Improved Methods for Generation, Sampling, and Recovery of Biological Aerosols in Filter Challenge Tests

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In preparation for filter efficiency tests and sampler comparison studies, methods of biological aerosol generation, sampling, and filter recovery were modified from previous studies. Methods described include (1) techniques for generating aerosols that reduced nuisance particles to negligible levels and increased the cell culturability of *Mycobacterium abscessus* by 30%, (2) sampling techniques that lowered the detectable range of biological particle size from 0.65 to 0.45 µm and reduced the sampling flow from the chamber from 28.3 to 1.5 L/min, and (3) development of methods to remove culturable organisms from respirator filter media. These methods were developed for filter challenge tests with *M. abscessus* and were applied to two other bacteria. They may also have application to a wider variety of organisms and bioaerosol assessments.

Keywords: aerosols, filter efficiency, *Mycobacterium abscessus*, particle size, samplers

There has been increasing interest in the use of respirators for control of exposure to infectious biological aerosols. Of particular importance are the issues of proper selection, reuse, and disposal. Selection of respirators for nonbiological particulate hazards requires knowledge of particle size and filter efficiency, as well as airborne concentrations and facial fit. These issues have been well-studied for nonbiological aerosol exposures,[1–7] but are less understood for biological ones. It is not clear, therefore, that respiratory protection selection criteria developed for nonbiological aerosols can be directly applied to bioaerosol exposures. In addition, reuse and disposal are problems unique to such exposures and have only been briefly studied for respirators or surgical masks.[8]

Several investigators have evaluated the efficiency of small numbers of respirator filters challenged with biological aerosols.[9–10] Research by Chen et al. challenged one manufacturer's disposable respirators and surgical masks with aerosolized *Mycobacterium abscessus* at 43 L/min.[11] Filter efficiency was evaluated by measuring concentrations upstream and downstream of the filter using a direct-reading particle counter and

sizer and a viable particle sampler. In general, these respirators were found to collect the aerosol efficiently. However, these results are not necessarily representative of all respirator and surgical mask filter media or for all types of biological aerosols. Therefore, this protocol was adapted for studies of a much wider range of respirator and surgical mask filters using three biological aerosols at several conditions of flow and relative humidity. The expanded experimental goals necessitated changes in the experimental apparatus and protocol, which are described in this article.

Bioaerosol viability, which can be compromised by stressors encountered during generation, sampling, and enumeration, was a concern throughout this research. Unexpected loss of culturability can lead to unknown bias in the final estimation of collection efficiency. Therefore, particular attention was given to preservation of culturability to minimize such effects.

BACKGROUND

A large set of experiments was designed to evaluate the capture efficiency of a wide range of respirator and surgical mask filters when chal-

lenged with three airborne bacteria (*M. abscessus, Staphylococcus epidermidis,* and *Bacillus subtilis* subsp. *niger)* at two flow and two relative humidity conditions. Survival of organisms following testing was then determined. Within this framework previously developed methods were modified to fit within the research parameters. Modifications included developments to allow comparison of a total and a viable particle sampler, improvements to the viable sampler, and development of a method to evaluate organism survival on filter media. This article focuses on experimental methods; results of the experiments are described elsewhere.[12–14]

Total Versus Culturable Particle Measurements

Airborne particles can be detected, counted, and sized using a variety of sampling techniques. Instantaneous sampling methods are available that utilize direct reading particle detection and have been used to measure aerosols with great success.[15] Such methods can measure total (culturable and nonculturable) particle concentration and size, but cannot distinguish culturable organisms from other particles.

At present, there are no instantaneous methods for sampling viable particle concentrations. Detection and analysis of such particles involve collection on nutrient media and enumeration of the resulting colonies.[16] These methods may yield information on both culturable particle concentration and size distribution, but are more time-consuming and give more variable results than total particle methods.

