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METHODS

Bioaerosol Sampler Choice Should Consider Efficiency and Ability of Samplers To Cover Microbial Diversity

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ABSTRACT Bioaerosol studies aim to describe the microbial content and increase understanding of the aerosolization processes linked to diseases. Air samplers are used to collect, identify, and quantify bioaerosols. Studies comparing the performances of air samplers have typically used a culture approach or have targeted a specific microorganism in laboratory settings. The objective of this study was to use environmental field samples to compare the efficiencies of 3 high-airflow-rate samplers for describing bioaerosol diversity using a next-generation sequencing approach. Two liquid cyclonic impactors and one electrostatic filter dry sampler were used in four wastewater treatment plants to target bacterial diversity and in five dairy farms to target fungal diversity. The dry electrostatic sampler was consistently more powerful in collecting more fungal and bacterial operational taxonomic units (OTUs). Substantial differences in OTU abundances between liquid and dry sampling were revealed. The majority of the diversity revealed by dry electrostatic sampling was not identified using the cyclonic liquid impactors. The findings from this work suggest that the choice of a bioaerosol sampler should include information about the efficiency and ability of samplers to cover microbial diversity. Although these results suggest that electrostatic filters result in better coverage of the microbial diversity among the tested air samplers, further studies are needed to confirm this hypothesis. While it is difficult to determine a single universally optimal air sampler, this work provides an in-depth look at some of the considerations that are essential when choosing an air sampler for studying the microbial ecology of bioaerosols.

IMPORTANCE Associating bioaerosol exposure and health problems is challenging, and adequate exposure monitoring is a priority for scientists in the field. Conclusions that can be drawn from bioaerosol exposure studies are highly dependent on the design of the study and the methodologies used. The air sampling strategy is the first methodological step leading to an accurate interpretation of what is present in the air. Applying new molecular approaches to evaluate the efficiencies of the different types of samplers used in the field is necessary in order to circumvent traditional approaches and the biases they introduce to such studies. The results and conclusions provided in this paper should be taken in consideration when conducting a bioaerosol study.

KEYWORDS air samplers, sampling biases, microbial diversity, next-generation sequencing

The microbial flora of aerosols, referred to as bioaerosols, consists of a combination of viable and nonviable microorganisms (e.g., bacteria, fungi, and viruses) and antigenic compounds of biological origin (e.g., animal and plant debris, endotoxins,

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toxins, proteins, and any other microbial metabolites) (1–3). From natural processes to industrial activities, microbes and their components can be aerosolized from any given source (e.g., humans, soil, and water) (4–6). Bioaerosols are recognized as one of the main transmission routes for infectious diseases (7–10) and are responsible for various types of health problems resulting from their inhalation and potential ingestion. Acute respiratory infections are the leading cause of death above all other infectious diseases (11). The dispersal of bioaerosols has a major impact on public health due to the presence of highly diverse and dynamic microbial communities in the air of urban and rural environments. Human exposure to bioaerosols is associated with a wide range of acute and chronic diseases ranging from allergies to asthma, rhinitis, sinusitis, and bronchitis. Bioaerosols also contribute to the dispersal of pathogens and other health effects due to occupational exposure (12–16).

Studying a link between bioaerosol exposure and health problems is challenging; therefore, adequate exposure monitoring is a top priority for aerosol scientists. Occupational exposure studies using only culture-based tools do not represent the overall microbial diversity found in bioaerosols. Those studies underestimate the large spectrum of microbes in bioaerosols, and therefore, the identification of new airborne etiological agents is inadequate. In 2010, Peccia and collaborators (17) highlighted the importance of incorporating DNA sequencing methods into the study of aerosol science. Application of these methods to samples from different environments allows for the identification and quantification of the microorganisms present and for a better understanding of human exposure. A next-generation sequencing (NGS) approach offers a thorough picture of the microbial content of a sample, leading to millions of sequences generated from a single sample (18–22).

Applying molecular methods to bioaerosols requires some modification of current protocols for optimum genome recovery. For example, using filtration instead of centrifugation of fungal cells from air samples prior to DNA extraction procedures leads to a better recovery yield and a more accurate description of fungal diversity using quantitative PCR (qPCR) and NGS approaches (our unpublished data).

Bioaerosol studies rely on representative air sampling regimes as a first step to adequately capture the microbial flora of aerosols in a particular area. Various aerosol collectors have been developed to capture and analyze airborne particulate matter for airborne pathogen detection and quantification and for biodiversity determination. The currently available samplers operate in one of two main ways, by using filtration or gravitational/inertial forces (23–25). In molecular biology studies, high-airflow samplers are the most used, as low-airflow samplers lead to low biomass concentrations that may alter molecular analyses. In filtration samplers, aerosols are collected using filters made from different types of materials, and then microbes are removed from the filters using an elution buffer (26). It is necessary to elute the particles to be analyzed from the filter to avoid any inhibition from filter materials in downstream molecular analyses (27). Of the samplers using gravitational/inertial forces, one of the most common types used in bioaerosol studies involves cyclones, where particles are directly impacted from the air into a liquid (28). After the microorganisms are concentrated in the liquid, molecular methods can be applied to assess microbial diversity (29). Electrostatic samplers can also be used to collect bioaerosols. Using these samplers, particles are collected due to their charge after passing through an electrostatic field (30). After being eluted, particles can be analyzed using multiple molecular techniques.

