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Recovery of Fungal Cells from Air Samples: a Tale of Loss and Gain

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ABSTRACT There are limitations in establishing a direct link between fungal exposure and health effects due to the methodology used, among other reasons. Culture methods ignore the nonviable/uncultivable fraction of airborne fungi. Molecular methods allow for a better understanding of the environmental health impacts of microbial communities. However, there are challenges when applying these techniques to bioaerosols, particularly to fungal cells. This study reveals that there is a loss of fungal cells when samples are recovered from air using wet samplers and aimed to create and test an improved protocol for concentrating mold spores via filtration prior to DNA extraction. Results obtained using the new technique showed that up to 3 orders of magnitude more fungal DNA was retrieved from the samples using quantitative PCR. A sequencing approach with MiSeq revealed a different diversity profile depending on the methodology used. Specifically, 8 fungal families out of 19 families tested were highlighted to be differentially abundant in centrifuged and filtered samples. An experiment using laboratory settings showed the same spore loss during centrifugation for *Aspergillus niger* and *Penicillium roquefortii* strains. We believe that this work helped identify and address fungal cell loss during processing of air samples, including centrifugation steps, and propose an alternative method for a more accurate evaluation of fungal exposure and diversity.

IMPORTANCE This work shed light on a significant issue regarding the loss of fungal spores when recovered from air samples using liquid medium and centrifugation to concentrate air particles before DNA extraction. We provide proof that the loss affects the overall fungal diversity of aerosols and that some taxa are differentially more affected than others. Furthermore, a laboratory experiment confirmed the environmental results obtained during field sampling. The filtration protocol described in this work offers a better description of the fungal diversity of aerosols and should be used in fungal aerosol studies.

KEYWORDS bioaerosols, centrifugation, filtration, fungi, recovery, taxon loss

Human exposure to diverse and dynamic airborne microbial communities has major impacts on public health in both urban and rural environments. These impacts range from allergies and asthma to the dispersal of pathogens and health effects from occupational exposure (1–4). Industrial environments are at the center of occupational health issues due to the many types of raw materials used, the prevalence of operations that release harmful bioaerosols, and the eventual high bioaerosol concentration in confined spaces (5–10). To better understand the potential risks associated with

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complex environments, it is essential to be aware of the nature of the airborne microorganisms in order to better control or prevent the potential health effects.

Fungal bioaerosols consisting of spores and hyphal fragments may be respirable and are potent elicitors of bronchial irritation and allergies, pulmonary inflammation, increased sensitivity to endotoxins, mucous membrane irritation syndrome, nasal congestion, sore throat, and irritation of the nose and eyes (11–21). The fungal impact on occupational health is largely underestimated (22). The incomplete portrait of fungal community documented using standard exposure assessment approaches still represents a barrier in establishing the link between respiratory problems and exposure to fungi (23, 24).

Typically, molds are collected from air using different sampling devices and studied through both culture- and/or non-culture-based methods. For instance, fungal load has been established by monitoring microscopic spore counts in aerosols (25, 26). Other studies have used culture methods for detecting specific fungal species (27–30). However, it is well documented that only 1% of fungi recovered from air samples are culturable; therefore, using these techniques may lead to an underestimation of the real fungal diversity and biomass in bioaerosols (31). Molecular approaches are a good alternative for getting around the nonviable/nonculturable limits of the commonly used methods mentioned previously. One widely used molecular approach is the specific real-time PCR amplification of targeted genes. This method is used to quantify specific fungi (32) and/or targets conserved regions of the 18S rRNA gene for the quantification of a greater diversity of fungi (33). Another molecular technique includes a high-throughput sequencing approach of taxonomically meaningful genes, which offers a more thorough analysis of the microbial content thanks to the millions of sequences that are generated. In the latter case, the eukaryotic ITS1 region, considered to be the universal fungal barcode, was amplified and analyzed through high-throughput sequencing (34). This allowed for an in-depth characterization of fungal diversity in the collected samples (35).

When working with air samples, microorganism concentration is commonly needed before applying DNA purification and further molecular techniques to a sample, especially with air samples from liquid impingers or filters eluted in a liquid. One of the common ways to recover fungal cells from the air for subsequent molecular analysis is to use a sampler where the fungal material is collected in a liquid or on a filter membrane and then eluted in a liquid buffer. It is then concentrated by centrifuging the liquid samples and resuspending the pellets in a smaller volume of buffer solution (36). Using centrifugation to concentrate microorganisms is a common practice in commercial column-based extraction kits, and it is often used with bacterial and archaeal specimens (37–39) for several types of samples, including environmental aerosols (7, 8, 40, 41).

Recently, as a part of a study describing bioaerosols in composting sites, *Aspergillus fumigatus* was quantified using culture and molecular approaches (quantitative PCR [qPCR]) (5). The comparison between culture and real-time PCR using a centrifugation protocol prior to DNA extraction led to inconsistent results, with higher concentrations of *Aspergillus fumigatus* found in samples using culture. This observation contradicts the actual detection efficiency of both methods and the results observed from bacteria successfully concentrated using centrifugation of air samples (7, 8, 40, 41). One hypothesis that might explain these results is that fungi have many distinct features, including charge (polarity) (42) and hydrophobicity (43, 44), that cause a different behavior during centrifugation. Hydrophobicity is caused by hydrophobins (45) or by hydrophobin-like proteins such as repellents (46). These surface proteins make fungal spores water repellent, which may cause these structures to be recovered less effectively through centrifugation, as a portion of them may be/are lost during the disposal of the supernatant. Spores with a lower density than water may also be lost when the supernatant is removed.

