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Evaluation of sampling methods for *Bacillus* spore-contaminated HVAC filters

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Abstract

The objective of this study was to compare an extraction-based sampling method to two vacuum-based sampling methods (vacuum sock and 37 mm cassette filter) with regards to their ability to recover *Bacillus atrophaeus* spores (surrogate for *Bacillus anthracis*) from pleated heating, ventilation, and air conditioning (HVAC) filters that are typically found in commercial and residential buildings. Electrostatic and mechanical HVAC filters were tested, both without and after loading with dust to 50% of their total holding capacity. The results were analyzed by one-way ANOVA across material types, presence or absence of dust, and sampling device. The extraction method gave higher relative recoveries than the two vacuum methods evaluated ($p \leq 0.001$). On average, recoveries obtained by the vacuum methods were about 30% of those achieved by the extraction method. Relative recoveries between the two vacuum methods were not significantly different ($p > 0.05$). Although extraction methods yielded higher recoveries than vacuum methods, either HVAC filter sampling approach may provide a rapid and inexpensive mechanism for understanding the extent of contamination following a wide-area biological release incident.

Keywords

HVAC sampling; Anthrax; *Bacillus anthracis*; biological agent; bioterror

1. Introduction

Although significant advances have been made over the last decade in the areas of detection, sampling, and decontamination following a biological terror incident, gaps remain in our

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ability to respond rapidly and recover from large-scale attacks (e.g., wide-area dispersal of a biological agent) (Calfee et al., 2013b; Campbell et al., 2012; Canter, 2005; Franco and Bouri, 2010; Wein et al., 2003). Particularly challenging will be the task of delineating the affected area and determining which buildings were contaminated following a wide-area release (Raber, 2011). Mapping the extent of contamination is important for the identification of zones of exposure as well as for orderly response and decontamination operations.

Numerous studies have demonstrated that particulates released outdoors can infiltrate buildings (National Institute for Occupational Safety and Health, 2002; Bennett and Koutrakis, 2006). Once inside, airborne contaminants are easily distributed throughout the building by the heating, ventilation, and air conditioning (HVAC) system (Thatcher and Layton, 1995; Sippola and Nazaroff, 2005; Krauter and Biermann, 2007; Krauter et al., 2005; Kowalski et al., 2003). Recently, it was proposed that sampling HVAC filters from buildings within the suspected contamination zone can help responders determine the affected areas rapidly (Ackelsberg et al., 2011; Van Cuyk et al., 2012; Hong and Gurian, 2012; Stanley et al., 2008). This approach, if effective, may offer numerous advantages over other sampling approaches. For instance, HVAC systems process large volumes of air and their filters likely collect representative samples of building particulates; HVAC filters are ubiquitous and abundant especially in urban environments; HVAC filters are also inexpensive, easy to access, and relatively easy to sample. Although several studies have investigated and optimized direct filter extraction methods for spore recovery, none (to our knowledge) have compared extraction methods to vacuum sampling methods when applied to HVAC filters (Solon et al., 2012; Farnsworth et al., 2006). Vacuum methods are the most commonly used by responders when sampling rough and porous surface types.

The goal of this current study was to compare two vacuum-based surface sampling methods and one extraction-based method for their relative abilities to recover *Bacillus atrophaeus* spores (surrogate for *B. anthracis*) from HVAC filters. Two filter types (electrostatic and mechanical) were each tested under two conditions (neat or loaded with dust).

2. Materials and methods

2.1. Test materials

Stainless steel (16-gauge, 304 or 316 stainless; Dillon Supply, Raleigh, NC) was cut into 10 cm × 15 cm coupons from larger pieces of stock material. During inoculation, six 10 cm × 15 cm stainless steel coupons were arranged in two rows of three (total area, 30 cm × 30 cm) on the surface of a larger (36 cm × 36 cm) piece of stainless steel (Fig. 1C). The six 10 cm × 15 cm steel coupons constituted one reference coupon (in sections for ease of extraction). Mechanical (MERV 8 Purafilter 2000 Blue Series, Las Vegas, NV) and electrostatic (MERV 8 Eco-Aire High Cap, Orlando, FL) HVAC filters (36 cm × 36 cm × 3 cm) were purchased directly from a commercial source. Both filters were pleated varieties, typical of commercial and residential buildings.

