PAPER

The effect of filter material on bioaerosol collection of *Bacillus subtilis* spores used as a *Bacillus anthracis* simulant

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The objective of this study was to determine filter materials and extraction methods that are appropriate to use for environmental sampling of *B. anthracis*. Four types of filters were tested: mixed cellulose ester (MCE) with a pore size of 3 μm, polytetrafluoroethylene (PTFE) with pore sizes of 1 and 3 μm, and gelatin with a pore size of 3 μm. *Bacillus subtilis* var. *niger* endospores (also known as *Bacillus globigii* [BG]) were used as a surrogate for *B. anthracis*. Endospores were collected into Button Inhalable Aerosol Samplers with sampling times of 15 minutes, 1 hour, and 4 hours. Physical collection efficiency was determined by measuring upstream and downstream *B. subtilis* concentrations with an optical particle counter. Vortexing with ultrasonic agitation and vortexing with shaker agitation extraction methods were evaluated. The MCE, 1 μm PTFE, and gelatin filters provided physical collection efficiencies of 94% or greater. The 3 μm PTFE filter showed inconsistent physical efficiency characteristics between filters. Epifluorescence microscopic analysis of the gelatin filter extraction fluid revealed the presence of contamination by non-culturable bacteria. Mean differences for microbial culturability were not statistically significant for filter materials and extraction methods. However, the vortexing with shaker agitation extraction method resulted in higher total microbial counts in the extraction fluids for MCE and 1 μm PTFE filters when compared to vortexing with ultrasonic agitation. In summary, the MCE and 1 μm PTFE filters in combination with vortexing and shaker extraction demonstrated the best performance for the filter collection and extraction of BG spores.

1. Introduction

Bioterrorism is defined as the use or threatened use of biologic agents against individuals to obtain an advantage for a specific purpose such as intimidation, ideological principles, or disruption of everyday activities.[1] Since October 2001 with the introduction of mail contaminated with *Bacillus anthracis* into several work environments, people worldwide have become increasingly aware of the potential for bioterrorism acts.[2] These events revealed the need to develop validated environmental sampling and analytical methods for specific biological agents to determine whether they are present and at what concentration to determine the potential health hazard.[3]

Traditionally, environmental monitoring for *B. anthracis* has been conducted using culture-based methods.[4] Culturable cell counts can be affected by a variety of factors such as the type of nutrient media selected; aerosolization, collection, and assay methods; and environmental conditions.[5] Filtration utilizes impaction, interception, and diffusion as the major collection mechanisms.[4] The primary advantages of using filtration collection for bioaerosol samples include potential to reach high collection efficiency, ease of sample collection and preparation, relatively low costs of collection equipment and supplies, and the ability to use various analysis techniques.

Several environmental monitoring evaluations using various sampling techniques were conducted to investigate the level of *B. anthracis* contamination in the *B. anthracis* affected work sites.[6–9] The National Institute for Occupational Safety and Health (NIOSH) provided technical assistance to the United States Postal Service at the Trenton Processing and Distribution Center in Trenton, New Jersey.[6] As part of the environmental assessment conducted at the facility, air samples were collected before and after a contaminated mail sorter was operated using different sampling techniques. For the gelatin filter samples, 27/36 (75%) samples were positive for *B. anthracis* spores after the contaminated mail sorter was operational. All the mixed cellulose ester (MCE), polytetrafluoroethylene (PTFE), and dry filter unit (DFU) air samples were positive for *B. anthracis* spores after the sorter was operational when the entire extraction sample was analyzed for optimum sensitivity.[6] An environmental survey was conducted at the Brentwood Mail Processing and Distribution Center Washington, DC in October 2001 after the building had been closed and the ventilation system turned off for 3 days.[7] Twelve air samples were collected for a time period of about 30 hours at a flow rate of 2 litres per minute (L min^{-1}) using open-faced 37-mm MCE filters and were negative for culturable *B. anthracis*.[7] Seven percent (8/114) of the sterile cotton gauze wipe samples and sixty-nine percent (27/39) of the vacuum dust samples were positive for *B. anthracis*.