Given these difficulties, the authors hoped to demonstrate that filter collection efficiency measured with a total particle sampler would be comparable to efficiency measured with a viable sampler. Future evaluations of filter efficiency using a biological aerosol challenge would be considerably simplified and accelerated if this goal could be met. The Aerodynamic Particle Sizer (APS) (TSI, Inc., Shoreview, Minn.) was selected as the total particle sampler for comparison with the Andersen Six-Stage Viable Particle Sampler (Graseby-Andersen, Inc., Atlanta, Ga.). Both of these instruments have been described in detail elsewhere.[15,17,18]

To compare total and viable particle measurements of bioaerosols it is of particular importance that the aerosol consist primarily of culturable particles. The presence of extraneous particles or nonculturable organisms, which may be detected by one method but not the other, will reduce the accuracy of bioaerosol measurements, particularly if they are in the size range of the culturable organisms or the size of the organisms is unknown. Organism culturability may be reduced during aerosolization due to desiccation, physical force, and osmotic shock.[19] This study focused on minimizing the effects of physical forces exerted on organisms during aerosolization. Additionally, an aerosol of single cells was desired; therefore, methods of dissociating cell clumps were evaluated. Methods used to minimize osmotic shock and to break up clumps, such as adding buffers and surfactants, respectively, were explored with *M. abscessus.*

In addition to preserving culturability, it is important that, to the greatest extent possible, a sampler collect particles within the entire range of particle size and that the two sampling methods (total and viable) measure particles in similar size ranges. For example, the particle aerodynamic diameter of *M. abscessus* may range from 0.3 to 1.1 μm.[20] The total particle sampler used in this study (the APS) measures particles with an aerodynamic diameter of 0.5–30 μm, while the viable particle sampler (the Andersen) collects particles with aerodynamic diameters ranging from 0.65 to greater than 10 μm.[17,18] Modifications to the viable sampler allowed a greater portion of the culturable particles to be

sampled by the Andersen and created greater equivalency in the lower range of particle sizes measured by the two samplers.

Measuring a Wide Range of Filter Collection Efficiency

When testing filters that are either very efficient or inefficient, adjustment of the upstream concentration alone may not be enough to ensure collection of samples within a sampler's limit of detection. In some cases other parameters, such as sample flow and time, may require adjustment. In the previous experimental setup it was possible to adjust bioaerosol concentration and sampling time but not sampling flow.[11] Therefore, the Andersen sampler was modified to allow adjustments in sampling flow from the chamber while keeping total flow through the sampler constant (at 28.3 L/min). This is necessary to assure proper operation of the Andersen sampler.

Since the concentration of particles downstream of a filter is a function of the concentration upstream, it is necessary to maintain a constant upstream concentration of culturable particles. A method was developed for maintaining solution homogeneity within the nebulizer, which produced a constant aerosol concentration and did not decrease cell culturability.

Recovery of Organisms Captured by Filters

Organisms that survive and are able to replicate after capture by respirator filter media may be released to the environment and present a health risk. Organism survival can be evaluated by removing, recovering, and then culturing captured organisms. To remove organisms, energy must be applied to overcome the forces by which cells are attached to the filter. However, this applied energy may decrease organism culturability.

An effective method of recovering organisms from filters involves the removal of attached particles by liquid elution. Elution is particularly useful because organisms can be recovered not only from the surface but from throughout the filter matrix. Agitation of the mixture improves recovery but also imparts physical stress to the organism, which may reduce culturability. Several methods of agitation were evaluated for minimum culturability loss, maximum cell recovery, and practicability.

CHANGES IN EXPERIMENTAL METHODS

As described above, three important features of this study required modifications in the experimental methods from those used in previous tests of filters with a biological aerosol, as well as the addition of a new protocol for recovery from filters. The reasons for these changes have been described above. This article describes the new and modified methods and how they relate to testing the efficiency of filters challenged with biological aerosols.

The majority of methods were developed using *M. abscessus* and were later applied to the other test bioaerosols (*S. epidermidis* and *B. subtilis* subsp. *niger*) when appropriate.[13] *M. abscessus* was obtained from the Centers for Disease Control and Prevention and cultured on Middlebrook-7H10 agar (Difco, Detroit, Mich.) at 36°C for 4 to 5 days for all tests.[11] The aerodynamic diameter for a single cell was estimated to range from 0.3 to 1.2 μm using equations derived by Cox.[21] The aerodynamic diameter was found to be 0.69 ± 0.03 μm using the APS (50 measurements were used to calculate the mean and standard deviation).