Filters within the dust treatment group were loaded with 25 g of dust, which was experimentally determined to be about 50% of the total holding capacity of the filter (data

not shown). Dust constituents, application procedures, and methods for determining the dust holding capacity were consistent with those outlined by the American Society of Heating Refrigerating and Air-Conditioning Engineers (ASHRAE) method for evaluating HVAC filter efficiencies (ASHRAE, 2007). The dust was a standardized test dust that consisted of carbon black, ISO fine dust, and cotton linters.

Stainless steel coupons were sterilized by autoclaving (121 °C, 103 kPa, for 1 h), and HVAC filters were sterilized by exposure to ethylene oxide (Anderson Products, Inc., Haw River, NC). Prior to testing, coupon sterility was confirmed by swab sampling (~25 cm^2) one coupon or HVAC filter from each sterilization batch, streaking the swab onto tryptic soy agar plates (TSA; Difco, Franklin Lakes, NJ) and incubating the plates at 35 ± 2 °C for 18 to 24 h. If contamination was detected, the entire batch was subjected to the sterilization procedure again.

2.2. Bacterial spore preparation and inoculation procedures

Spores of *B. atrophaeus* (ATCC 9372; formerly *B. subtilis* var. *niger* and *B. globigii*) (Nakamura, 1989) were used as surrogates for the biological agent *B. anthracis*. Spore preparations were obtained from the U.S. Army Dugway Proving Ground (Utah), and have been described previously (Brown et al., 2007a). These spores were prepared specifically for use as a *B. anthracis* surrogate during surface sampling studies (Brown et al., 2007a). Spores were suspended in 100% ethanol, combined with DYMEL® 134a (DuPont, Wilmington, DE) propellant, and loaded into metered dose inhalers (MDIs) by Cirrus Pharmaceuticals (Research Triangle Park, NC). Each MDI provides more than 100 consistent 50 μL doses, each dose containing ~10^8 aerosolized spores.

For stainless steel reference coupons, MDIs were attached to the apex of a pyramid-shaped deposition apparatus, and inoculations proceeded as described previously (Calfee et al., 2013a). For HVAC filters, the procedure was modified to conduct the inoculation under flow conditions, simulating the contamination of HVAC filters in buildings during an actual biological contamination incident. For this method, two pyramid-shaped deposition apparati were utilized, one in the typical upright orientation with an MDI attached to the apex, and the second inverted (open base oriented upward) and attached to the suction side of a high-volume air sampler (Thermo Scientific, VFC-PM10, Waltham, MA). The HVAC filter was oriented between the bases of the two pyramids. The high-volume sampler, operated at 50% power, provided ~500 cubic feet per minute of airflow during inoculation. Quartz filters (within the high-volume sampler) were positioned downstream of the HVAC filter, and captured particles escaping capture by the HVAC filter.

2.3. Surface sampling and test replication

Two vacuum sampling devices [vacuum sock, 37 mm mixed cellulose ester (MCE) filter cassette] and one extraction method were evaluated for their ability to recover aerosol-deposited spores from two pleated HVAC filter types (electrostatic and mechanical). Each filter type was evaluated with and without the addition of ASHRAE dust. Each of the three sampling methods was evaluated in a series of four trials; in each trial, all sampling methods were evaluated with one filter type (Table 1). During each trial, five replicate samples were

collected for each method and each filter type. In addition, extraction sampling of stainless steel surfaces (reference coupons) was conducted to normalize recoveries across trials. Normalization was necessary because trials were conducted on numerous days, using different MDIs to inoculate the test coupons. Surfaces were vacuum sampled or extracted after the 18 h allotted for spore deposition (gravitational settling in the chamber). Components for all sampling methods were assembled aseptically into sampling kits prior to experimentation.