Previous studies may have underestimated the fungal load and diversity in aerosol samples, probably due to the method used for aerosol sample treatment prior to DNA

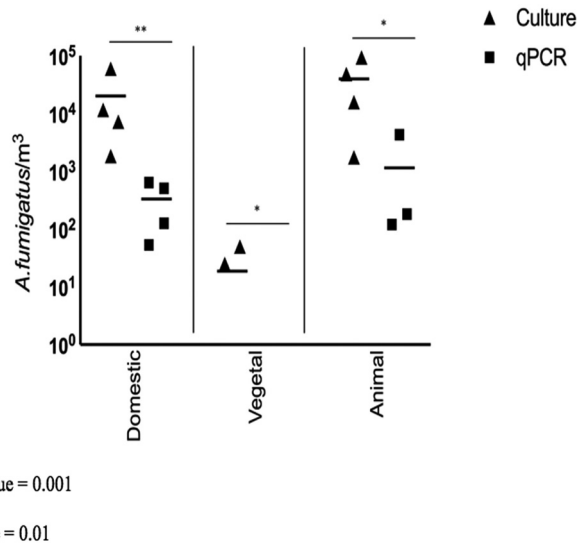

FIG 1 Concentration of *Aspergillus fumigatus* using culture and molecular (qPCR) methods in aerosol samples collected from three different composting facilities. Each point represents a different visit. The bars represent the mean value for each condition. The biological replicates are represented by the site sampled at each visit. Two visits in vegetal composting and one visit in animal composting were negative for *A. fumigatus*. Therefore, they do not appear on the figure.

extraction., e.g., centrifugation, vortexing/shaking, or enzymatic/chemical lysis (47–53). In some cases, fungi were underrepresented compared to bacteria in bioaerosols. This can lead to an underestimation of the fungal exposure in workplaces, schools, hospitals, and homes. Hence, the possibility of the health impact of fungi still can be undervalued.

The goal of this research is to develop a new molecular method-targeted protocol for concentrating mold spores recovered from air samples prior to nucleic acid extraction in order to overcome the loss during centrifugation, which is a widely used method for processing air samples. A real-time PCR and next-generation sequencing approach was applied to samples from two different environments. Results obtained using the new technique were compared with results using standard centrifugation protocols applying microbial ecology models. This research raises the question of fungal cell loss when recovered from air samples due to centrifugation and proposes an alternative method to better evaluate fungal diversity and human occupational exposure. This research is relevant for occupational and nonoccupational environments where liquid analysis is expected no matter what air sampling regimen is used. Also, studies involving bulk dust or settled dust sampling (vacuuming or electrostatic dust collection), including resuspension of dust into a liquid, will face the same problem of sample concentration.

RESULTS

Field work. (i) Compost. To study compost, culture methods and real-time PCR were used to quantify the concentration of *Aspergillus fumigatus* in aerosol samples collected with the Coriolis sampler during various visits to three different composting facilities (Fig. 1). The standard centrifugation protocol was used for fungal cell concentration prior to DNA extraction and real-time PCR quantification. In Fig. 1, the triangles and squares represent the concentration of *A. fumigatus* obtained from samples collected while workers were performing their normal duties. For samples from the domestic compost facility, culture methods yielded higher concentrations than qPCR during the four visits. Samples from two out of four visits were positive for *A. fumigatus* in the vegetal composting facility, and they were both results obtained using the culture method. qPCR analyses of the same samples were negative for *A. fumigatus*. A similar observation was made for samples from the animal composting facility. Results

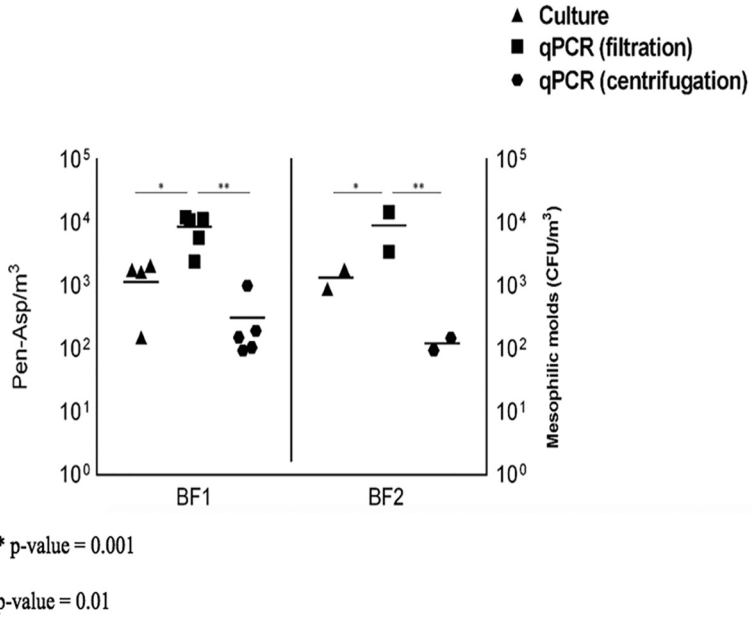

FIG 2 Concentrations of *Penicillium* and *Aspergillus* (Pen-Asp/m^3) using qPCR on filtered and centrifuged samples (left *y* axis) compared with concentrations of mesophilic molds (CFU/m^3) using culture counts (right *y* axis) from bioaerosol samples collected from two different biomethanization facilities. The points on the graph represent the sites sampled in each facility. The bars represent the mean value for each condition. The biological replicates are represented by the site sampled during the different visits. The bars represent mean concentrations.

from three out of four visits exhibited higher concentrations of *A. fumigatus* in cultured samples than those from qPCR.

(ii) Biomethanization. The bioaerosol samples collected from the two biomethanization facilities using the Coriolis sampler were separated into two groups. The first group was subjected to the new filtration-based protocol, and the second group was centrifuged using the standard protocol. After extracting the DNA from the concentrated samples, the efficiency of both concentration protocols was evaluated using qPCR targeting a region in the internal transcribed spacer (ITS) gene common to the *Aspergillus* and *Penicillium* genera (Pen-Asp). Figure 2 presents a comparison of the concentration of *Penicillium* and *Aspergillus* in the centrifuged and filtered samples. In samples from the first biomethanization facility, the five sites sampled showed a higher concentration of *Penicillium* and *Aspergillus* when filtration was used for concentrating the sample. Differences of up to 2 orders of magnitude (10^2 to 10^4) were observed between the filtration and the centrifugation methods. One data point is missing because one site in BF1 showed no mesophilic fungal growth. Similarly, the two sites sampled in the second biomethanization facility exhibited 100 times higher concentrations of *Penicillium* and *Aspergillus* when fungal cells were concentrated using the filtration protocol. Furthermore, concentrations of *Penicillium* and *Aspergillus* obtained by qPCR in the filtered samples were compared to the concentrations of other mesophilic molds obtained by culture counts. Figure 2 shows a difference of approximately 1 log between the concentrations obtained by the two methods, with the qPCR method yielding the higher concentrations. This difference is consistent throughout the seven sites sampled (5 in BF1 and 2 in BF2). These results strongly suggest that the centrifugation protocol applied to the compost aerosol samples led to the loss of *Aspergillus fumigatus* spores, and this is supported by the results presented in Fig. 1.