Total Versus Culturable Particle Measurements

The authors focused on the effects of aerosolization on organism culturability as a means of increasing the fraction of organisms that
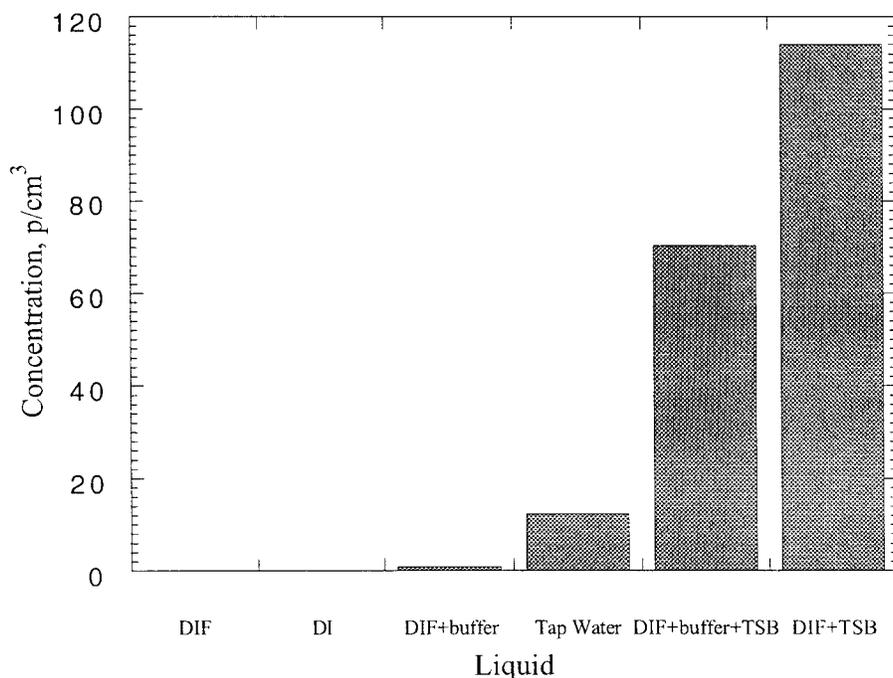
FIGURE 1. Concentration (particles/cm³) of nuisance particles generated by six nebulization liquids

could be cultured following the experiments. They also attempted to minimize the production of nuisance particles by eliminating materials that would create such particles from the nebulizer suspension.

Reducing physical stresses resulting from nebulization was a fairly simple process. The manufacturer recommends the nebulizer be operated at 120 kPa (20 psi), and previous studies successfully aerosolized *M. abscessus* at 49 kPa (7 psi).[1] By reducing the nebulizer operating pressure to 21 kPa (3 psi) the percentage of culturable particles of *M. abscessus* increased from a mean of 15% (at 49 kPa) to 45% (at 21 kPa). All three organisms used in the experiments were aerosolized with this lower pressure. The effects of reducing the pressure were not formally evaluated for *S. epidermidis* or *B. subtilis*; however, the culturability of both organisms was acceptable for testing.

The minimization of nuisance particles proved to be a more difficult problem. Almost anything added to water, as well as many of the materials commonly present in tap water, will produce an aerosol. Tap, deionized (DI), and deionized filtered water (DIF) are common nebulization liquids. In bioaerosol experiments, small quantities of substances are often purposefully or inadvertently added to the water. These may include buffers to prevent osmotic shock, surfactants to enhance mixing, and nutrients that may be added with viable cultures.
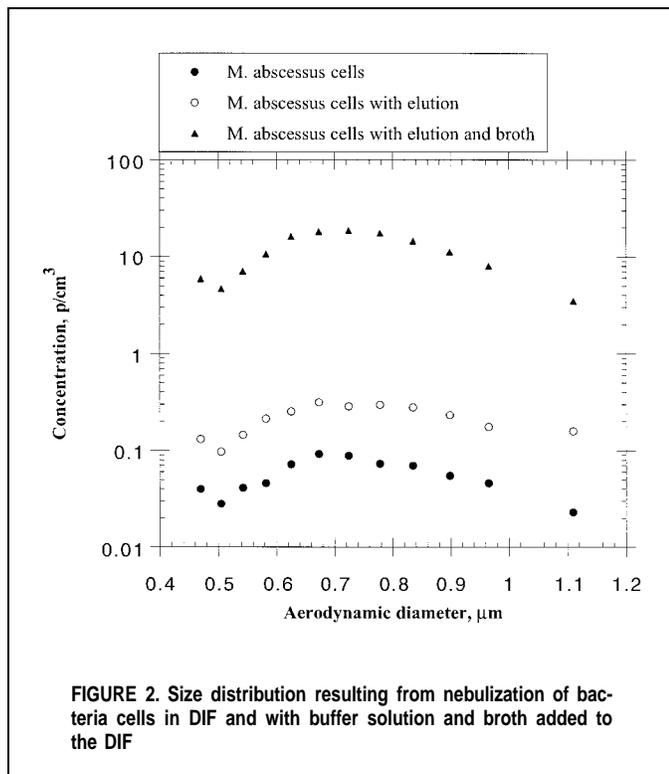
The production of nuisance particles (without organisms) was evaluated for a number of different liquids (Figure 1). In addition to tap water, DI and DIF, three other mixtures were also tested: (1) 45 mL DIF with 1 mL buffer solution (50 mL Tween 80 [FischerBiotech, Pittsburgh, Pa.] in 1 L buffered DI); (2) 50 mL DIF with 0.5 mL Tryptic Soy Broth (TSB; Difco, Detroit, Mich.); and (3) 50 mL DIF with 1 mL buffer and 0.5 mL TSB. It was found that both DI and DIF created very few nuisance particles, while all of the other liquids produced much larger quantities of