Vacuum socks (Midwest Filtration, Cincinnati, OH; Fig. 2A) were used to collect samples from surfaces according to procedures described previously (Brown et al., 2007b). Sterilized (gamma irradiated, 15 kGy) vacuum socks, packaged individually in vacuum-sealed bags were purchased from the vendor. The centermost 30.5 × 30.5 cm portions of HVAC filters were sampled. During sample collection, the sampler used the vacuum nozzle to traverse the filter surface at a pace of 3 to 5 s per linear foot. Surfaces were sampled in a back-and-forth manner with about 50% overlap of each sweep. Each HVAC filter was sampled first in the horizontal direction, then again in an orientation rotated 90° from the first (i.e., parallel with the pleats, and then perpendicular to the pleats). The OmegaVac (Atrix, Int.; Burnsville, MN), which supplied airflow of about 2000 L min^{-1}, was utilized with the vacuum socks. After sampling, the sock was removed from the cardboard tube holders, the opening secured with a zip-tie, and the sock was placed in a specimen cup for transport to the analysis laboratory.

Cassette filters, pre-loaded with 0.8 μm pore-size MCE filters (Fig. 2B), were utilized according to the methods reported previously (Calfee et al., 2013). For each sample, the centermost 30.5 × 30.5 cm portion of the inoculated HVAC filter was sampled by lightly pressing the angled tube to the filter surface and traversing the filter coupon at a rate of 3 to 5 s per linear foot. The Vac-U-Go pump (SKC Inc.; Eighty Four, PA), which supplied airflow of about 20 L min^{-1}, was utilized with the cassette filter device. Following sample collection, the Tygon® tube was removed from the cassette and placed into a 15 mL conical tube. The provided plastic plugs were used to seal the cassette, which was placed into a sterile sample transport bag.

2.4. Extraction sampling of HVAC filters

HVAC filters were excised from their cardboard frame using sterile scissors and further cut into two 15 cm × 30 cm pieces. Filter pieces were placed inside sterile wide-mouth 1 L containers, 700 mL phosphate buffered saline with 0.02% Tween 20 (PBST) was added aseptically, and the samples were extracted by agitation (300 rpm for 30 min) on an orbital shaker.

2.5. Extraction sampling of stainless steel reference coupons

For each replicate control coupon, the six 10 cm × 15 cm stainless steel coupons were aseptically transferred into a sterile 10 L Pyrex® beaker, with the inoculated side of each coupon facing upwards. To provide space for extraction liquid to flow around the sections, sterile bent glass rods were placed below and between each coupon. Sterile PBST (1.5 L) was added aseptically to the beaker, and the beaker was covered with aluminum foil. The

beaker was then placed into an ultrasonic cleaner (Branson model 8510; Danbury, CT), and the sample was agitated (40 kHz, 15 min). Immediately following agitation, 1 L of the extraction liquid was collected from the beaker and transferred to a 1-L specimen container. Before aliquots were collected from this container for analysis, the contents were homogenized by manual mixing/shaking.

2.6. Recovery of spores from vacuum samples

Spores were recovered from vacuum sock samples similarly to the method described by Brown et al. (2007b). Briefly, the collection portion of the vacuum sock was wetted by dipping into a sterile 133 mL specimen cup (VWR, Radnor, PA; part# 25384-144) containing 20 mL sterile PBST, then cut into segments with sterile scissors. The cups, containing the segmented sock, were then agitated (30 min, 300 rpm) on a rotating laboratory shaker to dislodge the spores from the sock material.

Spores were recovered from the 37 mm cassette devices by first adding 5 mL of PBST to the 15 mL conical tube containing the 2.5 cm Tygon® tube and PVC adapter. The tubes were then sonicated for 1 min at 40 kHz in an ultrasonic water bath (Branson Model 8510; Danbury, CT) and subsequently agitated by vortexing for 2 min. The 5 mL eluant was then transferred to a 60 mL jar (Container & Packaging Supply, Eagle ID, Louisville, KY; part# J037, lid part# L208). With the cassette outlet plug in place, the inlet plug was removed, 1 mL of PBST was pipetted into the orifice and the plug was returned. The cassette was then rotated such that the added liquid wetted all surfaces of the filter and cassette. Next, the filter cassette was opened using a specialized tool (SKC, Int.; part# 225-8372), with the cassette in the upright orientation so that no liquid was spilled. An additional 1 to 2 mL of PBST was then used to rinse the interior of the cassette, and all liquid inside the cassette was captured by pipette and transferred to the 60 mL jar. The filter was then aseptically removed from the cassette and placed into the 60 mL jar. With the filter removed, the remaining portion of the filter cassette was rinsed with an additional 3 – 4 mL of PBST and transferred to the jar. For each extraction, a total volume of 11 mL PBST was used (5 mL for nozzle extraction, 6 mL for cassette rinse). The volume of extract recovered was determined using a 10 mL serological pipette (graduated to 12.5 mL). The filter and both liquid fractions, now combined in the 60 mL jar, were sonicated for 3 min to dislodge spores; the jars were rotated 90° within the water bath after 1.5 min.