Additional monitoring was conducted on a mail sorting machine at the Brentwood facility that had handled two of the letters containing anthrax spores in October 2001.[8] Air sampling performed using slit agar samplers with TSA plates showed 1 colony forming unit (CFU) before the machine was activated and 6 CFU during simulated work tasks.[8] No colonies were detected from the respirator filter samples worn by the evaluation team.[8] Weis and associates investigated secondary aerosolization in an office contaminated from the October 2001 incidents.[9] The investigators found that

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re-aerosolization did occur during both levels of activities. All 10 of the personal air samples collected on gelatin filters in 37-mm open-faced filter cassettes were positive for *B. anthracis*.

The investigations referred to above have raised several questions concerning which environmental sampling and analysis methods are suitable for aerosolized biological agents and yield consistent, reproducible results. Extraction methodology is very important when working with microorganisms collected on a filter medium. Wang *et al.* evaluated the effectiveness of low frequency shaking, vortexing, and ultrasonic vibrating in eluting bacteria from respirator filter materials. Vortexing was found to extract the highest total and culturable bacteria counts from the filters.[10] Another series of experiments by Wang *et al.* explored the effect of sampling time, humidity, and extraction technique using five different microorganisms (*B. subtilis* endospores, *Penicillium melinii*, *Aspergillus versicolor*, *Pseudomonas fluorescens*, and *Serratia marcescens*).[11] They achieved the highest extraction efficiency (96%–98%) with 2 minutes of vortexing followed by 15 minutes of ultrasonic agitation with polycarbonate filters.[11] Increased sampling time was associated with a decrease in the culturability of the bacterial cells and spores.

Koller and Rotter looked at several issues concerning the use of gelatin filters for collecting airborne bacteria. They found that the gelatin filters had a collection efficiency of greater than 99.95% for particles between 0.5 and 3.0 μm in size.[12] The investigators also found that dissolving the filters in either isotonic saline or 1% peptone water yielded a higher bacterial count (1.6 and 2.3 times, respectively) when compared to bacterial counts from filters directly placed on the solid nutrient media.[12] They also found that shaking the dissolved filters in either isotonic saline or 1% peptone water with glass beads showed a higher bacterial count that was not a result of the natural growth of cells during the experimentation time. The authors concluded that this was likely due to the dispersion of aggregates.[12] They also found that exposing gelatin filters to sterile air showed a reduction in bacteria survivability that was a function of the exposure time. Macher and First conducted a laboratory study that compared the collection efficiency of personal (designed to collect air in the worker's breathing zone) samplers including liquid impingers, spiral sampler, gelatin and membrane filters, and a personal cascade impactor.[13] They used latex particles (2 μm in diameter), *B. subtilis* spores and *Escherichia coli* cells as the test particles. They reported that the gelatin filters had similar collection efficiencies when compared to membrane filters, but dehydration of the gelatin filter was a problem that affected the culturability of sensitive microorganisms.

In this study, protocols for filter sampling of *B. subtilis* endospores using different filter materials that have not been compared in a laboratory setting were investigated. The extraction efficiency of two standard methods was studied and compared: vortexing with ultrasonic agitation and vortexing with mechanical shaking. The effect of sampling time on the extraction efficiency and culturability of the endospores was evaluated. The results from this study can be used to select an appropriate filter and extraction method for bacterial endospores.

2. Materials and methods

2.1 Experimental set-up

Four commercially available 25 millimetre (mm) filters were used for this study: MCE filters with a pore size of 3 μm (Millipore Corporation, Billerica, Massachusetts); Zefon Corporation PTFE (Zefluor™) filters with a 1 μm pore size (obtained from SKC Inc., Eighty-Four, Pennsylvania); Pall Corporation PTFE (Teflo™) filters with a PMP support ring with a 3 μm pore size (obtained from SKC Inc.); and Sartorius gelatin filters with 3 μm pore size (obtained from SKC Inc.). The porosity of all filters ranged from 60% to 80%. Selected experiments were also conducted using polycarbonate filters with a pore size of 3 μm (GE Osmonics, Inc., Minnetonka, MN).

Each filter was used with the SKC Button Inhalable Aerosol Sampler operated at a flow rate of 4 L min^{-1} by the SKC Universal sampling pump (Model 224), which was connected to the sampler by Tygon® tubing. The Button Sampler was chosen for this study because it follows the ACGIH/ISO inhalability curve at 4 L min^{-1} and can be used to collect both stationary (area) and personal (breathing zone) air samples.[14] The Button sampler was used as designed with one o-ring above and one o-ring below the backing plate. The volumetric flow rate for each sampler was pre- and post-calibrated after each laboratory run using a Buck calibrator (A. P. Buck, Inc., Orlando, FL). The samples were collected for 15 minute, 1 hour, and 4 hour intervals.

