cell lines, researchers can screen collected bioaerosols by inoculating cells and looking for common cytopathic effects (CPEs) such as rounding of infected cells, fusion with adjacent cells and lysis of cells. Examples of well-known cell lines that are routinely used in viral diagnostics include primary rhesus monkey kidney (RhMK) cells, primary rabbit kidney cells, human lung fibroblasts (MRC-5), human epidermoid carcinoma cells (HEp-2), human lung carcinoma cells (A549) and Madin Darby kidney cells (MDCK) [Leland and Ginocchio 2007]. Selection of the appropriate cell line is based on the specimen source and the suspected causal viral pathogen. Certain viral pathogens may require several cell passages before CPEs can be observed. More information on enumeration assays for viable viruses can be found in standard virology reference texts, such Principles of Virology [Flint et al. 2009b] and Fields Virology [Fields et al. 2007].

1.) **Viral plaque assay**

A widely used approach for detecting viral pathogens and quantifying viral titers is the viral plaque assay (VPA) [Condit 2007]. Under biological safety controls, the appropriate cell line is propagated and plated, usually in a 6-well format, at a concentration at which cells form a monolayer. The cell monolayer is next treated for a defined period (30-60 minutes) with a specific volume (0.1 mL to 1.5 mL) of the collected bioaerosol and incubated at the specified temperature and CO2 levels for 24 to 72 hours. Throughout the incubation period, cells are routinely inspected and CPEs are documented. Upon completion of the incubation period, cells are chemically fixed, stained and plaques (zones of cellular clearing) are enumerated. The final concentration of the collected viral aerosols is calculated based on the number of plaques, dilution of the inoculum and volume plated, and is expressed in plaque forming units per mL (PFU/mL]). While the VPA is a cost-effective method of assessing sample viral loads, results can take anywhere from 3-5 days. Other limitations such as the inability to detect low viral titers and inactivated (noninfectious) virus and the failure of some viruses to form plaques, may compromise detection and underestimate the viral loads in an aerosol sample. Likewise, common indoor and outdoor contaminants (such as fungi and bacteria) can impair the VPA by disrupting the cellular monolayer or outcompeting for nutrients in the cell culture medium.

2.) **Tissue culture infectious dose assay**

Another cell culture-based approach to identifying viral aerosols is the Tissue Culture Infectious Dose assay (TCID50), also known as an endpoint dilution assay [Condit 2007]. As with the VPA, select cells are plated at a desired concentration in a 96-well format and inoculated with serial dilutions of the collected sample. Following a specified incubation period, cells are examined for CPEs. The TCID50 is defined as the dilution of virus required to infect 50% of the cell culture wells [Reed and Muench 1938]. Based on the number of cells that are infected at the designated virus dilution, viral titers are mathematically calculated. Limitations of the TCID50 are similar to those observed with the VPA.

3.) **Immunofluorescence antibody (IFA) assays**

To enhance viral detection and quantify viral loads, immunofluorescence antibody (IFA) assays (direct or indirect) are frequently used in combination with cell culture-based methods [Flint et al. 2009a; Tortora et al. 2013]. By combining infected cells with a fluorescently-labeled, antigen-specific antibody, it is possible to increase detection levels without the lengthy incubation periods that are typically necessary with VPAs and TCID50 assays.

c. Interpretation of data

In industrial hygiene surveys that evaluate bioaerosols, indoor bioaerosol levels are usually compared to outdoor levels or to a control area. In general, indoor bioaerosol levels are lower than outdoor levels, and the taxa are similar [ACGIH 1989; Burge et al. 1977; Hung et al. 2005; Macher 1999; Solomon et al. 1980]. However, elevated indoor bioaerosol levels may be a sign of dampness, water infiltration, or microbial contamination [Hung et al. 2005]. In 2010, The ACGIH published a variety of occupational exposure limits for aerosols that are derived from biological material in specific industries and include subtilisins derived from *Bacillus subtilis*, as well as cotton, grain, flour, wood, and organic dusts [ACGIH 2015; Eduard et al. 2012]. To date, no occupational exposure limits for specific fungal bioaerosols exist and these typically fall under particulate matter not otherwise regulated (10 mg/m3 for inhalable dust; [ACGIH 2015; Eduard et al. 2012]. Proposed limits developed in other regions of the world are provided in Eduard et al. [2012].

Although the quantification of viable fungal propagules can provide helpful datasets to evaluate differences between indoor and outdoor fungal diversity, the interpretation of results should be evaluated closely. Total fungal exposure will be underestimated as non-viable fungal bioaerosols are not captured in the analysis [Eduard et al. 2012]. Fungal genera, including *Cladosporium*, *Alternaria*, and *Epicoccum*, and Basidiomycetes are predominantly localized in outdoor environments and the presence of elevated concentrations may be an indicator of indoor fungal contamination [Hung et al. 2005]. Similarly, the presence of certain hydrophilic species including *Stachybotrys chartarum* and *Aspergillus versicolor*, and *Chaetomium globosum* may be signs of indoor fungal contamination and may require immediate inspection [Flannigan et al. 2011; Hung et al. 2005].

Where local amplification and dissemination of bacteria have not occurred in an occupied, indoor environment, Gram-positive cocci (e.g., *Micrococcus* and *Staphylococcus*) are normally dominant [Morey et al. 1986]. Airborne human skin scales and respiratory secretions may contain Gram-positive cocci [ACGIH 1989; Hung et al. 2005]. Detection of high levels of these microorganisms may be an indication of over-crowding and inadequate ventilation. Indoor air that tests high for Gram-negative bacteria indicates a need to identify and eliminate the source of contamination. Concentrations ranging from 4,500-10,000 CFU/m^3 have been suggested as the upper limit for ubiquitous bacterial aerosols [ACGIH 1989; Nevalainen 1989]. These exposure limits, however, do not apply to pathogenic microorganisms. Actinomycetes (mesophilic and thermophilic) are commonly found in agricultural areas. Their presence in indoor environments is an indicator of contamination [ACGIH 1989; Banaszak et al. 1970; Lacey and Crook 1988]. Thermophilic

Actinomycetes at concentrations above 70 CFU/m^3 in an affected person's work area have been regarded as the threshold for triggering remedial action [Otten et al. 1986].