particles with a median number aerodynamic diameter of 0.74 ± 0.04 μm.

M. abscessus organisms were added to each mixture, which was immediately aerosolized and measured using both a total particle analyzer (the APS) and a viable particle sampler (the Andersen). Total time of aerosolization was approximately 30 minutes. The results (Figures 2 and 3) corroborate the findings from the previous experiment: the addition of buffer and nutrients to the liquid increases the total particle concentration while the culturable particle concentration does not increase.
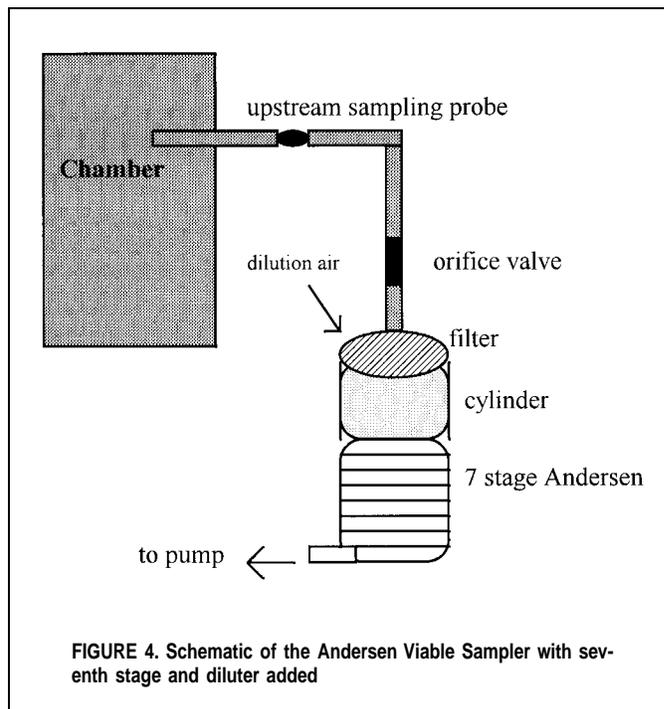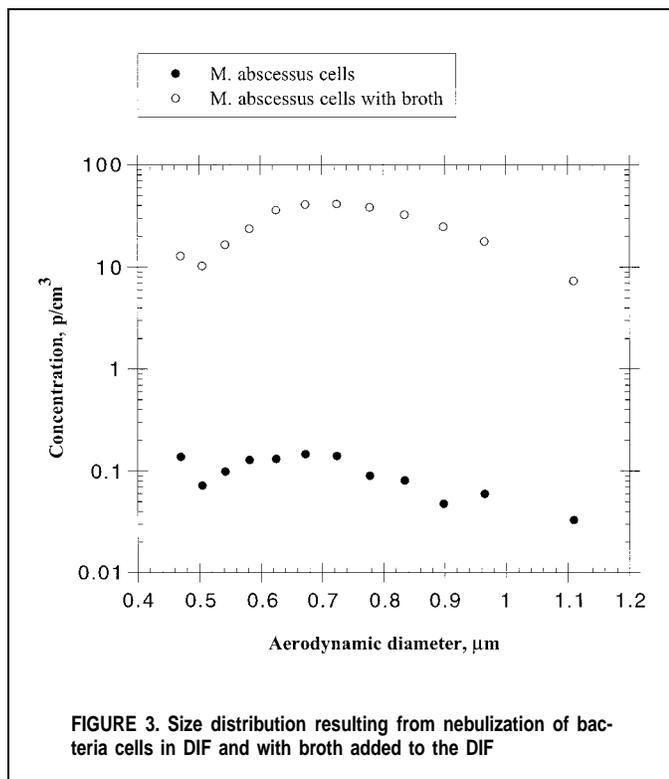
Thus, to eliminate nuisance particles, vegetative organisms were removed from solid agar with a wire loop. *M. abscessus* cultures were grown for 4 days before organisms were removed; *S. epidermidis* cultures were grown for 2 days before organisms were removed for nebulization; *B. subtilis* subsp. *niger* were stored in the dried form. Organisms were introduced to room temperature (21–24°C) DIF using a wire loop and mixed in the nebulizer jar. Nebulization, which occurred at room temperature, began immediately, and the maximum nebulization time for a given preparation was 3 hours. Airborne concentration was adjusted to the desired concentration using the APS.