Because the newly developed filtration-based protocol has multiple steps, it was necessary to set up control conditions to ensure that there is no bias due to the tungsten bead-beating step. In other words, the control combining centrifugation and tungsten bead beating confirmed that the spore gain observed (with qPCR and

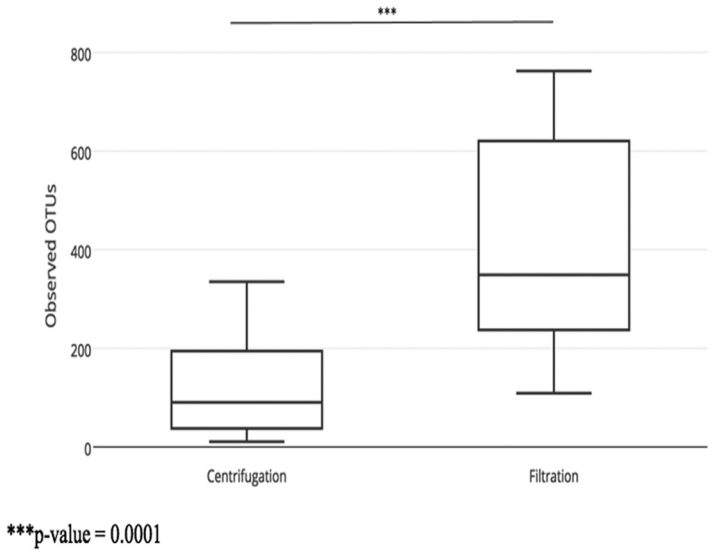

FIG 3 Boxplot representing the number of observed fungal OTUs in bioaerosol samples collected with the Coriolis sampler in biomethanization facilities. The samples were categorized into two groups, centrifugation and filtration, according to the concentration protocol used.

high-throughput sequencing) was not due to more cells being disrupted by the bead-beating step with the tungsten bead, but it is the result of the complete filtration protocol (see Fig. S1 to S3 in the supplemental material). More information on the control experiments is provided in the supplemental material (text under the section for tungsten bead control using two samplers).

(iii) High-throughput sequencing. Bioaerosol samples collected at the biomethanization facilities were sequenced using an Illumina MiSeq platform. The purpose of this analysis was to demonstrate the effect of centrifugation on the fungal diversity obtained from environmental samples. For biomethanization facilities, 32 samples were sequenced and 1,812,622 raw sequences were generated. After quality filtering and chimera checking, sequences clustered into 5,132 operational taxonomic units (OTUs).

(iv) Alpha diversity. The numbers of observed OTUs in each set of samples are presented in Fig. 3. The filtered samples contained a higher number of OTUs than the centrifuged ones. The maximum number of OTUs obtained from filtration was two times higher than the number obtained from centrifugation. Alpha richness (observed OTUs) was calculated at a value of 30,000 sequences. This number was chosen based on the lowest-depth sample parameter, which represents the lowest number of sequences in a sample. Samples with a lower number than what is chosen are excluded from the analyses. The higher the number, the more accurate the results are. In the currently presented case, all of the samples were included, as they have more than 30,000 sequences per sample, except for outdoor control samples. The negative controls are samples taken outside the facilities at each visit during the summertime. The gap between the number of sequences in samples taken from the working sites in the facilities and the outdoor negative controls was too large (more than 28,000 sequences). For this reason, outdoor controls were excluded from the analyses.

(v) Differential abundance. After observing a difference in the number of fungal OTUs between the samples treated with different concentration protocols, it seemed a logical next step to try to identify the OTUs that had significantly different abundances across the sample categories. To accomplish this goal, a statistical test designed specifically for differential analyses of count data was used. *DESeq2* is a statistical test package that estimates the variance-mean dependence in count data from high-throughput sequencing assays and tests for differential expression based on a model using negative binomial distribution (54). The *DESeq2* package is available at http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html. In this context, it

TABLE 1 Eight fungal families with the highest number of representative OTUs[a]

Taxonomy (family)	*P* value	No. of OTUs (genus or species level)
Davidiellaceae	7.41×10^{-6}–5.18×10^{-5}	17
Pleosporaceae	3.69×10^{-15}–2.40×10^{-5}	14
Cystofilobasidiaceae	3.29×10^{-9}–2.08×10^{-5}	13
Ganodermataceae	5.43×10^{-5}–1.15×10^{-5}	10
Meruliaceae	9.28×10^{-6}–2.22×10^{-3}	20
Polyporaceae	3.60×10^{-3}–3.85×10^{-2}	15
Psathyrellaceae	1.91×10^{-2}–1.28×10^{-2}	7
Ophiocordycipitaceae	1.84×10^{-2}–1.15×10^{-2}	8

[a]Shown are fungal families with the highest number of representative OTUs and the range of their adjusted *P* values, representing the statistical significance of their differential abundance between the groups of samples. The samples were separated according to the concentration protocol (centrifugation and filtration).

was used to test for differences in OTU abundance between groups of samples. The samples were grouped into two categories based on concentration method: filtration and centrifugation. To ensure the test was appropriate for these data, a diagnostic plot was generated to validate the fit line on the dispersion plot. The results showed that the data fit with the dispersion values when plotted against the mean of the normalized counts (Fig. S5). The detailed output was in the form of an OTU text file containing a list of all the OTUs in the raw input matrix and their taxonomic identification, along with their associated statistics. It also included *P* values representing the statistical significance of the differential abundance in the group of samples (Data Set S1). After a detailed analysis, eight families of fungi had significantly different abundances of OTUs between the centrifuged and filtered samples. The OTUs that were associated with unidentified taxa were not analyzed. Table 1 lists the eight fungal families and the range of adjusted *P* values obtained for their representative OTUs. The differential abundance was performed to the species level (whenever possible) or at least to the genus level. The detailed analyses refer to the fact that we scrutinized the OTUs that had the highest significant $\log_2$ fold change in the differential abundance analyses, and we summarized the findings to the family level to make it easier to visualize. In other words, the families that are listed in Table 1 had the highest number of OTUs (identified to the genus or species level) that had a significant differential abundance.