For all sample types, liquid extracts were serially diluted 10-fold (in PBST) and 0.1 mL spread-plated onto TSA plates in triplicate. Plates were incubated at 35 ± 2 °C for 18 to 24 h and colony-forming units (CFU) were enumerated visually. Only plates containing between 30 and 300 CFU were utilized for recovery estimates. Extracts were diluted and replated if none of the 10-fold dilutions resulted in all three plates containing colony counts within the acceptable range. All extracts were stored at 4 ± 2 °C. Total spore recovery was calculated by multiplying the mean CFU counts from triplicate plates by the inverse of the volume plated, by the dilution factor, and finally by the total volume of the extract.

2.7. Data treatment

To normalize spore recoveries across experiments, all recoveries from HVAC filters (extraction or vacuum sampling) were divided by the recoveries obtained by extraction of the stainless steel reference coupons collected during the same trial. Normalization was conducted to reduce bias associated with comparing samples from different test days. The resulting values, hereafter referred to as "relative recoveries," were ratios of test sample recovery to positive control recovery. These data were analyzed by one-way ANOVA across material types, dust presence, and sampling device. Bonferroni post hoc tests were subsequently conducted to evaluate each contrast. Significance was assessed using a p value equal to 0.05. SigmaPlot 11 (Systat Software Inc., San Jose, CA) was utilized for these abovementioned statistical analyses.

3. Results

Mean recoveries achieved by vacuum methods ranged from $3.4 \pm 1.3 \times 10^5$ CFU (37 mm MCE, Mechanical Filter, Neat) to $5.8 \pm 2.5 \times 10^6$ CFU (37 mm MCE, Mechanical Filter, with Dust) per sample (Table 1). Recoveries from extraction of HVAC filters ranged from $4.6 \pm 5.5 \times 10^6$ CFU (37 mm MCE, Mechanical Filter, Neat) to $9.8 \pm 5.1 \times 10^6$ CFU (37 mm MCE, Mechanical Filter, with Dust). Control sample (stainless steel extraction) recoveries were $1.8 \pm 0.5 \times 10^7$, $2.9 \pm 0.3 \times 10^7$, $2.2 \pm 0.7 \times 10^7$, and $3.1 \pm 1.1 \times 10^7$, for Trials 1 through 4, respectively (Table 1; Fig. 3). Relative recoveries achieved by the three sampling methods were highest for the extraction method (Fig. 4; $p \leq 0.001$). Relative recoveries achieved by the two vacuum methods were not significantly different (data pooled for all filter types and dust treatments; $p > 0.05$). The presence of dust on the filters did not significantly affect recovery (data pooled for all filter types and sampling methods; $p > 0.05$), nor did filter type (data pooled for all sampling methods and dust treatments; $p > 0.05$).

4. Discussion

Rapid yet accurate delineation of contaminated areas is crucial for effective response management following a biological terror incident (Franco and Bouri, 2010; Raber, 2011). A release of refined *B. anthracis* spores is generally thought to result in a slow decay of airborne spore concentrations following the initial release (Matsumoto, 2003). During such a scenario, it is likely that many of the airborne spores, if released outdoors, will infiltrate adjacent buildings and will subsequently be cycled through the HVAC system. Some fraction of these spores will be sequestered by the HVAC filtration system (Mead and Gressel, 2010). Although collection and retention of spores by HVAC filters is dependent upon numerous variables (e.g., filtration efficiency, flow rate, particle size, etc.), many agree that HVAC filters may provide a rapid and inexpensive way to begin to delineate the extent of contamination following a large-scale outdoor release (Ackelsberg et al., 2011; Solon et al., 2012; Stanley et al., 2008). Until now, no studies had been conducted to compare the two most logical sampling approaches for HVAC filter materials: direct extraction and vacuum sampling of HVAC filter media.