The laboratory chamber system was housed in a Biosafety Level II cabinet (SterilchemGARD, Baker Co., Sanford, ME). The set-up is similar to the one used by Wang *et al.*[11] A 6-jet Collison-type air-jet nebulizer (BGI Inc., Waltham, MA) generated viable aerosols for *B. subtilis* endospores at 10 L min^{-1}. The *B. subtilis* var. *niger* endospores (also known as *Bacillus globigii* [BG]), frequently used as a simulant for *B. anthracis*, were provided by the U.S. Army Edgewood Laboratories, Aberdeen Proving Ground, MD, in 2000 in a dried, powder form. The endospores have an aerodynamic diameter of approximately 0.9 μm.[11] The *B. subtilis* endospores were activated by suspending the dry spores in sterile deionized water, heating for 25 minutes at 55 °C and then washing by centrifugation twice in sterile deionized water at 7000 rpm. An initial concentration of 10^6 to 10^7 endospores per millilitre of the Collison nebulizer solution was established for the laboratory experiments and verified using a hemacytometer (Hausser Scientific, Horsham, Pennsylvania). If clumping was identified with the hemacytometer, the initial solution was agitated using a vortex for three minutes and re-checked with the haemocytometer before use.

The generated bioaerosol was mixed with high efficiency particulate air (HEPA) filtered laboratory air at 30 L min^{-1}. The mixture passed through an electrostatic charge neutralizer (TSI Aerosol Neutralizer Model 3012, TSI Incorporated, Shoreview, MN) before entering the bioaerosol chamber. Temperature and relative humidity were monitored by a direct reading thermohygrometer (Fisher Scientific International Inc., Hampton, NH) during the experiments. The tests were performed at ambient conditions: the average temperature in the set-up was 24.2 ± 1.8 °C and the average relative humidity (RH) was 33 ± 4.5%.

2.2 Physical collection efficiency

The physical collection efficiency (E_c) of the filters was determined by measuring the *B. subtilis* endospore concentration upstream (C_{up}) and downstream (C_{down}) of the filter sampler using an optical particle counter (OPC) (Grimm Model 1.108, Grimm Technologies Inc., Douglasville, GA). In each test, C_{up} was determined first, and then a directional switch was made to measure C_{down}. The initial two measurements collected when the direction was switched were not included in the analyses to allow the instrument to reach a consistent flow. A typical aerosol monitoring time was 2 to 3 minutes with each filter undergoing three consecutive replications to determine the average E_c. Three different filters were used for each series of experiments with the exception of the 3 μm PTFE for which eight filters were tested. To ascertain whether desiccation affected the E_c for the gelatin filter the E_c was additionally determined for gelatin filters after 4 hour sampling of HEPA filtered air.

The E_c was calculated as follows:

$$E_c = [1 - (C_{down}/C_{up})] \times 100\% \quad (1)$$

2.3 Experimental protocol for determining culturability and extraction efficiency

When determining the effect of sampling time on the measured bioaerosol concentrations and culturability, the test organisms were generated during a fixed period of 10 minutes in all the experiments to obtain similar loading for all filters. Clean HEPA-filtered air then was aspirated through the filters.

Each filter was removed from the Button Sampler immediately after sampling and soaked for 10 minutes in 20 millilitres of an extraction fluid of 0.1% (w/v) sterile peptone water with 0.01% Tween 80.[11] The samples in extraction fluid were vortexed for 2 minutes (Vortex-Fisher Scientific Inc.). The samples in solution then underwent an agitation step in either an ultrasonic bath (Fisher Ultrasonic Cleaners, Model FS20, 3 qt., 120 V 50/60 Hz, 1 A, 80 W, without heater, Fisher Scientific Inc.), or in a shaker (Burrell Wrist Action Shaker, Burrell Scientific, Pittsburgh, PA) for 15 minutes. The extraction fluid was then decanted into a new centrifuge tube to remove the filter and serial dilutions (10^{-1}) were made from each extraction fluid. The samples were analyzed for culturable and total microbial count as explained below.