(vi) Taxonomic distribution. To study differences in taxa between the centrifuged and filtered samples at the genus level, each sample was analyzed individually. Based on the OTUs, a list of genera was identified for each sample. Fifty taxa were identified in the 16 samples from the filtration protocol group, all of which were present in every single sample. Samples treated with the centrifugation protocol showed a different distribution. The most striking difference between samples from the two different protocols was that there were nine fungi not detected in any samples from the centrifuged group that were present in every filtered sample. The taxonomic distribution is described in Fig. 4. Briefly, 6 fungi were detected in 26% of the centrifuged samples, 18 fungi were detected in 40% of the centrifuged samples, and 17 fungi were detected in more than half of the centrifuged samples.

A literature review on the presence of hydrophobins or oil droplets in the 7 fungal genera that were constantly absent when using the centrifugation protocol is presented in Table 2. The majority of studies have reported the presence of oil droplets by direct observation of fungal cells using microscopy (*Davidiella*, *Alternaria*, *Fusarium,* and *Capnobotryella*). In some cases, oil droplets and lipid production were assessed by chromatography profiles (*Epicoccum* and *Cryptococcus*). For *Botrytis*, the presence of hydrophobin was confirmed by analyzing gene expression during the sexual development of the cells.

Laboratory settings. Strains of *Aspergillus niger* and *Penicillium roquefortii* grown in laboratory settings were tested for loss due to centrifugation, as observed for fungal species with environmental field samples. Spores of wild-type *A. niger* did not pellet upon centrifugation at 1,700 × *g* when harvested in H_2O on 14 March 2018 (calculated

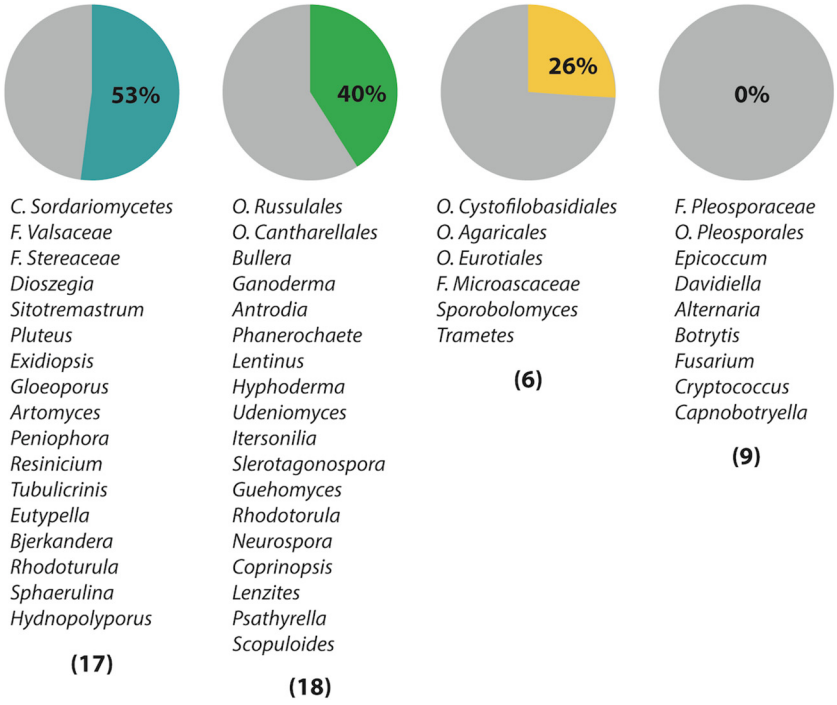

FIG 4 Distribution of fungi detected in the centrifuged samples. All 50 fungi listed were present in 100% of the filtered samples. Each pie represents the percentage of detection of the taxa listed under the centrifuged samples. The taxa listed under each pie chart were present in the percentage of centrifuged samples shown above. Numbers in parentheses represent the number of taxa listed above. C, class; O, order; F, family.

indoor relative humidity [RH], 30%; Table S1). However, spores did pellet after they had been in contact with aluminum. This indicates that static electricity prevented the spores from pelleting (data not shown). To expand on this result, tubes were rubbed with wool, creating a charged surface; this is the so-called triboelectric effect. Spore concentration was severely reduced by transferring the spores of *A. niger* wild-type and Δ*pptA* strains and *P. roquefortii* via a charged 15-ml polypropylene tube (Fig. 5). Addition of 150 mM NaCl did not alleviate the spore loss. These data indicate that spores had adsorbed to the charged surface of the 15-ml tubes mainly due to the triboelectric effect. In the next set of experiments, we counted spore loss (Table S2). This time, no effect was observed for the transfer via a charged 15-ml tube, possibly due to a higher RH (i.e., 48 and 56%) on the days of the experiments (May 15 and 23; Table S1). In the case of wild-type *A. niger*, about 50% of the spores were not recovered, explained by adsorption to the wall of the microtube during centrifugation. Notably, about 90% and 98% of the *A. niger* Δ*pptA* strain and *P. roquefortii* spores, respectively, were not recovered. These data show that spores adsorb to the wall of the tube during centrifugation and that melanin is not the cause of this effect. Spores may also adsorb to the tube wall during transfer, but this requires static electricity.