Aerosol samplers collect particles using a wide range of variables, such as collection size range, airflow rate, collection volume, and physical properties, that can all put stress on the microbes collected. Environmental factors, such as temperature, relative humidity, and UV radiation, coupled with different aerosol sources, influence the behavior and fate of the aerosolized particles. In addition, the nature and concentration of microorganisms, whether or not they are pathogenic, their relative resistance to stress, and the types of particles in which they are contained must all be considered when choosing the appropriate bioaerosol sampling method (31–35).

Since the emergence of bioaerosol science, studies comparing the performances of

air samplers have typically used a culture approach or have targeted a specific microorganism. Furthermore, the majority of these studies have used laboratory settings to create bioaerosols and scrutinize the performance of the samplers (31, 32, 36–39). One study also compared high-volume portable bioaerosol samplers on field environments (indoor and outdoor) using culturable airborne microorganisms, in addition to using laboratory settings to compare the samplers (36). Zhen and collaborators investigated the effect of air sampling stress on the measurement of 16S rRNA genes for bioaerosols in the laboratory and field experiments using qPCR and NGS with two devices (Button Aerosol Sampler and BioSampler [40]). The SKC BioSampler is one of the most commonly used samplers in comparison studies (41–47). As a high-volume aerosol collection tool, the Coriolis μ air sampler (Bertin Technologies, Montigny-le-Bretonneux, France) is also frequently used for comparison purposes (48–50). Very few studies included the high-airflow-rate liquid impingement SASS 2300 (Research International, Monroe, WA, USA) or the high-airflow-rate electrostatic filter SASS 3100 (Research International) in their comparisons (39, 47, 51).

The objective of this study was to use environmental field samples to compare the efficiencies of three high-airflow-rate samplers for describing bioaerosol diversity using a next-generation sequencing approach. A bioinformatics workflow was used for multivariate analysis, differential abundance, statistical analyses, and taxonomy identification. Samples collected from wastewater treatment plants were targeted for bacterial diversity, and samples from dairy farms were targeted for fungal diversity. It is necessary to combine the microbial biodiversity information obtained from samples with the physical processes of the samplers to collect those samples to make wiser choices on the type of samplers used. This paper provides new insights on the biases introduced by the use of specific air samplers and aims to suggest sampling strategies that lead to the broadest microbial diversity.

RESULTS

Summary of data processing. Sequencing the genomes of 15 aerosol samples from dairy farms yielded 786,705 sequences. After quality screening and discarding singletons, 21,802 unique sequences clustered onto 1,657 operational taxonomic units (OTUs). For wastewater treatment aerosol samples, sequencing the genomes of 48 samples led to 1,528,010 sequences. After quality screening and discarding singletons, 74,768 unique sequences clustered into 2,006 OTUs.

Rarefaction curves. To validate the sequencing depth and to confirm the effective sampling of the biological content of the aerosol samples collected at the dairy farms and the wastewater treatment plants, a rarefaction analysis was performed using the observed OTU alpha-diversity metric. The lowest-depth sample parameter was used to choose the sequencing depth for the rarefaction analyses. In other words, samples with a lower sequencing depth than the one chosen were excluded from the analyses. The higher the sequencing depth, the more likely the real diversity of the sample will be attained. In this case, the sequencing depth was 50,000 sequences per sample for dairy farm (DF) samples and 10,000 sequences for wastewater treatment (WWT) samples. All of the samples were included in the analyses, except the outdoor controls due to low sequence numbers. The points shown in Fig. 1 (DF) and 2 (WWT) were calculated as follows: 10 values from 10 to 50,000 sequences for DF samples and from 10 to 10,000 sequences for WWT samples were randomly selected. For each of these values, the corresponding number of OTUs observed was noted for all of the samples. Then, the average number of OTUs observed $\pm$ the standard deviation was calculated for each of the 10 values. To analyze the rarefaction data, samples were grouped according to the type of sampler used. The form of the curves in Fig. 1 and 2 indicates an efficient coverage of the fungal and bacterial diversity, respectively, as no more diversity was observed even with greater numbers of sequences per sample.