9 Sample analysis methods for non-viable and non-culturable bioaerosols

The collection and classification of nonviable and non-culturable microorganisms cannot be performed by using viable culture methods. A large proportion of fungal bioaerosols are non-viable and would not grow and proliferate on nutrient media [Eduard et al. 2012]. This fraction of the bioaerosol load is equally important to assess in industrial hygiene surveys that investigate the role of personal bioaerosol exposure on respiratory health [Brasel et al. 2005; Green et al. 2011; Mitakakis et al. 2003]. These bioaerosols can also contain antigens, allergens, microbial volatile organic compounds and even mycotoxins [Brasel et al. 2005; Eduard et al. 2012; Green et al. 2011; Green et al. 2006b]. Identification of nonviable or non-culturable microorganisms or components of microorganisms (such as cell wall fragments) can be performed using a variety of other available assessment strategies such as microscopy, immunoassays and, more recently, molecular biology techniques [Afanou et al. 2015; Brasel et al. 2005; Eduard et al. 2012; Flannigan et al. 2011; Green et al. 2011; Hung et al. 2005; Macher 1999; Rittenour et al. 2012].

Microscopy includes a variety of approaches that utilize bright-field, light, phase contrast, fluorescence or even electron-based approaches [Eduard et al. 2012; Macher 1999]. These methods enable the enumeration of both viable and nonviable microorganisms [Macher 1999] as well as other non-culturable bioaerosols including cell wall fragments, plant pollen and pteridophyte and bryophyte spores [Green et al. 2011; Rittenour et al. 2012]. These approaches provide a platform to visualize particle morphology and to identify reproductive fungal structures of individual genera that are based on a combination of propagule phenotypes [Flannigan et al. 2011; Macher 1999]. However, these approaches can be confounded by observer bias, especially when it comes to differentiating bioaerosols that contain similar morphological phenotypes such as amerospores (e.g. *Aspergillus conidia*) [Flannigan et al. 2011; Hung et al. 2005]. The ASTM has published a standard method for the use of optical microscopy to categorize and quantify fungal structures in samples collected by inertial impaction [ASTM 2009].

To overcome these methodological challenges, alternative methods based on the quantification of bioaerosol biomarkers (proteins or DNA) have been developed that enable the quantification of these bioaerosol sources [Eduard et al. 2012]. These include a variety of assessment methods that can be used to qualitatively and quantitatively assess bioaerosol exposure by detecting cell wall components, proteins, carbohydrates, or oligonucleotides (e.g.

endotoxin or β-glucan) [Eduard et al. 2012]. Other chemical and proteomic methods including HPLC, flow cytometry, and mass spectrometry-based approaches can also be used to detect and quantify cell wall components such as microbial volatile organic compounds and mycotoxins. These approaches have been reviewed more extensively by Flannigan et al. [2011].

Several modifications of classical biochemical procedures have been used in recent years to facilitate inoculation of media, decrease the incubation time, automate the procedure, and systematize the determination of species based on reaction patterns. Historically, clinical microbiological techniques have been used for analysis of environmental samples. However, clinical strains and environmental isolates may differ, requiring modification of clinically-based techniques.

a. Microscopy

1.) Bright-field or light

In bright-field or light microscopy, an ordinary microscope is used for the morphological observation and sizing of sampled bioaerosols. Visible light from an incandescent source is used for illumination and the specimen appears against a bright backfield. Objects smaller than 0.2 μm cannot be resolved. The image contrast (visibility) decreases as the refractive index of the substance/microorganism under observation and the mounting medium become similar. To maximize the contrast, the mounting medium should have the same refractive index as glass or the immersion oil. Membrane filters are often "cleared" by using the appropriate immersion oil or acetone vapor/triacetin combination. This method is commonly used to observe various stained specimens and to identify and count viable and non-viable bioaerosols. In addition, pollen grains are often identified and enumerated in this manner [Eduard et al. 1990].

Collection of fungal bioaerosols onto an adhesive surface followed by microscopic identification based on the morphological characteristics of the spores (size, shape, septation etc.) is another common method of assessment. This non-viable method overcomes limitations introduced in viable analyses and many genera can be differentiated based on differences in spore morphology [Eduard et al. 2012; Macher 1999]. Microscopic examination of fungi captured on filters or an adhesive tape are divided into seven spore morphological characteristics and include amero-, didymo-, helico-, stauro-, dictyo-, phragmo-, and scoleco- spores [Kendrick 2000]. Amerospores are the most common spore morphology encountered in air samples and are the most challenging to differentiate taxonomically [Kendrick 2000]. Amerospores are usually placed in a group represented as *Aspergillus/Penicillium* group [Hung et al. 2005].

Many other common environmental fungal bioaerosols share similar morphologies which can make taxonomic placement challenging for the untrained or inexperienced observer [Eduard et al. 2012]. Typical magnification used in the assessment of fungal propagules ranges from 400-1000X. A standard operating procedure for the assessment of microscopic non-viable samples is presented in Hung et al. [2005] and by ASTM [2009].

The confounding factors associated with traditional fungal exposure assessment methods have limited our understanding of the spectrum of fungal bioaerosols in industrial and occupational environments. Measures using these approaches also cannot be acquired in real time. Furthermore, identifying and quantifying the complete diversity of fungal bioaerosols using a standardized methodology is critical in the determination of fungal bioaerosols within occupational environments [ASTM 2009; Hung et al. 2005].