TABLE 2 Reported presence of hydrophobins or oil droplets in the seven fungal genera that were absent from the case of centrifugation

Fungal genus	Characteristic(s)	Reference(s)
Epicoccum	Production of fungal dye oil in water emulsions/hydrophobins absent	87, 88
Davidiella	Presence of small oil droplets in fungal cells/hydrophobins absent	89
Alternaria	Presence of numerous oil droplets in the vegetative hyphae/hydrophobins absent	90
Botrytis	Expression of hydrophobin genes in sexual development of fungal cells	91
Fusarium	Observation of oil droplets surrounding wild-type mycelium/hydrophobins present	92, 93
Cryptococcus	Microbial lipid production by fungal cells in different culture conditions/hydrophobins absent	94
Capnobotryella	Observation of oil droplets in fungal cells/hydrophobins absent	95

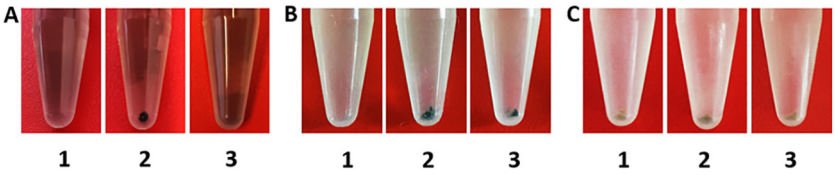

FIG 5 Triboelectric effect on spore pelleting of *A. niger* N402 (A), *P. roquefortii* FM164 (B), and *A. niger* Δ*pptA* strain (C) after centrifugation for 10 min at 1,700 × *g* in an Eppendorf fixed-angle rotor. Spores in H_2O (lanes 1 and 2) or 150 mM NaCl (lane 3) were added to a 1.5-ml tube either before (lanes 1 and 3) or after (lane 2) the transfer via a charged 15-ml tube.

The effect of adsorption to the wall of the tube during centrifugation was further assessed by using a fixed-angle, swing-out rotor. Fixed-angle rotors caused more spore loss due to the *g* force that brings the spores in close contact with the side walls of the tube (Fig. S4).

DISCUSSION

This study demonstrates an underestimation of fungal load and diversity in aerosol samples due to centrifugation steps prior to DNA extraction and proposes a new method to overcome this bias. The idea that fungal cells may be lost during the centrifugation step is supported by the water-repellent nature of fungi related to fungal hydrophobins and hydrophobin-like proteins (55). Fungal hydrophobins are secreted proteins that fold into amphipathic membranes when fungal cell walls come in contact with water, oil, or hydrophobic solid surfaces (56). This is the case when spores are produced on aerial reproductive structures such as conidiophores. The hydrophobic nature makes spores collect on the water surface or attach to the hydrophobic surface of plastic, such as that of Eppendorf tubes. After the centrifugation step, the supernatant, which contains centrifugation-resistant fungal cells, is discarded, resulting in an overall loss of spores. Another explanation of loss of spores after centrifugation is the presence of lipid droplets (LDs). These dynamic intracellular organelles can contain neutral lipids like triacylglycerols (TAGs) and sterol esters (SEs) (57–59). Apart from lipid storage, their roles have been extended to lipid metabolism and energy homeostasis, and their dysfunction is linked to many human diseases (60). LDs are ubiquitous in eukaryote and prokaryote cells (61, 62). In fungi, they are believed to support growth and propagation of mycelia and are implicated in fungal spore development (63). However, when and where the LDs are produced in fungal cells is still unclear. Their presence in the fungal cell could be an explanation for the loss of certain fungi in the supernatant during the centrifugation protocol by lowering cell density. The newly developed filtration-based protocol circumvents this issue, as all the liquid is filtered and its contents are fully recovered before the DNA extraction.

Using a next-generation sequencing approach, we aimed to characterize the fungal cell loss caused by the centrifugation protocol. Assessments of airborne fungal diversity in various environments have been the focus of several recent studies (64–66). There is an obvious need to document the effect of fungal cell loss due to centrifugation and to highlight the necessary precautions when working with fungi from aerosols sampled in liquid and used with molecular techniques/methods. As expected, the fungal cell loss was not limited to *Aspergillus* and *Penicillium* species. The Chao1 richness index and the Shannon/Simpson diversity indexes yielded results similar to those obtained by comparing the observed OTUs in Fig. 4. Centrifugation clearly affects the diversity/richness of fungal bioaerosols. These results are consistent with the qPCR quantification of *Aspergillus* and *Penicillium* from the same samples. Overall, the filtration protocol allowed for a better recovery of specific fungi (*Aspergillus* and *Penicillium*) and for a greater diversity (number of OTUs and Chao1 and Shannon/Simpson indexes).

Taxonomic analyses demonstrated very compelling results, as 100% of taxa detected by centrifugation were also detected by filtration. In contrast, taxa identified by filtration were present in portions of centrifuged samples ranging from 0% to 53%.

Although most of the identified fungal cells are filamentous and may be considered hydrophobic, the degree may vary from mildly to highly hydrophobic (67). This could explain the observed variations and losses among taxa depending on the concentration protocol used. For example, *Aspergillus fumigatus* conidia are considered to be highly hydrophobic compared to those of other fungi, such as *Cladosporium* (68). This can impact the efficiency of spore dispersion and, in this case, their behavior in a liquid/surface environment. Wösten et al. (43) described clearly how a fungus escapes the water to grow in the air and how this behavior is linked to the hydrophobicity of fungal cells. Hydrophobins have been identified in most, if not all, filamentous ascomycetes and basidiomycetes. However, they are absent from yeasts and other phyla of the fungal kingdom. It was shown that in the dimorphic fungus *Ustilago maydis*, surface hydrophobicity is not caused by hydrophobins but by hydrophobin-like proteins called repellents (46). This may also be the case for other yeasts or nonascomycete or nonbasidiomycete species. Spores with a wettable surface could be lost during the centrifugation procedure due to lipid droplets that lower the density of the cell. In contrast, some fungi may be preferentially centrifuged compared to other fungi due to their wettable nature or the absence of lipid droplets. The hypothesis of fungal cell loss due to hydrophobicity caused by hydrophobin and/or oil droplets was supported by previous studies reporting the presence of hydrophobins or oil droplets in the seven fungal genera identified in this study as the ones that are the most affected by centrifugation.

The *in vitro* laboratory experiment confirmed the loss of fungal spores observed during centrifugation of environmental air samples. Another mechanism of spore sorption acts via electrostatic interactions. Spores of *A. niger* have both a hydrophobic nature and a surface charge (69). Addition of salt reduces the long-distance electrostatic interactions, enabling the short-distance hydrophobic interactions with the microtube to occur. However, addition of salt did not increase the incidence of sorption to the wall, implying that the long-range electrostatic interactions are low compared to those of the short range of hydrophobic interactions. The presence of melanin also did not increase spore sorption. In fact, more spores adhered to the wall of the tube in a strain not producing melanin, likely explained by the fact that the Δ*pptA* spores are still hydrophobic (our unpublished results). Melanin contributes to spore surface characteristics in some fungal species, like *A. niger*. Thus, it is believed that melanin could play a role in the initial aggregation/adhesion of spores to pellet.