The current study sought to compare these two general approaches for sampling HVAC filters. In addition to extraction sampling, two vacuum sampling devices were evaluated; the vacuum sock and a 37 mm cassette filter device. Overall, the results suggest that the extraction sampling approach for contaminated HVAC pleated filters achieved higher recoveries than either vacuum sampling method.

Vacuum method recoveries were typically ~30% of the recoveries achieved by extraction of the HVAC filters (Table 1). Assuming that the efficiency of extraction of the vacuum devices is similar to the efficiency of direct extraction of HVAC filters, these findings suggest that vacuum methods collect about 30% of the filter-bound spores. This collection efficiency is similar to the values reported by Brown et al. (2007b), where vacuum methods were evaluated for collection of *Bacillus* spores from painted wallboard, stainless steel, concrete, and carpet (Brown et al., 2007b).

The low recoveries (relative to stainless steel controls) observed for the extraction and vacuum sampling methods during the current study may be due to the method utilized to inoculate HVAC filters. To mimic contamination mechanisms in real-world incidents, inoculation of the filters was conducted under flow conditions. Since the filters (MERV 8) used in this study were not expected to have high collection efficiencies for spore-sized particles, many of the spores likely passed through the filter media during inoculation. Because recoveries from HVAC filters were normalized by recoveries from extraction of stainless steel coupons (smooth, nonporous) inoculated under static conditions, it is logical that relative recoveries would be significantly less than 1.

Although extraction sampling demonstrated significantly higher relative recoveries than the vacuum methods, challenges in implementing this method in the field should be considered. First, accessing HVAC filters within a building and being able to excise portions of the filter media will likely be challenging for the sampler when wearing HAZMAT gear. Furthermore, many commercial buildings have numerous filter banks, and are equipped with more complex sock-type filters. Such complex filtration systems may pose difficulties for the collection of excised samples. Lastly, compared with vacuum methods, extraction methods require about 10-fold greater volume of extraction buffer and require considerably more time per sample for processing, thus reducing sample throughput. High occurrence of background (non-target) organisms and presence of significant PCR inhibitors may pose analytical challenges for all HVAC sampling methods. Future studies should determine the ability of these methods to detect and quantify a target organism seeded onto HVAC filters following a typical use period.

Following a building contamination incident, the contaminant will be partitioned over time to interior surfaces (floors, walls, objects, etc.), duct interior surfaces, plenum surfaces (if plenum return), exfiltration (removal from the building), and the HVAC filter media. The relative quantities of the contaminant within each of these compartments will be unique for every building. For this reason, sampling of HVAC filters should not be solely relied upon as a method for determining exact magnitudes of building contamination nor for determining risk of exposure. However, HVAC filter sampling may provide a rapid and inexpensive mechanism to indicate the extent of contamination following a wide-area incident. Vacuum

sampling approaches, although less efficient than extraction methods, still provide the same order of magnitude as the extraction recovery and may be sufficient for the purpose.

In summary, extraction and vacuum sampling methods were evaluated for their ability to quantify spore contamination on HVAC filter media. The extraction method demonstrated higher relative recoveries than the two vacuum methods evaluated. Both sampling approaches may have utility in delineating the extent of contaminated buildings following a large-scale incident.