2.) **Phase contrast**

Phase-contrast microscopy is used when the microorganism under observation (e.g., *Escherichia coli*) is hyaline and an alternative mounting medium is not possible. As light passes through the specimen, variations in the index of refraction of the components cause phase shifts in the light. A phase-contrast microscope uses a special condenser and diffraction plate that cause these phase shifts to appear as differences in brightness and contrast. One cannot see an object exactly matching the refractive index of the mounting liquid; however, very slight differences produce visible images. This type of microscope is commonly used to provide detailed examination of the internal structures of living specimens; no staining is required.

3.) **Fluorescence**

Fluorescence microscopy uses an ultraviolet or near-ultraviolet source of illumination that causes fluorescent compounds in a specimen to emit light. Fluorescence microscopy for the direct count of microorganisms has been described in a number of studies [Eduard et al. 2012]. Direct-count methods to enumerate microorganisms (especially bacteria) have been developed using fluorescence microscopy and some stains such as acridine orange, fluorescein isothiocyanate (FITC) and 4',6-diamidino-2-phenyl-indole (DAPI) [Macher 1999; Thermo Fisher Scientific 2014]. Utilization of stains such as calcofluor white can resolve fungal spores and hyphal structures [Haghani et al. 2013]. This stain binds to chitin; however, other plant-derived and insect bioaerosol sources (e.g. dust mite, plant pollen) may also be resolved using this stain. Viability stains also have been developed and are available commercially for the detection of viable fungi and bacteria bioaerosols in collected air samples [Thermo Fisher Scientific 2014].

4.) **Electron**

Electron microscopy consists of a beam of electrons that enable structures smaller than 0.2 µm, such as viruses, to be resolved. Scanning electron microscopy (SEM) and, more recently, field emission scanning electron microscopy (FESEM), are approaches used to study the surface features of prokaryote and eukaryote cells as well as viruses (usually magnified 1,000-10,000X). These bioaerosols are immobilized onto a semi-solid filter submicron membrane or in the form of a liquid suspension and a three-dimensional image of the area is generated. Images from SEM can provide vital information about the size, morphology and concentration of the collected bioaerosol [Afanou et al. 2014; Eduard et al. 2012]. However, SEM does not provide information on viability of the collected bioaerosol. FESEM has been recently used to resolve fungal fragments that are produced from fungal colonies following abiotic disturbance [Afanou et al. 2015; Afanou et al. 2014]. Transmission electron microscopy can also be used to examine viruses or the internal ultrastructure in thin sections of cells (usually magnified 10,000-100,000X), although the image produced is not three- dimensional. Compared to other methods of assessment described in this chapter, SEM and FESEM-based approaches require a highly trained technician to obtain images from bioaerosols captured on filter membranes.

b. Endotoxin assays

The lipopolysaccharide endotoxin is a virulence factor possessed by all Enterobacteriaceae (as well as other Gram-negative bacteria) that is found in the outer membrane of the cell wall. Airborne endotoxin has been found in high concentrations in agricultural, industrial, and office environments [Eduard et al. 2012; Milton et al. 1990; Rylander and Vesterlund 1982; Singh et al. 2011b]. Individuals may experience disseminated intravascular coagulopathy, respiratory tract problems, cellular and tissue injury, fever, and other debilitating problems. Endotoxin can be detected in air samples collected on glass filters using the Limulus amebocyte lysate (LAL) assay [Eduard et al. 2012]. This assay uses amebocytes from the blood-like circulating fluid of the *Limulus polyphemus* (horseshoe crab). After exposure to the lysed amebocyte cells, the chromogenic version of the LAL enables endotoxins to be quantified [Eduard et al. 2012]. Laboratories use this assay to test for contamination by Gram-negative bacteria [Baron and Finegold 1990].

Although widely used, endotoxin aerosol measurement techniques lack comparability between results obtained in different laboratories because of differing sampling, extraction, and analytical methods [Jacobs 1989; Milton et al. 1990; Olenchock et al. 1983; Rylander and Vesterlund 1982] and water-insoluble endotoxins are not detected [Eduard et al. 2012]. A monoclonal antibody assay has also been developed but it is less sensitive

than the LAL method. Similarly, chemical-based approaches are available to detect endotoxins including gas chromatography-mass spectrometry [Eduard et al. 2012].

c. Biochemical analysis methods

Because of the high frequency of isolation of Gram-negative rods in clinical settings, several commercial multi-test systems have been developed for identification of members of the family Enterobacteriaceae and other pathogenic microorganisms. These microorganisms are indistinguishable except for characteristics determined by detailed biochemical testing. These systems require that a pure culture be examined and characterized. A list of some commercially available identification kits is provided in Table III. All of these multitest systems have documented accuracies greater than 90% in clinical settings [Baron and Finegold 1990; Koneman 1988]. For fungi, API (Analytab Products, Plainview, NY) and BIOLOG can also be used to differentiate yeasts based on the respective biochemical and physiological profiles [Flannigan et al. 2011].

d. Chemotaxonomic approaches

Cellular fatty acids (CFA) of bacteria are structural in nature, occurring in the cell membrane or cell wall of all bacteria. When the bacteria are grown under standardized growth conditions, the CFA profiles are reproducible within a genus, down to the subspecies or strain level in some microorganisms. The Sherlock Microbial Identification System (MIS), developed by MIDI (Newark, DE), provides a chromatographic technique and software libraries capable of identifying various microorganisms based on their CFA composition [Sasser 1990a; Sasser 1990b]. The chromatographic technique is also known as gas chromatography fatty acid methyl ester analysis (GC-FAME). MIS has a database containing the analysis libraries for culturable Gram-negative and Gram-positive bacteria, and yeasts. In a comparison study [Amy et al. 1992], only 8 of 18 isolates, identified by either API multitest or MIDI MIS, were identified accurately using BIOLOG multitest. A prototype method for extracting and analyzing fungi is currently being distributed by MIDI.