To equalize the range of particle sizes measured by the two samplers, a seventh stage was added to the Andersen sampler using design criteria developed by Marple and Willeke.[22] This stage, with jet number and pattern identical to stage 6, has a jet diameter of 0.14 mm and was designed to collect particles in the 0.45–0.65 μm range. An average of 6 ± 7%, 55 ± 21%, and 38 ± 20% of the *M. abscessus* organisms were collected on the fifth (1.1–2.1 μm), sixth (0.65–1.1 μm), and seventh (0.45–0.65 μm) stages, respectively. These results indicate much greater equivalency in the sampling of small particle sizes by the two samplers, after modifications to the Andersen.

FIGURE 2. Size distribution resulting from nebulization of bacteria cells in DIF and with buffer solution and broth added to the DIF

FIGURE 4. Schematic of the Andersen Viable Sampler with seventh stage and diluter added

Measuring a Wide Range of Filter Collection Efficiency

Filter efficiency measurements require a constant upstream concentration and the collection of alternating upstream and downstream samples. Maintenance of a constant aerosol concentration requires a homogenous solution of liquid and particles in the neb-

FIGURE 3. Size distribution resulting from nebulization of bacteria cells in DIF and with broth added to the DIF

ulizer. When an attempt was made to nebulize the *M. abscessus* cells in DIF the initial airborne concentration was as expected, but then rapidly decreased to zero. It was suspected that this was due to the hydrophobic nature of the cells.[23] Substances such as surfactants, which would enhance mixing, could not be used because they would produce nuisance particles. Instead, a magnetic stirbar (Nalgene Star Head, Nalgene Co., Rochester, N.Y.) was used, which enhanced mixing without damaging cells or creating nuisance particles. The paddlewheel-style stirbar was placed in the nebulizer set upon a magnetic stirplate that was adjusted so the stirbar circled the nebulizer wand and rotated clockwise to agitate the liquid surface. This produced a constant airborne concentration ($\pm 10\%$) of *M. abscessus* cells for up to 3 hours. It was not necessary to use this method for the two other organisms because they readily mixed with the DIF and maintained similar consistency in airborne concentration without continuous mixing.