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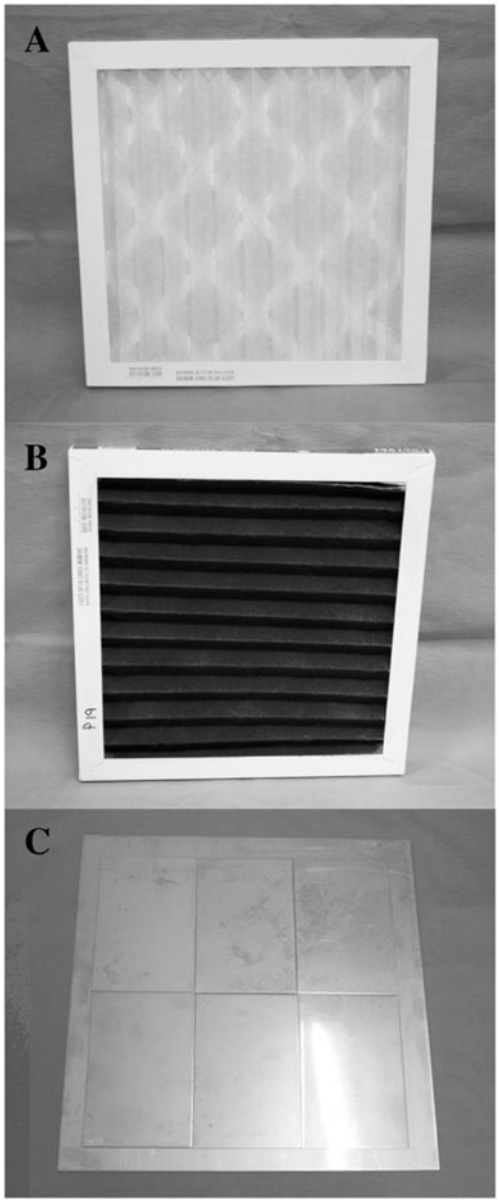

Fig. 1.

Photograph of representative neat HVAC filter (A), HVAC filter loaded with dust (B), and stainless steel reference coupon (C). HVAC filters (A and B) were 36 cm × 36 -cm × 2.5 cm, stainless steel coupons (C) consisted of six 10 cm × 15 cm sections.

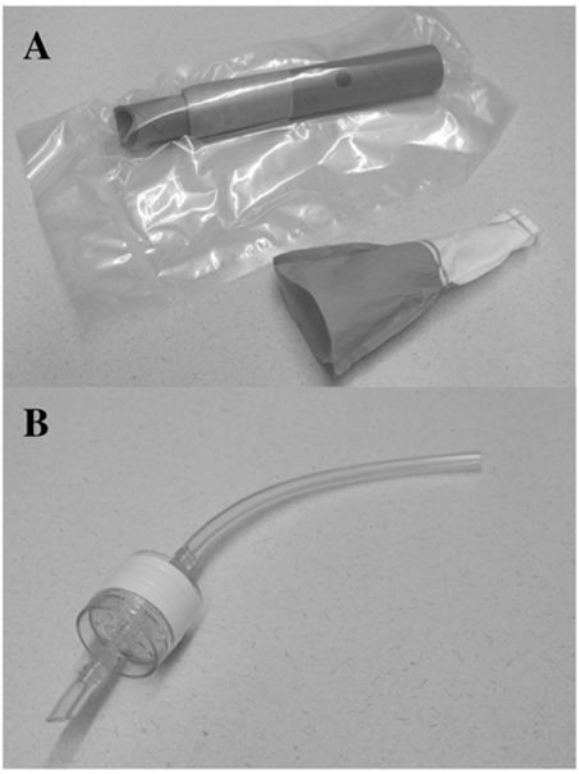

Fig. 2.
Photograph of the sock (A) and 37 mm cassette (B) vacuum sampling devices.

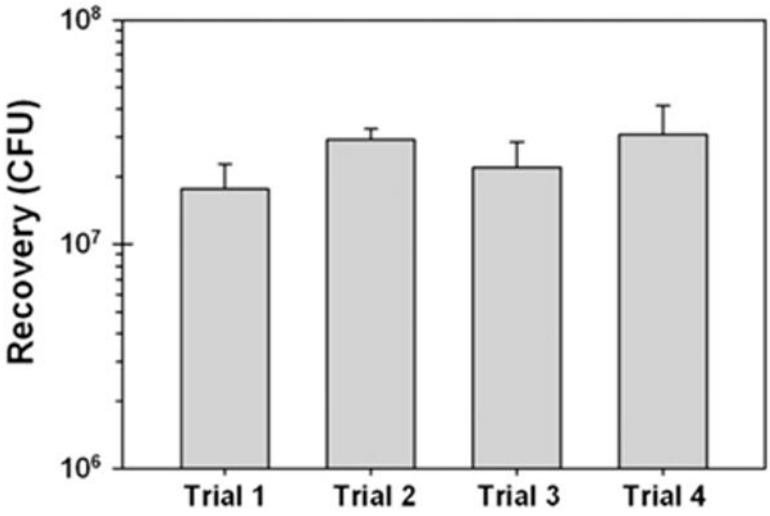

Fig. 3.