Alpha diversity as measured by richness and diversity indexes. The measurement of species diversity was first introduced by Whittaker and defined as the number of species and their proportion within one sampling site (52). There are different ways

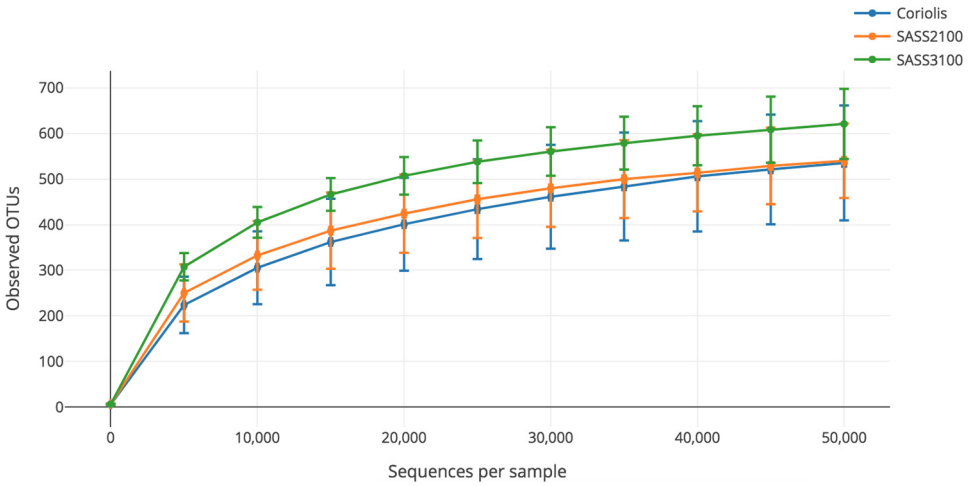

FIG 1 Rarefaction curves obtained from the number of observed OTUs and the sequences per sample for air samples collected from the five DFs visited. Samples were grouped according to the type of sampler used. An average of the OTUs observed in each sample was calculated for all the samples ± the standard deviation.

to measure alpha diversity depending on the context of the study. A list of indexes is presented in a book by Magurran and McGill (53) and was used to help make an informed choice about which indexes to apply to the data set in this study.

In this study, the following four indexes were used to measure alpha diversity: Chao1, observed OTUs, Shannon, and Simpson. More specifically, Chao1 is a richness estimator. The higher the number of different OTUs in a sample, the higher the value of the Chao1 index. For Shannon and Simpson indexes, the species richness is combined with the abundance to give one diversity measure. The Simpson index represents the probability of two randomly selected OTUs from the same sample being the same species. The output values are bounded between 0 and 1, where 1 represents the highest diversity. Shannon output values are bounded between 0 and 10, where 10 represents the highest diversity. All diversity measures were obtained using the alpha-diversity QIIME script.

The four diversity indexes were used to compare the diversity of the microbial communities in bioaerosol samples collected by three different high-flow-rate air samplers. Figures 3 and 4 show the results of the comparisons of samples from DFs and

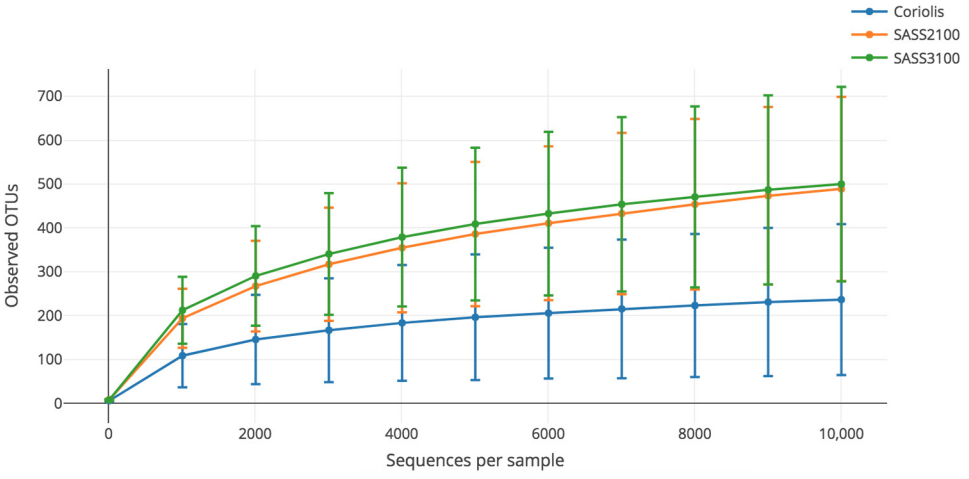

FIG 2 Rarefaction curves obtained from the number of observed OTUs and the sequences per sample for air samples collected from the four WWT plants visited. Samples were grouped according to the type of sampler used. An average of the OTUs observed in each sample was calculated for all the samples ± the standard deviation.