Applying the standard centrifugation protocol when studying fungal bioaerosols may lead to an underestimation of some species, including the taxa identified in this research. In certain studies fungal load has been underestimated using the centrifugation method (47–49). Studies using settled dust sampling (via an electrostatic dust collector [EDC]) that also require handling samples in liquid buffers and centrifugation may also be affected by fungal loss. In addition to being underestimated in diversity and exposure studies, these fungi may represent greater health risks because of the characteristics associated with their hydrophobicity. In this context, a study showed that a hydrophobin layer may affect immune recognition of fungi (70). Of the nine taxa not detected by the centrifugation protocol, some are quite common, including *Fusarium*, *Cryptococcus,* and *Alternaria*. *Fusarium* species are responsible for a broad range of health problems, from local and systemic infections to allergy-related diseases, in immunodepressed individuals (71). Lipid droplets seem to have a particular role in *Fusarium* species virulence (72, 73). Also, lipid droplets in *Cryptococcus* affect the host-pathogen interaction (74).

Further research can help us better understand fungal aerosol behavior. Future *in vitro* studies could be extended to include all the taxa identified in this research to determine if *in vitro* samples constantly yield the same results as environmental samples. Additionally, during the centrifugation protocol, the pellet and supernatant could be used for lipid droplet identification. It would confirm the hypothesis of fungal taxon loss during centrifugation due to lipid droplet production.

Conclusions. Although this paper seems to be a collection of three different studies with many variables, the storyline and the controls used tells the story of a particular fungal cell behavior when retrieved from air and concentrated using liquid medium. Here, the three environments studied represent biological replicates of the observed tendency of the fungal cell behavior. Even more striking, the same conclusions were drawn when the experiment was repeated in a laboratory setting using strains cultured in medium instead of air samples collected from the field. The data presented in this research provide a unique framework for understanding the role of the concentration protocol prior to DNA extraction on fungal composition obtained from bioaerosol samples. We believe that this work identified the issue of fungal cell loss when recovered from air samples and propose an alternative method to better evaluate fungal exposure and diversity. Based on the results of this investigation, the newly developed filtration protocol should be used to achieve the highest possible fungal cell recovery from air samples.

MATERIALS AND METHODS

Field work. (i) Environmental field samples. The samples used for this study were collected as part of two other different studies. The common goal was to assess occupational exposure to bioaerosols. Thus, the sampling sites were chosen according to workers' activities. Below is a summary of the field sampling methods.

(a) Compost. The compost samples come from a year-long sampling schedule of compost piles from three different composting plants in order to monitor their composting processes. Each plant treats different raw materials: household green waste (domestic), manure and hay (vegetal), and pig carcasses and placenta (animal). All of the composting plants were located in the province of Quebec, Canada. Outdoor temperature varied from 7°C to 10°C during spring visits, 19°C to 28°C during summer, and 1°C to 5°C during the fall visit. Sampling took place in the morning and could last until 1 pm, depending on workers' activity. At each visit, three samples were taken in the beginning, middle, and end of the working shifts. Detailed information about the sampling schedule is presented in the original composting study report (5).

(b) Biomethanization. Samples were collected from two different facilities as a part of a biomethanization study. One facility (BF1) processes primary and secondary sludge from wastewater treatment plants as well as industrial waste. The second one (BF2) handles municipal waste from domestic sources. Both facilities were visited once during summer and once during winter. Five sites were sampled in the first BF and two sites in the second one. At each site, three samples were taken in the beginning, middle, and end of the process. Samples were then pooled to obtain a final volume of 45 ml for each site sampled. The time of the sampling campaign was dependent on workers' activity and occurred between 8 am and 4 pm. During the summer sampling time, temperature ranged from 22°C to 26°C. During winter, temperatures ranged between 18°C and 23°C. Detailed information about the sampling sites can be found in to the original study report (6).

(ii) Air sampling. A liquid cyclonic impactor (Coriolis μ; Bertin Technologies, Montigny-le-Bretonneux, France) set at 300 liters/min for 10 min was used to collect bioaerosols for this study. Fifteen milliliters of sterile 5 mM phosphate-buffered saline (pH 7.4) was used to fill the sampling cone of the Coriolis. In both environments and in all sampling sites, the sampler was placed within 1 to 2 m from the source.

(iii) Culture approach. One milliliter of the 15-ml Coriolis sampling liquid (5 mM phosphate-buffered saline) was used to perform a serial dilution from 10^0 to 10^{-4} concentration/ml. The dilutions were made using a saline and Tween 20 solution and were performed in triplicate. One hundred microliters of each triplicate was plated on Rose Bengal medium with chloramphenicol at a concentration of 50 μg/ml. Half of the petri dishes were incubated at 25°C for mesophilic mold growth and the other half at 50°C for thermophilic mold growth, specifically *Aspergillus fumigatus*. After 5 days of incubation, molds were counted and the counts were translated into CFU per square meter. The concentrations were obtained after calculating a mean count of the triplicate plated dilutions. *Aspergillus fumigatus* was identified according to specific macroscopic and microscopic features. Spores were observed with a Leitz Laborlux S microscope and lactophenol blue staining.

(iv) Fungal cell concentration prior to DNA extraction. *(a) Standard centrifugation protocol*. In this paper, the centrifugation protocol is referred to as the standard because of its use in most prior bioaerosol studies, including molecular biology approaches. Each sample was divided into three aliquots of 1.5 ml each, which were centrifuged for 10 min at 14,000 $\times$ *g*. The supernatant was discarded and the pellets were kept at −20°C until DNA extraction.