e. Chemical-based approaches

A variety of chemical-based approaches are available for the detection and quantification of bacteria and fungi in environmental samples [Flannigan et al. 2011; Hung et al. 2005]. Common approaches include thermal desorption - gas chromatography - mass spectrometry (TD-GC-MS), high performance liquid chromatography, gas chromatography-tandem mass spectrometry, and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry [Flannigan et al. 2011]. These methods require the formation of a library of markers or spectral signatures that are used to discriminate between various prokaryote and eukaryote species. Examples

of spectral signatures that have been used for the detection of fungi include microbial volatile organic compounds (mVOCs), mycotoxins, ergosterol, 3-hydroxy fatty acids, muramic acid as well as intracellular and extracellular proteins. NIOSH Method 2549 is a TD-GC-MS based approach that allows for the detection of mVOCs in environmental samples [NIOSH 2003d].

f. High performance liquid chromatography

High performance liquid chromatography (HPLC) is commonly used for bioaerosol "fingerprinting" and biomass determination. Techniques such as proteomics and identifying variations in chromatographic patterns can be used to determine the source of airborne material. For example, ergosterol has been used for decades to detect fungal contamination [Seitz et al. 1979] and even determine taxonomy [Axelsson et al. 1995; Pasanen et al. 1999; Schnurer 1993]. Keratin analysis can be used to identify bioaerosols derived from vertebrates and possibly the habitat within an environment [Staton et al. 2013]. Detection can be as straightforward as using UV absorbance or as complex and specific as employing an ion trap mass spectrometer.

HPLC can be adventitious compared to other methods of analysis; it is an established technology, fairly inexpensive after initial equipment costs, fast and accurate. Its disadvantages include a lack of specificity inherent with detectors using UV absorbance (especially at lower wavelengths), and that complex matrices associated with bioaerosols can prove to be troublesome and may require multi-step enhancement procedures such as solid-phase extraction. Buffered solvent systems are sometimes required, which can be technically difficult to use.

1.) Mycotoxins

Fungal contamination is a concern in food production because it can modify the nutritional content of feed and cereal grains and introduce potentially adverse mycotoxins. Before the use of HPLC, methods of identifying fungal contamination were time consuming or missed non-viable organisms which still contributed to the biomass [Seitz et al. 1979]. HPLC can be used to detect ergosterol, which is a structural sterol nearly universally present in fungi but not naturally present in grains [Pasanen et al. 1999]. HPLC has also been used to detect ergosterol, mannitol and arabitol in bioaerosols [Buiarelli et al. 2013].

Ergosterol is extracted using a liquid-liquid extraction and concentrated. The clean samples are then analyzed by HPLC with UV detection. The ergosterol UV spectrum varies significantly from the UV spectrum of higher plant sterols, making it specific to fungal contaminants. Though the method is still fairly time consuming, it eliminates

the need for fungal culturing and can detect the presence of non-viable fungi. When combined with other sterols this information can be used to help determine fungal species [Schnurer 1993].

In addition to using sterols to identify fungal contamination, mycotoxins can also be analyzed by liquid chromatography with mass spectrometry (LC-MS). Identification of specific mycotoxins can help identify the species of fungi present [Bennett and Klich 2003; Castillo et al. 2016].

Mycotoxins can play a role in indoor air quality (IAQ), food safety and possibly bioterrorism. The use of LC-MS as a screening tool to identify mycotoxins can reduce the use of more intensive molecular techniques for identification and quantitation. When using mass spectral analysis it is important to have a reliable and accurate database for identification and a qualified analyst to correctly interpret data.

2.) **Other biomolecules**

More recently, HPLC has been proposed as a forensic tool to identify and track vertebrate species using keratin profiles. Like ergosterol in fungi, keratin is found mainly in dander left by vertebrates [Plowman 2007]. If patterns can be established, it may be possible to identify what species had been present in a specific dwelling and possibly even track movements [Staton et al. 2013]. Bacterial contamination can also be tracked using endotoxin analysis and bacterial peptidoglycan fingerprinting [Staton et al. 2013].

3.) **Sample preparation and enhancement**

Bioaerosols can have complex matrices with many interfering constituents. Ultraviolet absorption detectors are fairly inexpensive and straightforward to use, but they can suffer from a lack of specificity and sensitivity. Although mass spectrometry detectors do not suffer from a lack of specificity or sensitivity, a dirty sample can still present challenges.

Another potential pitfall is the presence of large particles in bioaerosol samples. In general, analytical HPLC systems and detectors use small diameter tubing and small orifice injectors that are easily clogged or contaminated by particles. Most analytical systems have some sort of less-expensive trapping or pre-column that can be sacrificed in order to spare the more expensive analytical columns. However, trap columns are still very much an expense and should only be considered if other options are not available. A multitude of cleanup procedures are available to lessen or eliminate problems due to particles, the most common of which are simple centrifugation and

filtration. Both methods will lessen the likelihood of clogging or damaging the system, but neither offers target analyte enrichment.

4.) **Liquid-liquid extraction**

In order to enrich analytes, samples can be concentrated, chemical interferences can be removed, or a combination of both methods can be used. One of the oldest methods of enrichment is a liquid-liquid extraction, which separates analytes based on relative solubility in a given solvent [Koncsag and Barbulescu 2011]. In general, two immiscible solvents are mixed, one solvent containing the whole extract (called the "feed") and one that ideally solubilizes the analyte of interest preferentially. Based on solubility, the chemical constituents will either stay in the feed solvent or partition into the other solvent. Once the solvents are allowed to phase-separate, the solvent containing the enriched analyte (called the raffinate) is removed.

Liquid-liquid extraction is usually done using an aqueous solvent and an organic solvent. Solvent selection is critical and can be difficult. The goal of the extraction is to choose a solvent that leaves behind as much of the interfering matrix as possible in the feed solvent while also being able to preferentially solvate the analyte of interest. The solvent also must be compatible with the analytical instrumentation. Liquid-liquid extractions tend to use large amounts of solvent, which can result in the analyte of interest being below analytical levels of detection and/or levels of quantitation. Many organic solvents can be easily concentrated by evaporation, but this can pose problems with labile or volatile analytes.