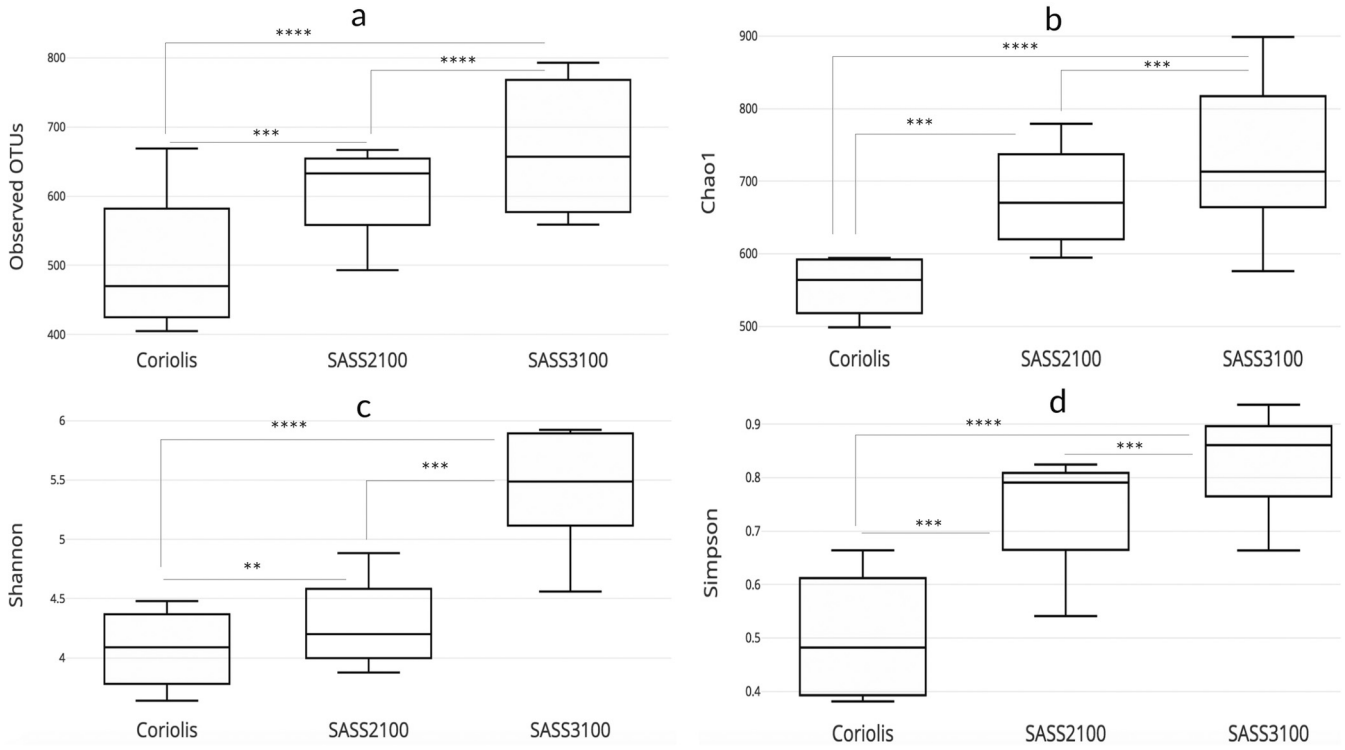

FIG 3 Comparison of alpha-diversity metrics of observed OTUs (a), Chao1 index (b), Shannon index (c), and Simpson index (d) for bioaerosols collected with three different high-airflow-rate samplers (electret-SASS 3100, liquid-SASS 2300, and liquid-Coriolis) at five dairy farms. Asterisks show the statistical significance of the Mann-Whitney U test (ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

WWT plants, respectively. In both environments, samples collected by the electrostatic dry sampler SASS 3100 showed a greater estimation and diversity of species richness than those collected by the cyclonic liquid samplers SASS 2300 and Coriolis. The lowest values were obtained by the Coriolis sampler, while the SASS 2300 sampler falls in the middle of the three samplers. These results were consistent throughout both environments in the study (fungal bioaerosols in DF and bacterial bioaerosols in WWT). In some cases, the minimum values were almost equal for the SASS 2300 and SASS 3100 samplers, while the median and maximum values obtained by the SASS 3100 sampler were higher in all cases for the four richness and diversity indexes. The large standard deviation values obtained in some cases are indicative of an important variation of species richness between the samples taken from the five DFs and the four WWT plants.

Multivariate analysis. After estimation of the diversity within samples (alpha), microbial community studies seek to compare the diversity between samples (beta). One common technique used to achieve this relies on the creation of a (dis)similarity matrix to calculate the distance between samples. It should be noted that different methods for calculating a dis(similarity) matrix can yield different results. Depending on the type of data set, the type of analyses, and the objectives of the study, some metrics are more suitable/appropriate than others (54–57). The UniFrac distance metric was used to compare bacterial communities in the WWT samples using the bacterial OTUs inferred from 16S rRNA sequences. This was done by creating a distance matrix made up of the calculated distances between pairs of samples. The Bray-Curtis dissimilarity measure was used to compare fungal communities in the DF samples using fungal OTUs from the internal transcribed sequence 1 (ITS1) regions. The reason for using a different distance metric was that UniFrac differs from the Bray-Curtis index in that it incorporates information on the phylogeny of the observed species in the samples. Thus, the use of a reliable phylogenetic tree is necessary when using the UniFrac distance metric (58). As ITSs are subject to intraspecific variability (59), the construction