To sample culturable particles while staying within the range of detection, a dilution-air system was added to the Andersen sampler, which reduced the sampling flow from the chamber from 28.3 to 1.5 L/min. This was achieved by inserting an orifice valve between the upstream sampling probe and the inlet to the Andersen sampler. An aluminum cylinder 6 inches in height with the same diameter as the sampler was attached to the top of the first stage. The top of this cylinder was covered with a high-efficiency filter through which dilution air entered the sampler. The sampling probe, containing the orifice valve, extended from the top of the cylinder to the test chamber. The 1.5 L/min drawn from the chamber was diluted with filtered air to bring the total flow through the Andersen to 28.3 L/min, which was necessary to preserve the particle size-selective characteristics of the sampler (Figure 4). The losses in the dilution system were evaluated using the APS and comparison sampling with an unaltered Andersen sampler and determined to be undetectable. These changes in sampler configuration allowed both longer upstream and shorter downstream sampling times, which were necessary with very efficient (i.e., high-efficiency particulate air filters) and inefficient
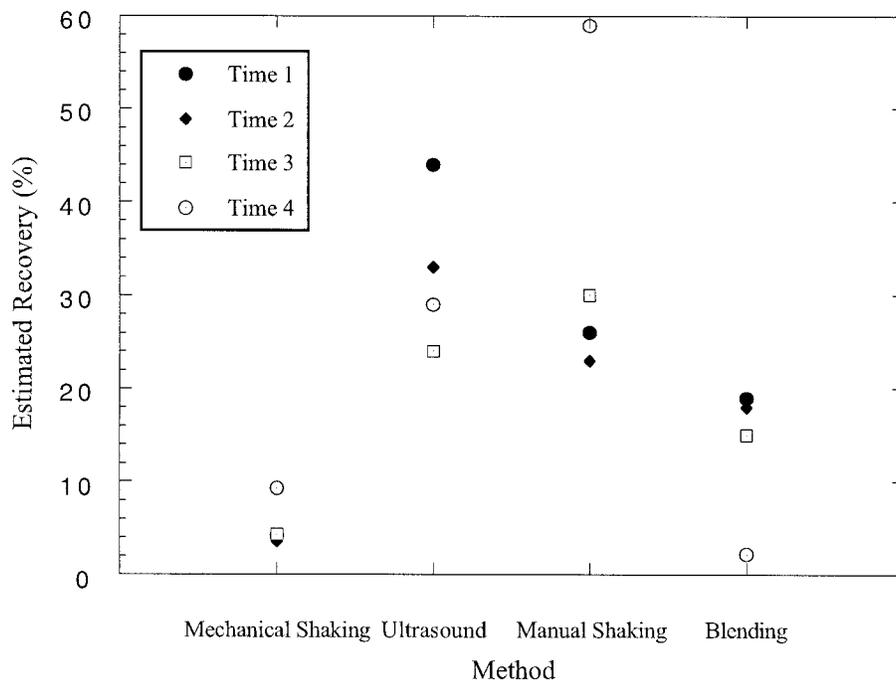
FIGURE 5. Estimated bacteria recovery from a respirator filter using four agitation methods for four periods

(i.e., surgical masks) filters, respectively. This minimized possible overloading and desiccation of organisms collected on agar plates.

Recovery of Organisms Captured by Filters

Two experiments evaluated four agitation methods for recovery of organisms from loaded filters: (1) manual shaking, (2) mechanical shaking, (3) ultrasonic vibration, and (4) blending. The first experiment determined the agitation time for each method that recovered the greatest number of culturable organisms, while the second compared the four methods. Recovery was evaluated by the number of organisms recovered compared with the number of organisms loaded on the filter.

The loading, elution, and agitation methods were similar for both sets of experiments. Filters were loaded with 1–2 particles/cm³ of aerosolized *M. abscessus* during challenge tests performed at a system flow of 45 L/min.[12] Exact loading concentration and the total loading time (17–32 min) were recorded for each filter. Immediately following loading, 1 inch² (6.45 cm²) of filter was cut from the respirator or mask and combined with 10 mL of liquid (which consisted of a 0.005% Tween 80 buffer solution) and placed either directly into a blender (Waring Products, Winstead, Conn.) or in a 50-mL polypropylene centrifuge tube (Corning, Inc., Corning, N.Y.). The centrifuge tubes were then either manually shaken (45° arc from the elbow), set on the mechanical shaker operated at 250 rpm (New Brunswick Scientific, Edison, N.J.), or placed into an ultrasonic cleaner (Mettler Electronics, Pasadena, Calif.). The maximum time of agitation was 3 minutes. Immediately following agitation duplicate 100-L aliquots were plated on Middlebrook 7H10 Agar (Difco), and incubated at 36°C for 4 days. Results were reported as colony forming units (CFUs) per square inch of filter.

The first set of experiments determined the agitation time for

each method that recovered the greatest number of culturable organisms from a loaded dust/mist filter (Survivair 10100, Survivair, Santa Ana, Calif.). Seven filters were each loaded with 1–2 × 10⁵ organisms; only one agitation method was tested on each filter. The number of organisms loaded on the filter was estimated by subtracting the concentration of culturable particles penetrating the filter (downstream concentration) from the concentration of culturable particles approaching the filter (upstream concentration) multiplied by the time of loading and the system flow.

Only one filter was used for evaluation of the blending method; there were two replicates for the other three methods. Separate vials containing the filter and liquid were agitated for four periods per method: manual shaking (50, 100, 200, and 500 shakes), mechanical shaking (30, 60, 90, and 180 sec), and ultrasonic vibration (30, 60, 90, and 180 sec). For the blending method, the filter and liquid were mixed at low speed for 20 sec after which a portion of the liquid was plated. The filter was then blended for an additional 20 sec (40 sec total) and the liquid was plated again. This was repeated two additional times for totals of 60 and 120 sec.