5.) **Solid-phase extraction**

Another option that can help avoid these pitfalls is the use of solid-phase extraction (SPE) [Sigma-Aldrich 1998]. SPE exploits the analyte affinity (or lack thereof) for a solid material packed inside a column. Sample enrichment can occur by the column retaining interfering compounds and the analyte of interest passing through, or by the column retaining the analyte and the interfering chemicals passing through. Concentration of the analyte of interest can be achieved by eluting the analyte in a smaller volume of solvent, and filtration can be achieved simultaneously. SPE may make it possible to achieve analyte concentration and avoid potential losses that could arise from concentrating a large volume of solvent. Appropriate solvent selection is also critical to successful SPE.

g. Immunoassays

The immunoassay is an analytical technique for measuring a targeted antigen, which is also referred to as an analyte. A critical component of the immunoassay is the antibody or ligand, which binds a specific antigen or binding site. The binding of the antibody or ligand forms the basis for the immunoassay, and numerous formats have been devised which permit visual or instrumental measurements of this reaction. Antibodies include either monoclonal or polyclonal antibodies and these are commonly employed to detect organisms by binding to antigens, usually proteins, polysaccharides or other cell wall components [Hung et al. 2005; Macher 1999]. The analysis is usually performed following extraction of the analytes to form a heterogeneous matrix. In most immunoassays, there is little need for extensive sample cleanup. Following the development of radioimmunoassays, many immunoassays that use monoclonal antibodies are now readily available from commercial sources, permitting laboratories to use standardized immunoassays or rapidly develop in-house immunochemical assays. In addition, commercially available immunoassays or multiplex platforms are available to quantify a variety of indoor or occupational bioaerosol sources [King et al. 2013]. Some of the more widely used immunoassay formats are as follows:

1.) **Enzyme immunoassays (EIA)**

Enzyme Immunoassays (EIA) are composed of a variety of assay formats that can be used to quantify bioaerosols in an air or dust sample. The binding of an antibody or antigen to an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), is the basis of EIA techniques. Enzymatic activity, in the presence of a chromogen, results in a colored end-product that is quantified using a spectrophotometer. Many, if not most, commercially available EIAs are enzyme-linked immunosorbent assays (ELISAs). There are four types of ELISAs which include direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA.

The sandwich ELISA method is typically used for the detection for airborne viruses and aeroallergens [Hung et al. 2005]. In this assay, a capture antibody is bound to a solid surface, usually a 96-well plate. The bioaerosol extract is added to the plate containing the capture antibody and incubated for a specified length of time, washed with a phosphate buffer solution and probed with an enzyme-labeled antibody that enables detection and quantification, either through colorimetric changes or fluorescence emissions. The advantages to using a sandwich-based ELISA method are that it is highly specific and can be used on complex samples such as aerosols. Some disadvantages to using a sandwich-based ELISA are poor antibody recognition and/or minimal detection due to low sample concentration. Lastly, it should be noted that ELISA, like any protein-based assay, does not distinguish between viable and non-

viable viral aerosols. Multiplexed technologies that enable the detection and quantification of multiple allergen sources in one sample are also available from a variety of commercial sources [King et al. 2013].

EIA methods to assess fungal bioaerosols based on the detection of fungal cell wall components, enzymes, antigens and allergens have become commercially available. β-1,3-D glucan, extracellular polysaccharides, mycotoxins and a variety of fungal antigens can now be quantified in air and dust samples using immunochemical, enzymatic and chemical detection platforms [Chew et al. 2001; Douwes et al. 1997; Eduard et al. 2012]. Although many of these biomarkers and methods serve as a proxy measure for total fungal biomass, these approaches can be confounded by limitations associated with component extraction biases. In addition, complex extraction, washing, amplification or immunochemistry steps are required that may add hours or even days before a dataset is finalized for analysis and interpretation.

To date, there are a number of commercial companies that have developed ready to use EIA kits for the detection and quantification of a variety of indoor and occupational biomarkers including dog, cat, dust mite, fungal and rodent allergens [Filep et al. 2012]. These EIA approaches enable the collection and quantification of these biomarkers in the work environment and provide a ready to use platform for the industrial hygienist.

2.) **Fluorescent immunoassays (FIA)**

Utilization of fluorescent-labeled antibodies to detect bacterial antigens was introduced by Coons et al. [1941; 1942]. Various FIA techniques have now evolved and are commonly utilized in laboratories. These include: (1) direct FIA, to detect cell-bound antigens using a fluorescent antibody; (2) indirect FIA to detect cell-bound antigens using a primary antibody and a fluorescent secondary antibody; and (3) indirect FIA to detect serum antibodies using an antigen, serum, and a fluorescent antibody. Various fluorescent dyes, such as fluorescein, fluorescein isothiocyanate, and rhodamine isothiocyanate, may be employed [Thermo Fisher Scientific 2014]. A fluorescent or confocal microscope is used to evaluate the samples and to count the number of fluorescently stained organisms [Garvey et al. 1977; Popp et al. 1988]. Similarly, flow cytometry-based approaches using fluorescent-labelled antibodies have also been employed to evaluate a variety of bioaerosol sources including bacteria, pollen and fungi [Rittenour et al. 2012; Rule et al. 2007; Rydjord et al. 2007]. Multiplexed approaches have been developed for the detection of multiple allergens in the same extracted sample [King et al. 2013].