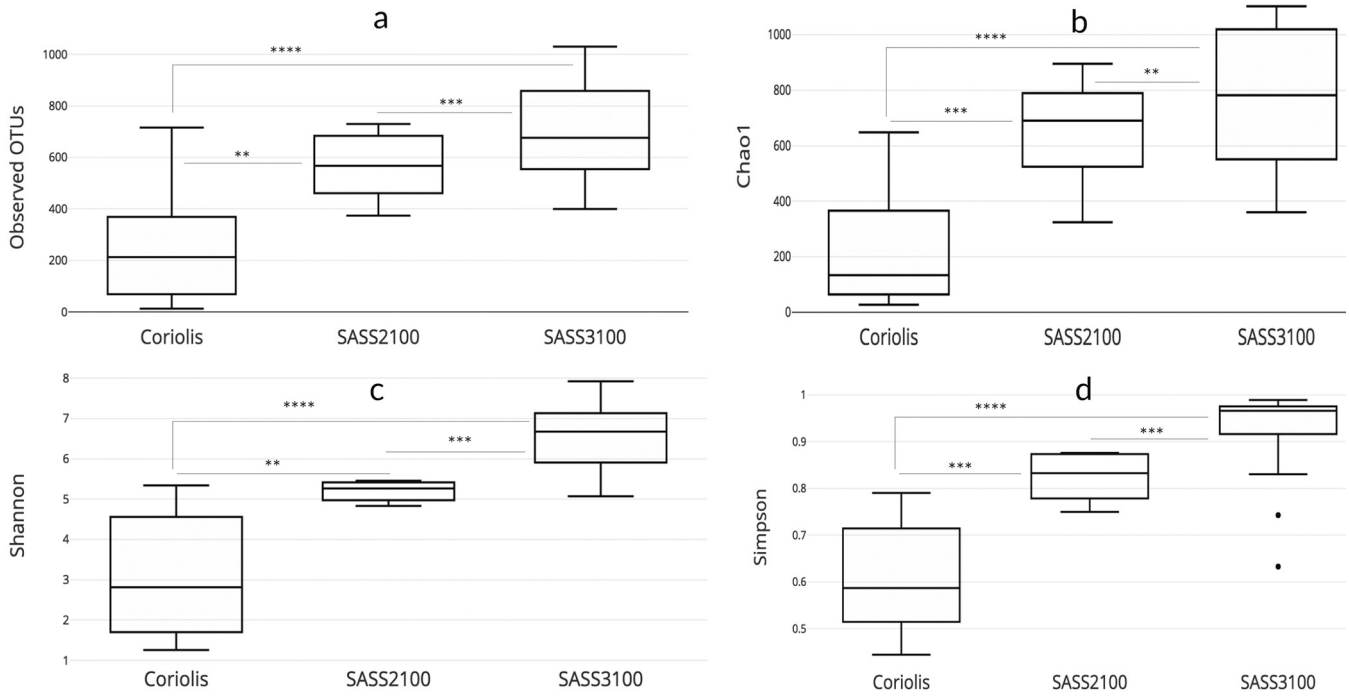

FIG 4 Comparison of alpha-diversity metrics of observed OTUs (a), Chao1 index (b), Shannon index (c), and Simpson index (d) for bioaerosols collected with three different high-airflow-rate samplers (electret-SASS 3100, liquid-SASS 2300, and liquid-Coriolis) at four wastewater treatment plants. Asterisks show the statistical significance of the Mann-Whitney U test (ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

of a phylogenetic tree is not recommended due to the possibility of obtaining different results using the same data set with different tree construction methods (our unpublished data). The Bray-Curtis dissimilarity index is a good alternative, as it does not consider phylogeny when calculating the distance between samples. The output from the Bray-Curtis index is bounded between 0 and 1, where 0 means that the two samples have the same composition and 1 means that they do not share any species. QIIME scripts for beta-diversity analyses were used to produce the quantitative input for the UniFrac (weighted) and the Bray-Curtis dissimilarity indexes, both of which include the abundance of OTUs. Because the Bray-Curtis dissimilarity index uses the absolute abundance of the OTUs, it is necessary to use a rarefied OTU table as an input for this calculation.

One purpose of multivariate analyses is to attempt to represent between sample distances in a 3-dimensional space using ordination (60). To evaluate ordination models, the principal-coordinate analysis (PCoA) is a common visualization tool used for this matter. A dissimilarity matrix is used as an input for ordination calculation and clustering. Distance matrices obtained from Bray-Curtis and weighted UniFrac indexes are both well suited for PCoA (58, 61). The matrices were transformed to coordinates and then plotted using the QIIME principal-coordinates script. Figures 5 and 6 show the three principal-coordinate axes capturing more than 64% of the variation in the input data from DFs and over 37% of the variation in WWT data, respectively. For DF samples (Fig. 5), the following three variables were tested to try and explain the fungal variability in the data set: (i) the dairy farm visited (DF1 to DF5), (ii) the air sampling method used (liquid or dry), and (iii) the type of air sampler used (Coriolis, SASS 2300, or SASS 3100). Samples were colored according to the three variables to visualize sample clustering. Samples closer to one another are more similar than those ordinated further apart. The best sample grouping can be observed in Fig. 5a, as samples from several farms were grouped together. That is the case for samples from DF1 (blue), DF2 (orange), and DF5 (purple). No particular pattern was observed when samples were colored according to the type of air sampling method and the type of air sampler used. All of the data points in Fig. 5b and c are randomly dispersed.