It was found that agitation times of 180 sec of mechanical shaking, 30 sec of ultrasonic vibration, and 20 sec of blending recovered the greatest number of culturable organisms (Figure 5). Five hundred manual shakes resulted in the greatest recovery. However, this method was not considered practicable to perform many times a day; therefore, 200 was the number of manual shakes selected for the second experiment.

In the between-method comparison experiments, material from one filter was agitated using each of the four methods. Agitation was performed on three replicates each of two dust/mist filters (Survivair 101000 and Moldex 2200, Moldex, Culver City, Calif.). Following loading, filter material was agitated as described
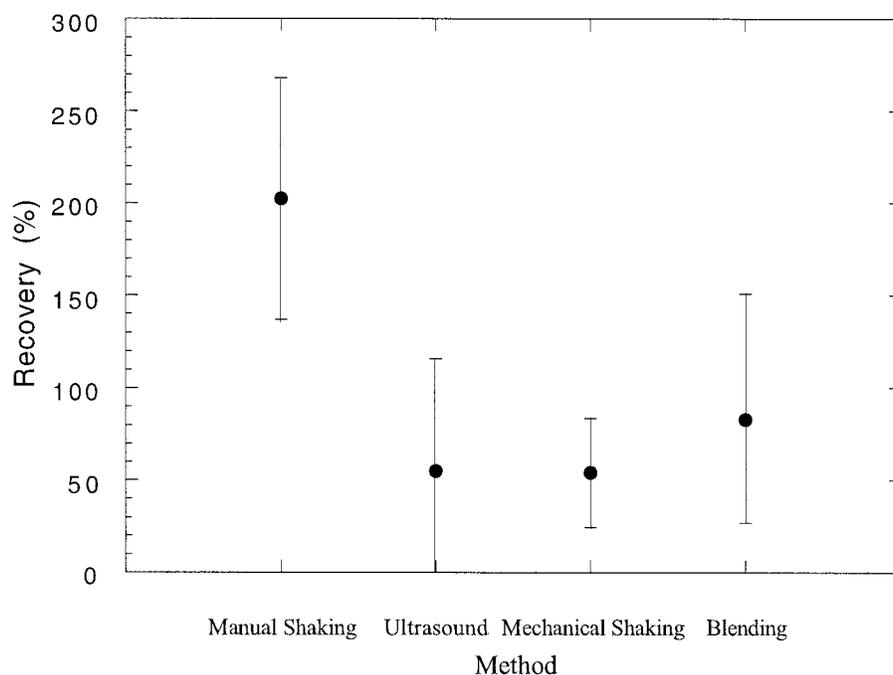
FIGURE 6. Bacteria recovery from a Survivair DM filter using four agitation methods (mean ± 2 standard deviations)

earlier with 200 manual shakes, 180 sec of mechanical shaking, 30 sec of ultrasonic vibration, and 20 sec of low-speed blending. The percentage recovery (R) for each method was determined by

$$R = (O/L) \times 100 \qquad (1)$$

where O = number of organisms recovered and L = number of organisms loaded on the filter.

The greatest recovery occurred when the Survivair filter was manually shaken (Figure 6). Recovery from the Moldex filter was highest and statistically similar for the blending and manual shaking methods (Figure 7). In one case, recovery was greater than 100%. A likely explanation is that a fraction of the organisms are rendered nonculturable during sampling with the Andersen, resulting in underestimation of the number of loaded organisms. It is also possible that aggregates were broken up during agitation, although the mean measured particle aerodynamic diameter was within the range expected for particles composed of single cells.

A protocol was chosen that involved elution of 1 inch^2 of filter in 10 mL of liquid followed by manual shaking 200 times. This method showed higher recovery in comparison with mechanical shaking and ultrasonic vibration, and it was considered feasible to perform many times a day. Blending proved to be infeasible because of the cleaning and sterilization required between each test.

DISCUSSION AND CONCLUSIONS

Some important improvements in experimental apparatus and protocol have been described, as well as the development and evaluation of filter recovery methods. These methods will allow the comparison of two sampling methods in experiments with different biological aerosols. The improvements may be applicable to other laboratory assessments requiring the use of biological aer-osols as well, such as filter efficiency tests and sampler characterization research.