Recovery from stainless steel reference coupons for each trial. Triplicate stainless steel reference coupons were inoculated during each trial to determine the abundance of spores dispensed by the inoculation device. These data were used to normalize recoveries across trials. Data are reported as mean recovery (CFU) ± standard deviation for each trial.

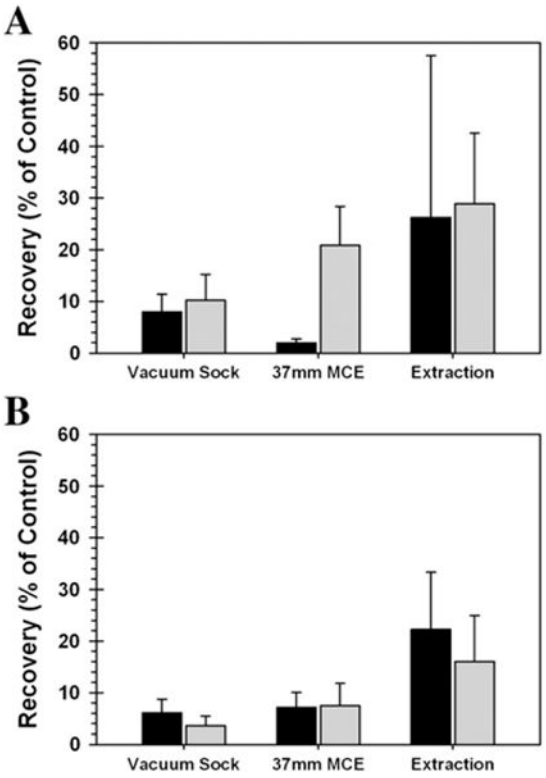

Fig. 4.

Spore recoveries following vacuum sampling or extraction of HVAC filters. Graph A reports recoveries from mechanical filters, Graph B reports recoveries from electrostatic filters. Black bars represent recovery from neat filters, gray bars reflect recovery from filters containing dust. Recoveries from HVAC filters are reported as a percentage of positive control (reference coupon) recoveries. Error bars indicate one standard deviation about the mean.

Table 1

Summary of Test Variables and Total Recoveries.

Trial	Surface sampled	Dust	Sampling method	Area sampled (cm^2)	Replicates (*n*)	Total recovery (CFU ± SD)
1	MERV8 Mechanical filters	No	Vacuum sock	929	5	$1.4 \pm 0.6 \times 10^6$
		No	37 mm MCE	929	5	$0.3 \pm 0.1 \times 10^6$
		No	Extraction	929	5	$4.6 \pm 5.4 \times 10^6$
	Stainless steel	No	Extraction	929	3	$1.8 \pm 0.5 \times 10^7$
2	MERV8 Mechanical filter	Yes	Vacuum sock	929	5	$2.8 \pm 1.6 \times 10^6$
		Yes	37 mm MCE	929	5	$5.8 \pm 2.5 \times 10^6$
		Yes	Extraction	929	5	$9.8 \pm 5.1 \times 10^6$
	Stainless steel	No	Extraction	929	3	$2.9 \pm 0.3 \times 10^7$
3	MERV8 Electrostatic Filter	No	Vacuum sock	929	5	$1.3 \pm 0.6 \times 10^6$
		No	37 mm MCE	929	5	$1.6 \pm 0.6 \times 10^6$
		No	Extraction	929	5	$4.9 \pm 2.4 \times 10^6$
	Stainless steel	No	Extraction	929	3	$2.2 \pm 0.7 \times 10^7$
4	MERV8 Electrostatic filter	Yes	Vacuum sock	929	5	$1.3 \pm 0.5 \times 10^6$
		Yes	37 mm MCE	929	5	$2.6 \pm 0.1 \times 10^6$
		Yes	Extraction	929	5	$4.8 \pm 2.7 \times 10^6$
	Stainless steel	No	Extraction	929	3	$3.1 \pm 1.1 \times 10^7$

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