For each set, a series of control and blank samples were collected. To check for contamination in the chamber set-up, a 15 minute sample of HEPA filtered air was collected on the appropriate filter without aerosolizing endospores. The resultant filter was then processed using the same laboratory techniques. A filter media blank was also included and analyzed for each experimental set. Aliquots of the extraction fluid were also analyzed to assess potential laboratory contamination.

2.3.1 Culturable count. For the culture-based analysis, aliquots (0.1 mL) of the original extraction fluid solutions, 10^{-1} dilution extraction fluid solutions, 10^{-3} and 10^{-4} dilutions of nebulizer solution, and extraction solutions from the blank samples were placed on TSA agar (Becton, Dickinson and Company, Sparks, MD) plates. Three replicates were made for each solution. The plates were incubated at 28 °C for 18 hours and the resultant colonies were counted on each culture plate that had the dilution with about 30–100 colonies. The culturable counts in the extraction fluid ($N_{cfu-Extraction}$) and in the Collison nebulizer fluid ($N_{cfu-Collison}$) were calculated as follows:

$$N_{cfu} = (cfu/10^{-n}) \times (V_1/V_2) \quad (2)$$

where cfu is the average number of colony-forming units on the three replicate agar plates, n is the dilution factor, V_1 is the extraction fluid volume, and V_2 is the volume of dilution applied to the plate.

2.3.2 Total count. Two methods were used to determine the total count of bacterial endospores on the filter: OPC and microscopic counting. An OPC was used to determine the aerosol concentration of endospores in the laboratory chamber based on the reading for the spore particle size range obtained during the experiment as a 1 minute average value using all particles greater than 0.65 μm. $N_{Total-OPC}$ is the total particle count for the generation period:

$$N_{Total-OPC} = C_{Total-OPC} \times Q \times t \quad (3)$$

where $C_{Total-OPC}$ is an integrated particle concentration for the sampling period based on the average of C_{up} over the sampling time, Q is the sampling collection flow rate (4 L min^{-1}), and t is the sampling time in minutes.

The following samples were analyzed for total count using an epifluorescence microscope (Model Laborlux S, W. Nuhsbaum Inc., McHenry, IL) at a magnification of 1000×: the original extraction fluids from the filter samples, 10^{-3} and 10^{-4} dilutions of the Collison nebulizer solution, and the blank samples. The slides were prepared by first filtering sterile phosphate buffer through a black 25 mm polycarbonate filter with a pore size of 0.2 μm in a filter holder using a vacuum. A 1 mL extraction fluid subsample was then filtered and stained using 3 mL of acridine orange solution for 10 minutes. The excess stain was removed by adding sterile phosphate buffer through the filter. The filter was mounted on a glass slide using immersion oil and a cover slip, and the edges were sealed with clear nail polish. The counting procedure was based on the acridine orange method described by Palmgren and associates.[15] Forty randomly chosen fields were counted on each slide. The total microbial count in the extraction fluid ($N_{Total-Extraction}$) and in the Collison nebulizer fluid ($N_{Total-Collison}$) was calculated using the same expression from the average microscopic field count (N):

$$N_{Total} = N(\pi R^2/A)\ (V_1/V_3) \quad (4)$$

where R is the effective filter radius (8.5 mm), A is the microscopic field area (0.02404 mm^2 for Laborlux S, Leitz Inc. microscope), V_1 is the extraction fluid volume (20 mL), and V_3 is the volume of dilution used for analysis (1 mL). The total microbial concentration ($C_{Total-Microscope}$) in the air sample (endospores m^{-3}) was calculated as:

$$C_{Total-Microscope} = N_{Total-Extraction}/(Q \times t) \quad (5)$$

and related to the $C_{Total-OPC}$.

2.3.3 Relative culturability. The relative culturability (RC) was defined as:

$$RC = [CF_{Extract}/CF_{orig}] \times 100\% \quad (6)$$

where $CF_{Extract}$ is the culturable fraction in the extraction fluid ($N_{cfu-Extraction}/N_{Total-OPC}$) and CF_{orig} is the initial culturable fraction in the Collison nebulizer ($N_{cfu-Collison}/N_{Total-Collison}$). The initial culturability for this experiment ranged from 0.075 to 0.373 with an average of 0.25 and a standard deviation of 0.07.