(b) Newly developed filtration-based protocol. Ten-milliliter aliquots of Coriolis suspension were filtered through a 2.5-cm polycarbonate membrane (0.2-mm pore size; Millipore) using a vacuum filtration unit. The filters were placed in a 1.5-ml Eppendorf tube with 750 μl of the extraction buffer (bead solution) from a MoBio PowerLyser Powersoil isolation DNA kit (Carlsbad, CA) and a tungsten bead with a diameter of 0.3 cm. The filters were flash-frozen by placing the Eppendorf tube in a 99% ethanol solution and dry ice. The frozen filters next were pulverized using tungsten steel beads in an Eppendorf tube in a bead-beating machine (Mixer Mill MM301; Retsch, Düsseldorf, Germany) at a frequency of 20 revolutions

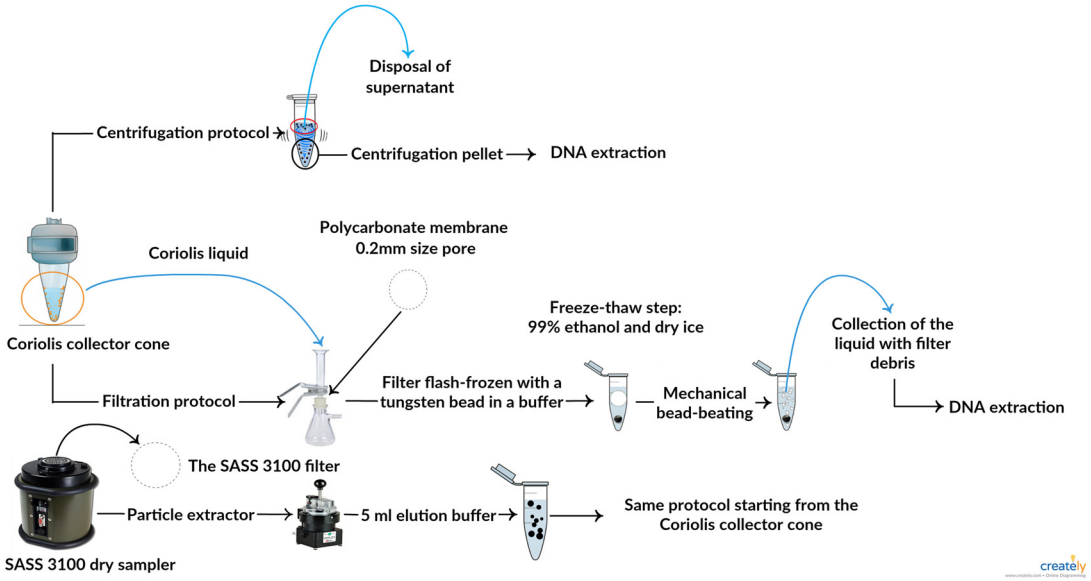

FIG 6 Diagram of the fungal cell concentration protocols prior to DNA extraction. The diagram shows each step from sampling to DNA extraction. The red circle shows the hypothesis of fungal cell loss during the disposal of the supernatant in the centrifugation protocol. The SASS 3100 dry sampler was used as a control condition (Fig. S1 to S3). After particle collection on the electret filter, a SASS 3010 particle extractor is required to elute the captured particles in a buffer. The particles are trapped in the filter via electric charges, and the use of the buffer changes the charges of the particles, which are collected in the liquid buffer.

per s for 20 min. An aliquot of 1.5 ml of the liquid containing the pulverized filter particles was used for the first step of the DNA extraction kit (Fig. 6).

(v) DNA extraction. A second bead beating using glass beads was conducted at a frequency of 20 revolutions per second for 10 min. A MoBio PowerLyser Powersoil isolation DNA kit next was used to extract the total genomic DNA from the samples by following the manufacturer's instructions. After DNA elution, samples were stored at −20°C until subsequent analyses.

(vi) Real-time PCR quantification. A Bio-Rad CFX 96 thermocycler (Bio-Rad Laboratories, Mississauga, Canada) was used for DNA amplification. The PCR mixture contained 2 μl of DNA template, 0.150 μmol/liter each primer, 0.150 μmol/liter probe, and 7.5 μl of 2× QuantiTect probe PCR master mix (QuantiTect probe PCR kit; Qiagen, Mississauga, Ontario, Canada) in a 15-μl reaction mixture. Standard curves were made with a strain of *Aspergillus fumigatus* (isolated from an environmental sample and identified with key identification tools). After extraction of the genomic DNA, a NanoDrop spectrophotometer (Thermo Scientific, MA, USA) was used to quantify the concentration of the extracted DNA. Dilutions from 10^6 to 10^0 then were used in triplicates for the standard curve calculations. The results were analyzed using Bio-Rad CFX Manager software, version 3.0.1224.1015 (Bio-Rad Laboratories), including efficiency and R^2 of the standard curve slope. Table 3 lists the primers, probes, and PCR protocol used for this study.

(vii) High-throughput sequencing. Amplification of the fungal ITS1 region, equimolar pooling, and sequencing were performed at the Plateforme d'Analyses Génomiques (IBIS, Université Laval, Quebec City, Canada). Briefly, amplification of the ITS1 region was performed using the sequence-specific regions (75 and references therein), using a two-step dual-indexed PCR approach specifically designed for Illumina instruments. In the first step, the gene-specific sequence was fused to the Illumina TruSeq sequencing primers, and PCR was performed on a total volume of 25 μl containing 1× Q5 buffer (NEB), 0.25 μM each primer, 200 μM each deoxynucleoside triphosphates, 1 U of Q5 high-fidelity DNA polymerase (NEB), and 1 μl of template cDNA. The PCR started with an initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 10 s, extension at 72°C

TABLE 3 Primers and probes used for qPCR quantification of selected microorganisms

Microorganism[a]	Primers and probe[b]	PCR protocol
Penicillium, *Aspergillus*, and *Paecilomyces variotii*	PenAsp1mgb (Taqman); PenAspR1, 5′-GCCCGCCGAAGCAAC-3′; PenAspF1, 5′-CGGAAGGATCATTACTGAGTG-3′; PenAspP1mgb, 5′-FAM-CCAACCTCCCACCCGTG-TAMRA-3′	Activation, 94°C for 3 min; denaturation, 94°C for 15 s; annealing/extension, 60°C for 60 s; cycles, 40
Aspergillus fumigatus and *Neosartorya fischeri*	Afumi (Taqman); AfumiR1, 5′-CCGTTGTTGAAAGTTTTAACTGATTAC-3′; AfumiF1, 5′-GCCCGCCGTTTCGAC-3′; AfumiP1, 5′-CCCGCCGAAGACCCCAACATG-3′	Activation, 94°C for 3 min; denaturation, 94°C for 15 s; annealing/extension, 60°C for 60 s; cycles, 40