FIA can be used to detect viruses. An example of a direct FIA assay for the detection of virus-laden aerosols is the Focus Forming Assay (FFA) [Flint et al. 2009a]. With the

FFA, fluorescent microscopy is used to visualize immunostained cells and viral titers are quantified as focus forming units per milliliter, or FFU/mL. While the FFA is more sensitive than culture-based methods alone, samples with low viral titers may weakly fluoresce and possibly be considered undetectable. To overcome weak detection levels, an indirect IFA assay may be more appropriate [Leland and Emanuel 1995; Madeley and Peiris 2002]. With indirect IFA assays, a primary, unconjugated antibody is used in combination with a fluorophore-conjugated, secondary antibody directed against the primary antibody. Because the secondary antibody is able to bind to multiple epitopes on the primary antibody, it increases fluorescence and enhances overall detection. While IFA is a trusted method of viral detection and quantification, it is fraught with limitations including excessive cost and the necessity of a skilled technician experienced in the reading of immunofluorescence. Likewise, because viruses are constantly undergoing antigenic drift and occasionally antigenic shift, changes in viral antigens can affect the binding affinity of the primary antibody and may result in false negatives.

3.) **Ligand-based assays**

As an alternative to cell culture and immunofluorescent-based assays, there are several protein-based methods of detection that can be used to quantify viral loads in an aerosol sample. The hemagglutination (HA) assay is a non-fluorescence quantitative assay that is based upon the ability of certain viral pathogens to agglutinate species-specific erythrocytes [Condit 2007]. In a serial twofold dilution, viral samples are mixed with a 1% solution of erythrocytes and incubated at room temperature for 30-60 minutes. Viral samples which form an agglutinated lattice are able to prevent red blood cells from precipitating out of solution by the binding of the hemagglutinin protein (present on the surface of the viral pathogen) to the sialic acid receptors (present on the surface of red blood cells). The titer of the sample is based on the well with the last agglutinated appearance, immediately before the well in which the red blood cells have settled out of solution. Hemagglutination units (HAUs) are typically used to quantify the viral concentration.

One of the major limitations of the HA assay is that it does not distinguish between infectious and non-infectious viral particles. Likewise, certain bacteria and fungi possess hemolytic activity and when present in a collected bioaerosol, can alter the HA assay and result in false positive readings. To circumvent this issue, a variation of the HA assay, known as the Hemagglutination-Inhibition test, can be performed [Stewart et al. 1967]. The HA inhibition test measures serum antibodies that are directed against the viral pathogen. When present in sufficient concentration, the serum antibodies are able to prevent agglutination of red blood cells thereby providing an alternative means of quantifying viral loads.

4.) **Direct and indirect immunostaining**

Direct and indirect immunostaining methods have been previously described for the detection of bioaerosol sources. Popp et al. [1988] developed a staining technique to enumerate bioaerosol samples directly captured on microscope slides. These approaches have enabled the identification of specific bioaerosol sources, especially those that do not contain morphological phenotypes that would be used by a trained microbiologist to resolve and identify a specific microorganism [Popp et al. 1988]. Alternatives to this approach have been developed and utilized in a variety of indoor and occupational environments. A press blotting approach that included immobilizing proteins from collected bioaerosols captured on an adhesive tape provided insight into the bioaerosols that contain allergen in the outdoor environment [Takahashi et al. 1993; Takahashi and Nilsson 1995]. An alternative method, called the Halogen Immunoassay, enables the immunostaining of allergen and antigen around bioaerosols captured on a protein binding membrane such as PVDF or mixed cellulose ester [Green et al. 2006c; Tovey et al. 2000]. This immunoassay approach has been used in a variety of indoor and occupational settings to evaluate allergen sources including, cat, dog, latex, rodent, plant, and fungi [Green et al. 2006a; Green et al. 2003; Green et al. 2005a; Green et al. 2011; Green et al. 2005b; Green et al. 2005c; Green et al. 2006b; Green et al. 2006c; Mitakakis et al. 2001; Poulos et al. 2002; Poulos et al. 1999; Razmovski et al. 2000; Renstrom 2002; Tovey and Green 2004]. Recently these approaches have been adapted to FESEM applications and have been used to detect morphologically indiscernible fungal fragments [Afanou et al. 2015].

5.) **Biosensors**

To overcome some of the technical challenges associated with traditional methods to assess bioaerosol exposure, real-time sensor technologies are being developed for the detection of bioaerosols [Fronczek and Yoon 2015; Hook-Barnard et al. 2014]. The sensor technologies are based on a variety of signal detection strategies that include optical, mechanical, electrical, or magnetic sensing approaches. These methods have resulted in platforms that have enabled the rapid detection of microbial pathogens and in an automated format. Using this technology requires little technological skill and can be developed into an automated handheld device. These developments have resulted in the fabrication of remote monitoring units in the agricultural sector that can process data remotely and apply it to geographical information systems or forecasting models.

Biosensors have been developed for a variety of applications including the detection of microbial pathogens [Fronczek and Yoon 2015; Hook-Barnard et al. 2014]. Typically, a biologically derived analyte (such as fungal spores, hyphae, antigens, peptides or nucleotides) is collected and interacts with a selected bioreceptor immobilized on a

sensor surface. Examples of bioreceptors include oligonucleotides, monoclonal/polyclonal antibodies, enzymes, cells, and even phages. The bioreceptor system consists of a physiochemical transducer that produces a measurable signal. Transducers can be optical and include surface plasmon resonance (SPR), which is a method that measures changes in the refractive index during molecular binding events [Unser et al. 2015; Usachev et al. 2014]. In contrast, electrochemical transducers measure changes in current, potential, impedance, and conductance across an electrode surface for detection events [Patolsky et al. 2006]. Examples of electrochemical detection include the measurement of electrical conductance produced by antibody-antigen binding events [Patolsky et al. 2004]. Both optical and electrochemical transducers have been developed for the detection of a variety of pathogens in the biosecurity, medical and agricultural sectors.

h. Gene-based assays

Cell culture, protein-based, and immunological-based assays are invaluable diagnostic tools. However, the evolution of nucleic acid-based molecular diagnostics are rapidly becoming the preferred method for detecting and quantifying bioaerosols [West et al. 2008]. Nucleic acid-based molecular diagnostics can be divided into two categories, (1) Direct sample analysis and (2) Indirect sample analysis, such as the Viral Replication Assay (VRA), which requires cultivation of the target microorganism prior to molecular analysis [Blachere et al. 2011]. Regardless of which approach is taken, there are three steps involved: extraction and purification of nucleic acids; amplification of the gene target; and detection of the amplicon. For viral, bacterial, plant and fungal nucleic acid extraction and purification, a number of kits are commercially available. Such kits generally rely upon either silica adsorption (spin-column) or affinity purification (magnetic separation) methodologies. Because bioaerosols are typically dilute in nature, investigators should determine which method yields the greatest amount of nucleic acids while minimizing sample handling, contamination and degradation. These are important variables to consider and can vary depending on the selected approach.