2.3.4 Physical extraction efficiency. The physical extraction efficiency (E_E) was defined as:

$$E_E = [N_{Total-Extraction}/N_{Total-OPC}] \times 100\% \quad (7)$$

E_E was determined to compare the mechanical extraction of the *B. subtilis* endospores from the filter materials.

2.4 Data analysis

Data analysis was performed using the SAS statistical package version 8 (SAS Institute, Inc., Cary, NC). A general linear model (GLM) procedure was used to look at the differences in relative culturability between filters, sampling times, and extraction efficiencies because there were unequal observations for some experimental conditions. Paired t-tests were performed to compare the average total count obtained by the OPC to the one obtained by microscopic counting. Standard t-tests were used to compare the physical collection efficiencies of gelatin filters obtained during two sampling periods. A one-way ANOVA was used to compare E_c-values obtained with different filter types. General linear models were utilized to assess the effects of sampling time, filter type, and extraction method for the E_Es. A significance level of 0.05 was used for all statistical tests.

Table 1 Physical collection efficiency for different filter materials using *B. subtilis* endospores with Optical Particle Counter (OPC)

Filter material (pore size)	Physical collection efficiency (%)
Mixed cellulose ester (3 μm)[a]	97.6 ± 4.1
Polytetrafluoroethylene (1 μm)[a]	94.2 ± 2.3
Polytetrafluoroethylene (3 μm)[b]	63.6 ± 32.3
Gelatin (3 μm)[a]	97.9 ± 3.7
Gelatin (3 μm)[c]	94.3 ± 6.5
Polycarbonate (3 μm)[a]	61.4 ± 24.6

[a] Average of 3 repeats for 3 different filters with standard deviation. [b] Average of 3 repeats for 8 different filters—showed leakage around filter when used with metal back-up pad and two o-rings. [c] Four hour sampling period prior to measurement—average of 3 repeats for 3 different filters.

3. Results and discussion

3.1 Physical collection efficiency

Table 1 presents the physical collection efficiencies (E_c) for the five filters used in this study. The MCE, 1 μm PTFE, and gelatin filters had similar average physical collection efficiencies of 94% or greater. Due to the low collection efficiency of the 3 μm PTFE filter along with the wide range of variability between the filters, this filter type was not used for the rest of the experiments. Further laboratory investigation showed that there was leakage around the filter when used with the Button Sampler metal back-up pad and two o-rings. Almost one hundred percent collection efficiency was obtained for one filter out of six using two o-rings that had a snug fit. A one-way ANOVA analysis of the MCE, 1 μm PTFE, and gelatin filters found no significant differences between the three filter types for E_c ($p = 0.41$). No significant differences were found when the E_c of the gelatin filters, measured directly after insertion into the Button Sampler, was compared to the E_c measured after the filtered laboratory hood air passed through the filter for 4 hours (*t*-test: $p = 0.47$). However, the gelatin filters were found to be brittle after the 4 hour air sampling period and could be easily broken. The 3 μm polycarbonate filter showed an average physical collection efficiency of 61%.

3.2 Comparison of the total count to the optical particle count

Fig. 1 shows the concentrations obtained by the OPC and by the microscopic counting for MCE and 1 μm PTFE filters. Paired *t*-tests comparing the two enumeration techniques showed no significant differences ($p = 0.13$ and $p = 0.37$ for vortex and ultrasonic agitation and vortex and shaker agitation, respectively). Linear regression models also showed strong positive correlations between the two enumeration methods ($r^2 = 0.96$ for vortex and ultrasonic agitation and $r^2 = 0.89$ for vortex and shaker agitation). This demonstrates that the average OPC particle count can be used instead of microscopic total count as the denominator in the relative culturability calculations.

The microscopic analysis of the extraction fluid obtained from the gelatin filters revealed the presence of bacteria, other than *Bacillus subtilis*, which were also found in the media blanks. These species, however, were not present in the samples collected using other filters. No growth other than *B. subtilis* was found on the culturable plates, indicating that the bacteria were rendered non-viable during the gamma sterilization process. This microbial contamination made the gelatin filter samples unsuitable for performing accurate total counts under the microscope. Thus, the data were not used. The microscopic analysis of some MCE filter extraction samples showed stray