[a]For all organisms, refer to http://www.epa.gov/microbes/moldtech.html.
[b]FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

for 30 s, and a final extension at 72°C for 2 min. The PCR was purified using the Axygen PCR cleanup kit (Axygen, New York, NY). The quality of the purified PCR products was checked on a 1% agarose gel. A 50- to 100-fold dilution of this purified product was used as a template for a second PCR step with the goal of adding barcodes (dual-indexed) and missing sequences required for Illumina sequencing. Cycling for the second PCR was identical to that for the first PCR but with 12 cycles. PCRs were purified as described above, checked for quality on a DNA7500 Bioanalayzer chip (Agilent), and then quantified spectrophotometrically with a NanoDrop 1000 (Thermo Fisher Scientific). Barcoded amplicons were pooled in equimolar concentrations for sequencing on the Illumina MiSeq. The oligonucleotide sequences that were used for amplification are the following: first-PCR primers, ITS1Fngs (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGCGTTCTTATCGATGC-3′); second-PCR primers, generic forward (5′AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGAC-3′) and generic reverse (5′CAAGCAGAAGACGGCATACGAGATGTGACYGGAGTTCAGACGTGT-3′). Note that primers used in this work contain Illumina specific sequences protected by intellectual property (oligonucleotide sequences © 2018 Illumina, Inc., all rights reserved).

(viii) Bioinformatics workflow. Briefly, after demultiplexing the raw FASTQ files, the reads generated from the paired-end sequencing using mothur v 1.35.1 were combined (76). Quality filtering was performed using Mothur, discarding reads with ambiguous sequences. Reads shorter than 100 bp and longer than 450 bp were also discarded. Similar sequences were gathered together to reduce the computational burden, and the number of copies of the same sequence was displayed. This dereplication step was performed using USEARCH (version 7.0.1090) (77). The selected region of fungal origin was then extracted from the sequences with ITSx, which uses HMMER3 (78), to compare input sequences against a set of models built from a number of different ITS region sequences found in various organisms. Only the sequences belonging to fungi were kept for further analyses. Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE (version 7.1) (79). The similarity threshold (97%) is commonly used in all OTU-based analyses and was shown to be optimal when using ITS for fungal identification (80). UCHIME was used to identify and remove chimeric sequences (81). QIIME (version 1.9.0) (82) was used to assign taxonomy to OTUs based on a UNITE fungal ITS reference training data set for taxonomic assignment and to generate an OTU table. The microbial diversity analysis conducted in this study was achieved by using QIIME commands described in the QIIME scripts (http://qiime.org/scripts/).

Laboratory experiments. (i) Strains and culture conditions. Wild-type *A. niger* strain N402 (83) and wild-type *P. roquefortii* strain FM164 (also known as LCP6094) (84) were grown in 20 ml minimal medium (MM) (85) with 2% glucose and 1.5% agar at 30°C and 25°C, respectively. The melanin-deficient *A. niger* Δ*pptA* strain was grown in 20 ml MM–siderophore spent medium (50/50) with 2% glucose, 20 mg/ml L-leucine, and 1.5% agar (86). Plates were inoculated by spreading conidia confluently over the agar surface. After 7 days, conidia were harvested with 10 ml H_2O per plate and filtered over Miracloth (Millipore). Spores were counted in a 100-fold dilution in saline–Tween 80 (0.9% NaCl, 0.005% Tween 80) (S/T). Accordingly, spore suspensions were brought to a final number of 1×10^8/ml. Spores were used in experiments within 2 h after harvesting at a concentration of 1×10^7/ml H_2O or 150 mM NaCl.

(ii) Centrifugation analysis. The effect of triboelectricity was assessed qualitatively by charging polypropylene tubes by rubbing with wool. Charging was confirmed by attraction of a slip of paper to the tubes. Two aliquots of 1 ml were transferred via a charged 15-ml polypropylene tube (62.554.502.PP; Sarstedt) to a 1.5-ml polypropylene microtube (72.690.001; Sarstedt), while another aliquot was directly added to the microtube. Spores were centrifuged for 10 min at 1,700 × *g* at 4°C in an fixed-angle Eppendorf rotor. The experiments were performed on the 3rd (*A. niger* N402), 8th (*P. roquefortii* FM164), and 9th (*A. niger* Δ*pptA* strain) of May 2018. In the next set of experiments, the effect of triboelectricity was assessed quantitatively by performing the same experiments on the 15th (*A. niger*) and 23rd (*P. roquefortii*) of May 2018. The supernatant was carefully pipetted off by first taking the upper 900 μl followed by the lower 100 μl. The pellet was resuspended in 100 μl H_2O by pipetting and transferred to a clean microtube. Spores were counted using a hemocytometer. When necessary, 10- or 100-fold dilutions were made using S/T.

(iii) Statistical analysis. Descriptive statistics were used on qPCR data to highlight significant differences. The normality was verified by the D′Agostino and Pearson omnibus normality test. The normality assumption on data was not fulfilled. Nonparametric Mann-Whitney *U* test (two-tailed) analyses were performed to highlight significant differences showing a *P* value of ≤0.05. All results were analyzed using the software GraphPad Prism 5.03 (GraphPad Software, Inc.).

For sequencing data, the differences in the observed OTUs shown with boxplots were also analyzed with nonparametric Mann-Whitney *U* test using the software GraphPad Prism 5.03 (GraphPad Software, Inc.). *Deseq2* statistical tests were used to determine the statistical significance of the differential abundance of OTUs across samples. *Deseq2* test was used as part of the QIIME script for differential abundance analyses. Detailed information about the performance of the test is presented in the differential abundance section of Results. Outdoor control samples were excluded from the analyses due to the low number of sequences and the low number of OTUs exhibited. This way, the rarefaction depth is high, and we eliminate the possible biases related to samples clustering according to the number of sequences rather than diversity.

Data availability. Raw sequence reads of every sample used in this study and that support its findings have been deposited with the National Center for Biotechnology Information (NCBI) under BioProject identifier PRJNA450069.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02941-18.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.4 MB.

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We have no competing financial interests to declare.

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