With the advent of molecular assays such as the Polymerase Chain Reaction (PCR) and Real-Time Quantitative PCR (RT-qPCR), which detect specific genetic sequences in the sample DNA or RNA, it has been possible to provide standardized assays, reduce turnaround time, and enhance assay sensitivity and detection specificity [Cella et al. 2013; Life Technologies 2014; Mahony 2008]. Using gene-specific oligonucleotides coupled with either an intercalating fluorescent dye (e.g. SYBR green) or a fluorogenically labeled gene probe (e.g. VIC, 6FAM), industrial hygienists are able to monitor indoor and outdoor bioaerosols for the presence of microorganisms. Likewise, multiplexing PCR and bead-based multiplexing PCR, which couples PCR and flow-cytometry, can be used for high-

throughput screening of multiple respiratory viruses in a single reaction mixture. It should be noted that poor assay design, primer and probe base-pair mismatches and degraded template nucleic acids can lead to false negatives or reduced detection sensitivity. Investigators should ensure optimal assay design, validate limits of detection, and run valid PCR controls in parallel.

Within the last two decades, a variety of molecular technologies have been used to quantify eukaryotic biomass including fungi and plant pollen in occupational, health, residential, and industrial samples [Rittenour et al. 2012; Scott et al. 2011; Summerbell et al. 2011]. Examples of these technologies include molecular based methods to evaluate specific or conserved gene loci (internal transcribed spacer region of ribosomal RNA) such as Sanger [Rittenour et al. 2014] or next generation sequencing [Kettleson et al. 2015], denaturing gradient gel electrophoresis [Johansson et al. 2014] and quantitative PCR [Eduard et al. 2012; Vesper et al. 2007]. The latter approach includes examples such as a DNA-based mold specific quantitative PCR (msQPCR) method that enables the detection and quantification of 36 indicator fungal species [Vesper et al. 2007]. This msQPCR method has been used to develop an Environmental Relative Moldiness Index (ERMI) to quantify the mold burden in homes. Originally developed by the U.S. Environmental Protection Agency (EPA) [Vesper et al. 2007], this approach has been licensed to a variety of companies in the commercial sector and is widely used to evaluate indoor fungal bioaerosol particles in settled dust during investigations of indoor air quality [Bolaños-Rosero et al. 2013; Kettleson et al. 2015; Reponen et al. 2012; Reponen et al. 2011a; Taubel et al. 2016; Vesper et al. 2013; Vesper et al. 2007]. The development of this methodology has provided the first step towards a standardized approach to quantify fungal bioaerosol sources within the indoor environment. Other metagenomic molecular methods including Sanger, 454, and Illumina miSeq sequencing platforms have also provided new insights into the complete diversity of bacterial and fungal bioaerosols in indoor, outdoor and occupational environments.

10 Limitations of bioaerosol sampling and characterization

Bioaerosol sampling can be a useful tool to study occupational exposures, potential health hazards, and the transmission of infectious diseases. However, bioaerosol sampling has significant limitations, and these need to be kept in mind when deciding whether or not to collect bioaerosol samples, preparing a sampling plan, and interpreting the results.

The first and most important limitation is the lack of standards and guidelines for acceptable bioaerosol exposure limits. NIOSH and other organizations have set recommended exposure

limits for several organic materials which may contain microorganisms and their fragments, such as cotton dust, grain dust, starch and wood dust [NIOSH 2010]. Although numerous studies have suggested a connection between exposure to various bioaerosols and respiratory illnesses, exposure limits do not currently exist in the US for airborne pollen, fungi, protozoa, bacteria, viruses, or their fragments. These limits have not been established largely because it is not possible to definitively state that a particular bioaerosol concentration will or will not lead to adverse health outcomes [Eduard et al. 2012; Heederik 2013; Morey 2007; Nevalainen et al. 2015; NIOSH 2012a]. This is true for several reasons: bioaerosols are often a complex mixture of microorganisms and organic materials; thousands of species of microorganisms exist, and most have not been studied; microorganisms and their fragments can cause illnesses in a variety of ways, including allergic reactions, infections and toxicity; the health effects of biological materials can vary substantially from person to person; and sampling and analytical procedures are not standardized, which makes it difficult to compare results [Eduard et al. 2012; Heederik 2013; Morey 2007; Nevalainen et al. 2015; Taubel et al. 2016]. Although research is ongoing, no standards for acceptable levels of bioaerosols in the environment have been established by the US government or organizations such as the ACGIH or the AIHA.

In addition to the lack of occupational exposure limits for bioaerosols, measuring and interpreting bioaerosol concentrations are more complex than is often appreciated. Bioaerosol concentrations can vary significantly from location to location within a building, especially if the bioaerosol has one or a few localized sources. A study of bioaerosol exposure in a large engine plant found that levels of airborne fungi, bacteria and endotoxin varied from location to location within the plant [Thorne et al. 1996]. A study of airborne fungi in two residences found significant differences between two rooms sampled at the same time [Hyvarinen et al. 2001]. In healthcare settings, patients with certain respiratory infections expel bioaerosol particles containing infectious pathogens. Because of the dispersion of the aerosol and the settling of larger droplets, the bioaerosol concentration decreases rapidly as the distance from the patient increases [Jones and Brosseau 2015]. A study of airborne influenza in a healthcare clinic found that the concentrations were much higher in examination rooms containing patients with influenza than other locations, and that the airborne influenza concentration also varied from location to location within the waiting room [Lindsley et al. 2010a].