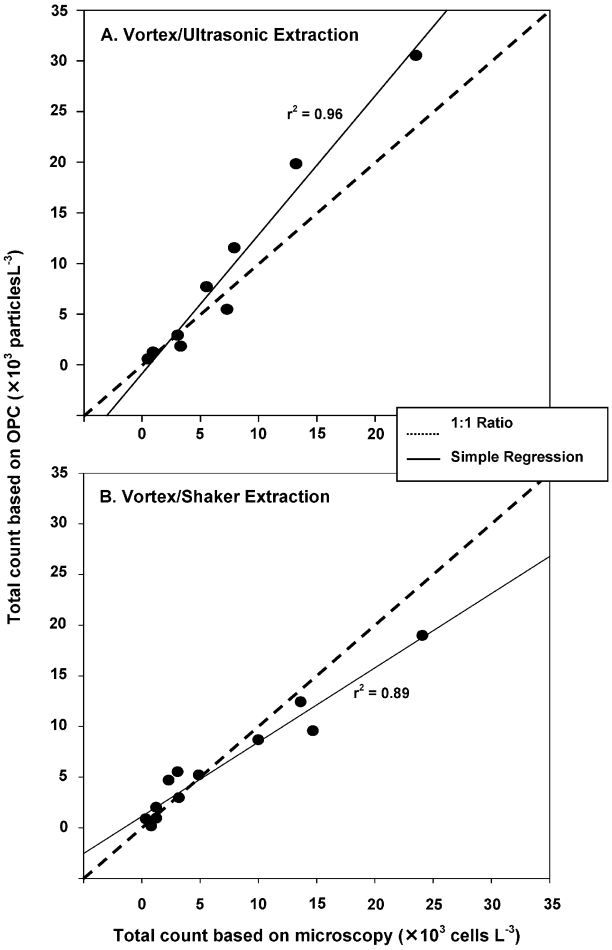

Fig. 1 Comparison of total particle concentration from microscopic count to optical particle counter (OPC) count (total count was average of four replicate samples; OPC count was the average of one-minute measurements during each consecutive experimental run) for the two extraction methods.

fibers that obscured some of the bacteria cells but did not interfere with counting procedures.

3.3 Relative culturability

Fig. 2A shows that the relative culturability using the vortex and ultrasonic extraction method for the MCE, 1 μm PTFE, and gelatin filters ranged from 72% to 130%; 93% to 100%; and 87% to 126%, respectively. The corresponding values when using the vortex and shaker extraction method ranged from 24% to 88%; 59% to 130%; and 72% to 100% (Fig. 2B). The data were examined for outliers and one data point was removed. The general linear model analyses indicated that mean differences were not statistically significant for time, extraction technique, or filter material. The vortex and shaker method showed more variability than the vortex and ultrasonic extraction method for the three filter types, but the differences between the two extraction methods were not statistically significant ($p = 0.071$). A relative culturability count of up to 126% was found for gelatin filters when dissolved in extraction fluid. Koller and Rotter also found a higher bacterial count than expected when comparing dissolved gelatin filter extract counts to tradition culture techniques.[12] The investigators used two extraction fluids (isotonic saline and 1% peptone water) in their experiments.

The culturability did not decrease over a 4 hour period for MCE, 1 μm PTFE, and gelatin filters as was expected from the polycarbonate (PC) results reported by Wang *et al.*[11] Overall, culturability obtained in this study is higher than that observed

Table 2 Average and standard deviation of physical extraction efficiencies as a percentage for MCE and 1 μm PTFE filters

	Physical extraction efficiency					
	Vortex with ultrasonic agitation (%)[a]			Vortex with shaker agitation (%)[a]		
Filter	15 minute sampling time	1 hour sampling time	4 hour sampling time	15 minute sampling time	1 hour sampling time	4 hour sampling time
MCE	66 ± 8	72 ± 12	75 ± 31	121 ± 25	101 ± 22	162 ± 25
1 μm PTFE	77 ± 16	69 ± 20	87 ± 50	123 ± 13	115 ± 11	108 ± 54

[a] Average of four sample replicates with standard deviation.

by Wang *et al*. who collected *B. subtilis* endospores on 0.2 μm PC filters and found that the culturability decreased from 17% to 5% with an increase in sampling time from 5 minutes to 4 hours at RH = 30%. The referenced study utilized a different filter and different batch of *B. subtilis* endospores than those tested in this experiment, which could partially explain the difference in results. In addition, the Wang study used endospores with a wider initial culturability rate (30% to 70%).