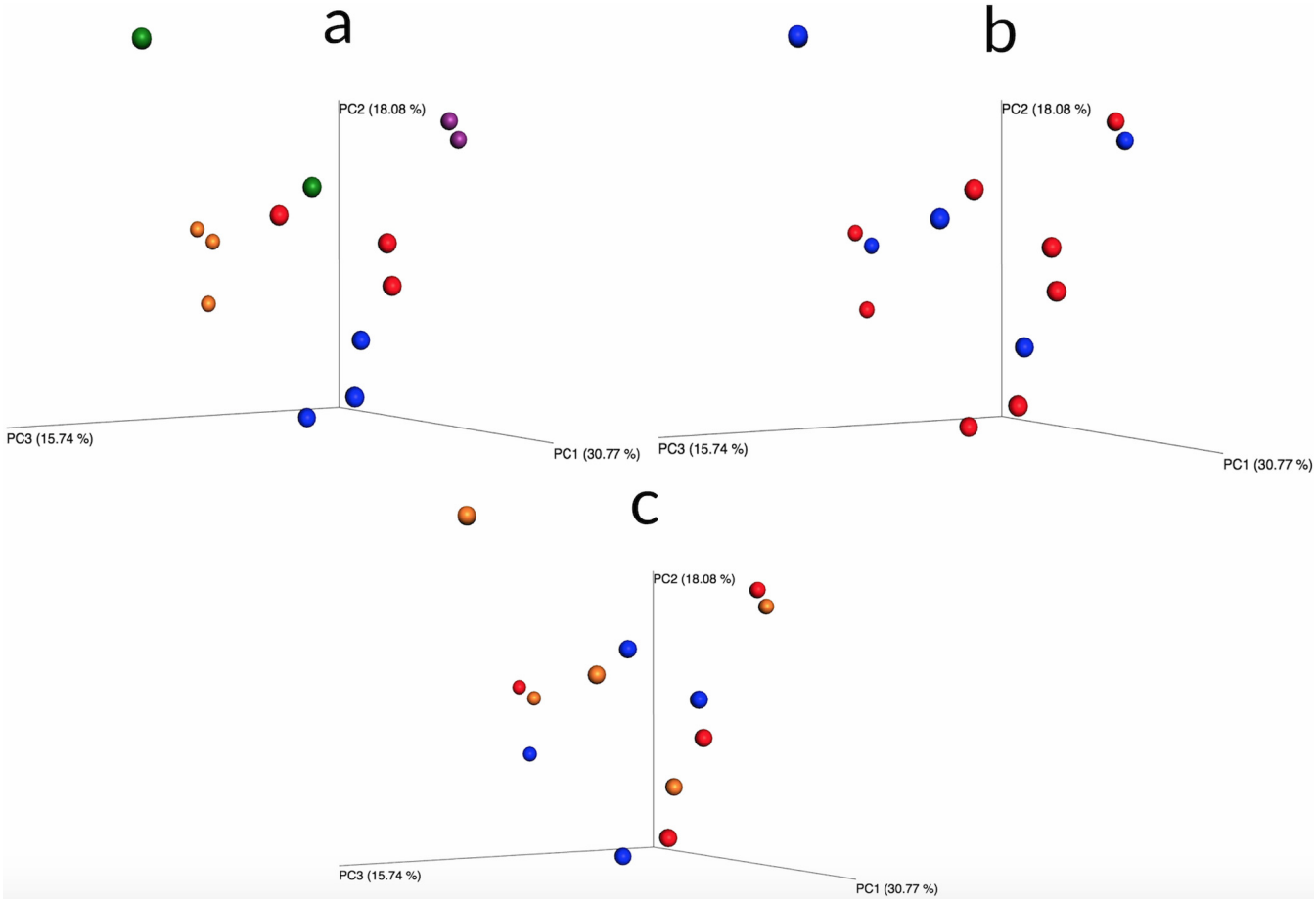

FIG 5 Principal-coordinate analysis of air samples collected from five dairy farms using three different high-airflow-rate samplers. The PCoA was calculated using the Bray-Curtis dissimilarity on ITS1 sequences. (a) Samples colored according to the DF visited (DF1 in blue, DF2 in orange, DF3 in red, DF4 in green, and DF5 in purple). (b) Samples colored according to air sampling method (dry electrostatic sampling in blue and liquid cyclonic impinger in red). (c) Samples colored according to the type of air sampler used (SASS 3100 in orange, SASS 2300 in blue, and Coriolis in red). Separation was noted in panel a between DF1 (blue), DF2 (orange), and DF5 (purple).