For example, to employ a "total" particle sampling method as a surrogate for a method that evaluates only culturable biological particles, it is important to demonstrate that these measurements are comparable. In addition, to assure that such comparability is maintained throughout an experiment, it is important to eliminate stresses that can lower culturability and to create an aerosol that is largely biological in nature. Preparation of the organism and the process of aerosolization can introduce significant stress and considerably lower culturability.[9] It has been demonstrated that the addition of materials such as nutrients, surfactants, broths, and so forth can lead to the production of significant numbers of nonviable, nonbiological particles, which can serve to mask the biological aerosol. It is important to minimize, assess, and report the effects of aerosolization on the culturability and overall nature of any biological aerosol used for laboratory studies of filters and samplers.

A common organism preparation method employed by investigators in bioaerosol research involves eliminating cell debris by "washing" organisms (i.e., repeated centrifuging and washing of the pellet).[9,24,25] However, such practices may place considerable stress on organisms and cause cell death. Subsequent generation of the diluted pellet could create an aerosol containing large numbers of nonviable organisms, including organisms that have been injured or broken apart by the centrifugation process. Such an aerosol may be acceptable for studies of health effects caused by both intact and damaged cells but is not useful when conducting filter or sampler comparison studies. The latter require a well-characterized, homogeneous, consistent aerosol to ensure that each filter or sampler is challenged with the same aerosol.

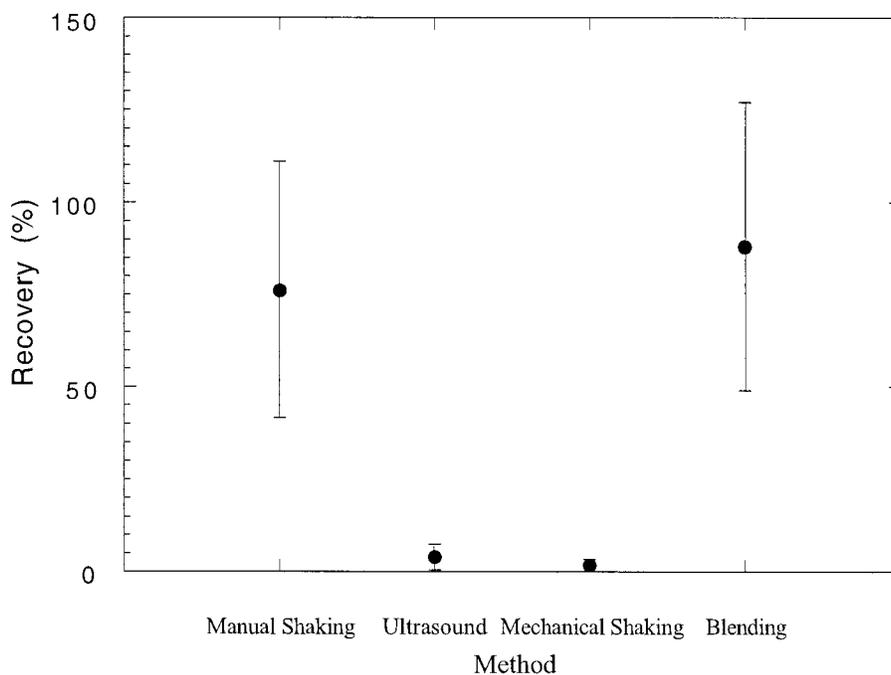It has also become common for investigators to employ a

FIGURE 7. Bacteria recovery from a Moldex DM filter using four agitation methods (mean ± 2 standard deviations)

"total" particle sampler (such as the APS and the similar Aerosizer [Amherst Process Instruments, Inc., Hadley, Mass.]) for measuring airborne concentrations of biological aerosols.[9,10] It cannot be assumed, however, that these provide useful surrogate measures of the biological aerosol until it is assumed that the biological aerosol is well characterized (size distribution and percentage viability). If the biological aerosol contains a large number of nonviable or nonbiological particles, measurements made with a total particle sampler may not be assessing the particles of interest, i.e., viable ones. It is important to fully characterize and understand the nature of the biological aerosol of interest prior to selecting a nonbiological surrogate.

Survival of organisms captured by filters can be evaluated with the methods developed to recover organisms captured by filters. Information regarding organism survival on respirator and surgical mask filters may be relevant when developing policies regarding use, handling, storage, and disposal of respirators and surgical masks. Additionally, application of this method may not be limited to respirator and surgical mask filters. Evaluating the fate and survival of organisms collected on filters in home, general, and local ventilation, and heating, ventilation, and air-conditioning systems may lead to the development of better maintenance and disposal procedures for these systems as well.

ACKNOWLEDGMENTS

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