Most bioaerosol collection methods provide a snapshot of the environmental bioaerosols at a specific time. Temporal variations in bioaerosol concentrations are commonly observed, especially if the bioaerosol generation occurs during episodic events rather than continuously. One study of indoor airborne mold in a residence found that day-to-day concentrations of airborne fungi varied considerably, and that levels were 26 times higher in the summer than in the winter [LeBouf et al. 2008]. Another residential study found that more airborne fungi were present in the morning than the afternoon and earlier in the winter compared to later [Hyvarinen et al. 2001]. In the influenza study mentioned above, day-to-day levels of airborne

influenza virus varied considerably depending upon the number of influenza patients present [Lindsley et al. 2010a].

Other factors also influence bioaerosol concentrations. Building airflow patterns and the operation of the HVAC system can affect bioaerosol levels, particularly if the HVAC system is a source of bioaerosol particles [Macher 1999]. Areas occupied by people show increased levels of bioaerosols compared to empty spaces, both because people themselves shed bioaerosol particles and because human activities such as walking and sitting can re-suspend dust from floors and furniture [Buttner and Stetzenbach 1993; Ferro et al. 2004; Hung et al. 2005; Qian et al. 2012]. Outdoor air is an important source of airborne fungi in many indoor environments due to fresh air being drawn in by HVAC systems and infiltration through cracks and openings [Eduard 2009].

Because of these issues, if bioaerosol sampling is to be conducted, it needs to be a part of a well-planned and comprehensive sampling strategy. The development of a sampling plan should begin with a thorough inspection and understanding of the workplace, including the building, HVAC system, and possible sources of bioaerosols. The sampling plan should integrate other types of data collection with the bioaerosol sampling, such as bulk sampling of possible source materials, surface sampling of settled dust, and health surveys of workers. Collections will need to be carried out at multiple locations and multiple time points, and even then it must be kept in mind that such samples may not fully characterize the exposure and that false negative results are quite possible [ASTM 2014a; Hung et al. 2005; Macher 1999; Morey 2007]. The verification code for this document is 277454

Bioaerosol sampling can be beneficial when done in the appropriate context [Hung et al. 2005; Macher 1999; Morey 2007]. It can be helpful to compare indoor and outdoor levels of bioaerosols to identify possible indoor problem microorganisms. Sampling for specific microorganisms of concern can be useful, especially if there is a known source, such as a composting operation or an aeration tank in a sewage treatment facility [Environment Agency 2009; Masclaux et al. 2014]. Bioaerosol sampling is also a valuable research tool for better understanding sources and exposures. On the other hand, sampling for bioaerosols will not be helpful if there is not some basis for interpreting the resulting data. For example, because of the lack of dose-response information and the variability associated with bioaerosol sampling, NIOSH does not recommend routine air sampling when investigating possible respiratory illness due to exposures in damp buildings. Instead, NIOSH recommends inspections of the building and its HVAC systems to locate moisture and microbial growth problems, followed by remediation [NIOSH 2012a].

11 Safety considerations

Investigators should use appropriate personal protective equipment (PPE) and practice good personal hygiene when conducting indoor environmental quality, disease outbreaks, and agricultural health investigations that have resulted in medically diagnosed symptoms. PPE may include respiratory protection to prevent inhalation of microbes and microorganism-resistant clothing to prevent transmission to investigators by bodily contact with microorganisms. Good personal hygiene practices include washing exposed skin and clothing thoroughly and refraining from eating, drinking, or smoking in a contaminated area. These simple steps will help minimize the ingestion, inhalation, or uptake of microorganisms.

All samplers, culture plates, and other equipment should be handled aseptically to prevent contamination of the samples and, more importantly, to prevent the spread of potential human pathogens to the worker or the work environment. All surfaces, including washed hands, may harbor microorganisms or spores unless they are specifically sterilized. Practically speaking, however, not all objects can be sterilized. While disinfection with an oxidizing chemical or alcohol destroys most vegetative cells, these agents do not destroy all spores. Samplers should be disinfected or, if possible, sterilized after each sample collection. Special care should be given to samplers with convoluted inlets or air pathways where microorganisms may accumulate.

Information on the safe handling of biological specimens can be found in in the free online manual "Biosafety in Microbiological and Biomedical Laboratories" from the Centers for Disease Control and Prevention at (http://www.cdc.gov/biosafety/publications/bmbl5/index.htm) [CDC 2009]. Information on the handling of some specific pathogens can also be found at the CDC website (www.cdc.gov).

12 Resources

The NIOSH Manual of Analytical Methods has several chapters discussing other aspects of aerosol sampling, including general considerations and factors affecting aerosol sampling, an explanation of filter pore size, sampling airborne fibers, sampler wall losses, and avoiding bypass leakage in filter cassettes [NIOSH 2003e]. NIOSH also maintains a web page on indoor environmental quality with more information and links to additional resources at http://www.cdc.gov/niosh/topics/indoorenv/. The American Industrial Hygiene Association (AIHA; https://www.aiha.org) has reference materials and resources on indoor air quality and bioaerosols, as does the American Conference of Governmental Industrial Hygienists (ACGIH; http://www.acgih.org). ASTM International (http://www.astm.org) publishes numerous standards and guides on the evaluation of indoor air quality, including developing

an air sampling strategy and the collection and evaluation of bioaerosols [ASTM 2009; ASTM 2014a; ASTM 2014b; ASTM 2014d; ASTM 2014e]. The Centers for Disease Control and Prevention has guidelines on environmental infection control in healthcare facilities that include recommendations on environmental sampling [CDC 2003].

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