For WWT samples (Fig. 6), the following four variables were tested in order to explain bacterial variability: (i) the wastewater treatment plant visited (WWT1 to WWT4), (ii) the air sampling method used (liquid or dry), (iii) the type of air sampler used (Coriolis, SASS 2300, or SASS 3100), and (iv) the sampling season (summer or winter). Again, applying a color to each of the four variables tested, the most obvious grouping is associated with the type of air sampler used (Fig. 6c). Samples collected with the Coriolis (orange) are grouped with the samples collected with the SASS 3100 (red). Figure 6a, b, and d show scattered dots with no particular color grouping.

Statistical significance of sample grouping. To determine the statistical significance of the sample grouping visualized with the PCoA, a permutational multivariate analysis of variance (PERMANOVA) was applied on the Bray-Curtis dissimilarity matrix and the UniFrac distance matrix. PERMANOVA divides the dissimilarity matrix according to different potential sources of variation and calculates the statistical significance that a variable has in explaining the differences observed between samples. It is inspired by the statistical ANOVA but, because it is a nonparametric test, it analyzes the variance and determines the significance using permutations (62). Whereas analysis of variance/multivariate ANOVA (ANOVA/MANOVA) assumes normal distributions, PERMANOVA is more appropriate for sequencing data where distribution is hard to determine. The same variables used for grouping in the PCoA were used with the PERMANOVA. The results were consistent with the observations made from the PCoA. For DF samples, the only significant *P* value was associated with the type of DF visited ($P = 0.001$). The two