In this study, PC filters of 3 μm pore size using the vortex and ultrasonic extraction method in conjunction with a 15 minute sampling time were evaluated. The relative culturability was 18 ± 17%. Thus, the relative culturability results with 3 μm PC filters are in general agreement with those reported by Wang *et al*.[11] The data obtained in the two studies suggest that PC filters have a lower culturability than the other filters.

3.4 Physical extraction efficiency

The E_{ES}s for the MCE and 1 μm PTFE filters are presented in Table 2 for three sampling periods: 15 minute, 1 hour, and 4 hour. Each number represents the average percentage of endospores that were extracted from the filters relative to the total number of endospores collected on the filters. The data were examined for outliers and three data points were removed. The GLM analysis showed that the extraction method had a significant effect on the total number of spores extracted from the filter ($p < 0.001$). The vortex with shaker method showed higher extraction efficiencies for both filters over the three sampling times. This may be due to the higher mechanical forces that the filter undergoes when the shaker device is used. As described above, the relative culturability did not differ between the extraction methods. Thus, the two methods had similar effects on the culturability of *B. subtilis*, but the overall efficiency of vortexing with shaker agitation extraction was higher than that of vortexing with ultrasonic extraction. The E_{ES}s for the gelatin filters were assumed to be 100% because the gelatin filters dissolved into the extraction fluid.

The extraction efficiency for the 3 μm PC filter was 88 ± 12% when the vortexing with ultrasonic agitation extraction method was used. Wang *et al*. found that the extraction efficiency was 98 ± 1% for 0.2 μm PC filters when vortexing for 2 minutes and agitating ultrasonically for 15 minutes.[11]

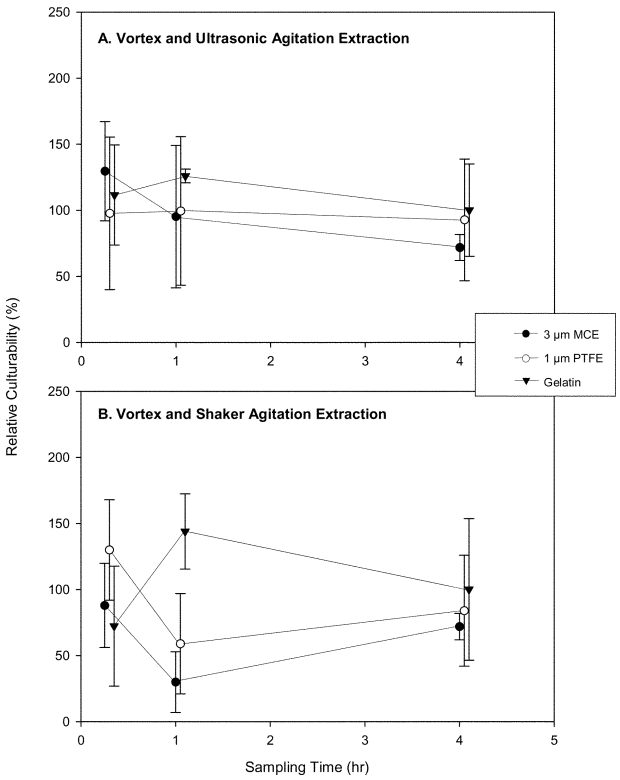

Fig. 2 Percent relative culturability comparison between vortex and ultrasonic and vortex and shaker agitation extraction methods for mixed cellulose ester, polytetrafluoroethylene, and gelatin filters based on the average of at least three replicates for the two extraction methods with standard deviation bars.

4. Conclusions

The two extraction methods showed a similar effect on the bacterial culturability, but the vortex with shaker agitation extraction method showed a significantly higher physical extraction efficiency for MCE and 1 μm PTFE filters than the vortex with ultrasonic agitation extraction method. Relative culturability and the extraction of *B. subtilis* off the sampling filters were not affected by sampling times of up to 4 hours. The 3 μm PTFE filters, which were thinner than the other filters, showed a wide range of physical collection efficiencies that would limit the ability to collect consistent environmental samples. The gelatin filter extraction fluid contained contamination by non-culturable bacterial cells that made total microscopic counting unfeasible. The results show that among the tested filters and extraction methods, the MCE and 1 μm PTFE filters and the vortex with shaker agitation extraction method had the best performance when sampling and analyzing *B. subtilis* endospores.

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