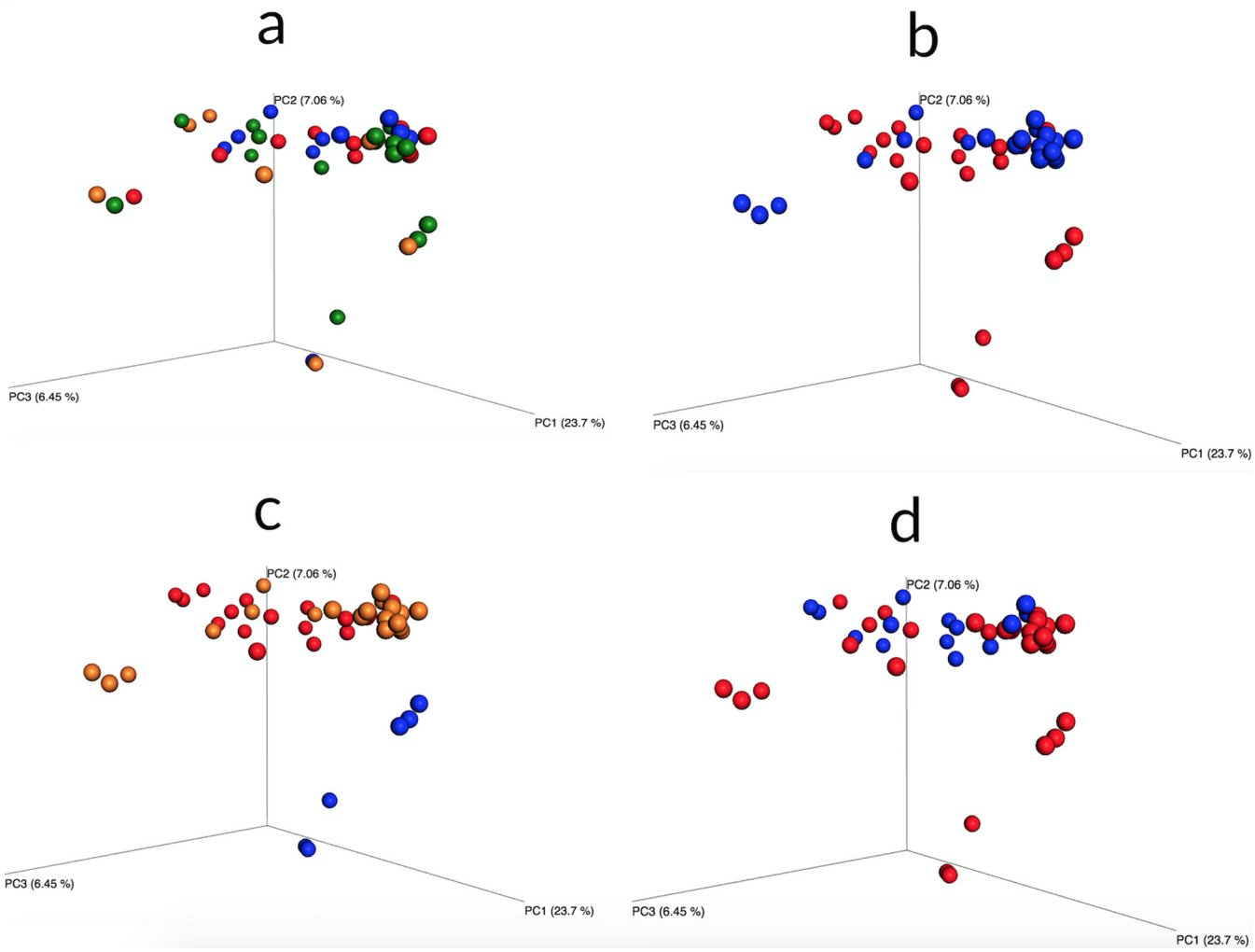

FIG 6 Principal-coordinate analysis of air samples collected from four wastewater treatment plants using three different high-airflow-rate samplers. The PCoA was calculated using the weighted UniFrac distance metric on 16S rRNA sequences. (a) Samples colored according to the WWT visited (WWT1 in red, WWT2 in blue, WWT3 in orange, and WWT4 in green). (b) Samples colored according to the type of air sampling method used (dry electrostatic sampling in blue and liquid cyclonic impinger in red). (c) Samples colored according to the type of air sampler used (SASS 3100 in orange, SASS 2300 in blue, and Coriolis in red). (d) Samples colored according to the sampling season (summer in blue and winter in red). Clusters were noted in panel c between samples collected with the Coriolis and the SASS 3100 samplers.

other variables tested showed no significant difference, as observed in Fig. 5b and c (air sampling method, $P = 0.76$; type of air sampler used, $P = 0.88$).

For the WWT data, the most significant *P* value was obtained for samples grouped according to the type of air sampler used ($P = 0.0003$). This relationship can also be observed in Fig. 6C. Groupings based on season resulted in a *P* value of 0.06. Comparisons of groupings based on the WWT visited had a *P* value of 0.18, and those based on air sampling method had a *P* value of 0.08.

OTU frequencies across samples. After observing differences in the number of fungal and bacterial OTUs between air samples collected with different air sampling equipment, the next step was to try to identify the OTUs that had significantly different abundances depending on the air sampling method used. As two samplers used in this study are liquid cyclonic samplers and one is an electrostatic dry sampler, samples collected from the DFs and WWT plants were separated in two groups, liquid and dry. To accomplish this goal, a statistical test designed specifically for differential analyses of count data was used. The test allows the comparison of OTU frequencies in groups of samples and to ascertain whether there are statistically different OTU abundances in the different sample groups. The G-test uses absolute data count rather than relative

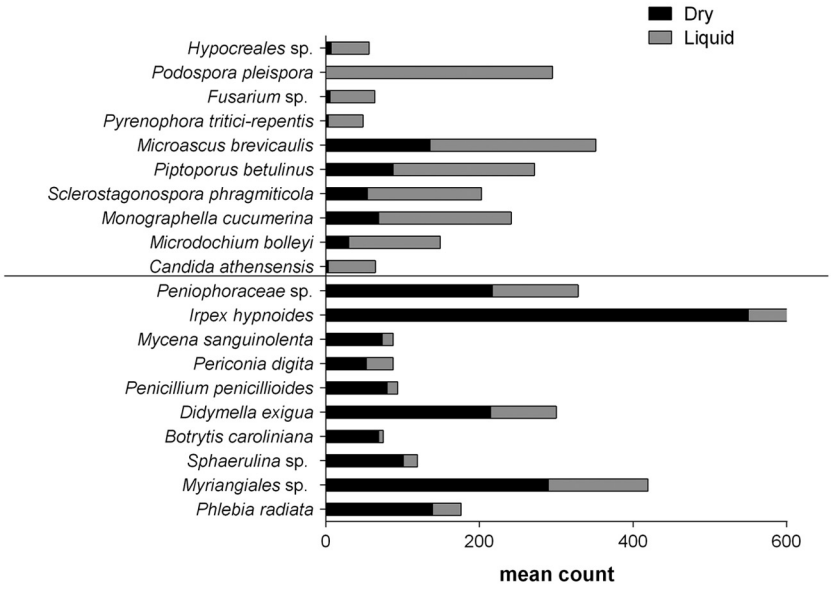

FIG 7 Fungal OTUs with statistically significant differential abundances across the samples collected with a dry electrostatic sampler (SASS 3100) and two liquid cyclonic samplers (SASS 2300 and Coriolis) from five DFs. The line in the middle separates the taxa that were more abundant in either dry sampling or liquid sampling.

abundances. More specifically, the output of the test contains the test statistic, the *P* value corrected for multiple comparisons, and a mean count for each OTU in a given sample group. The tested null hypothesis states that the frequency (count) of any given OTU is equal across all sample groups. The G-test was performed using the group significance QIIME scripts. Figures 7 and 8 show the 10 OTUs that had the most significantly different abundances in the dry and liquid samples at DFs and WWT plants, respectively. All the OTUs presented in Fig. 7 and 8 have large differences in mean counts between dry and liquid sampling. However, not all of the OTUs with differential abundances between the two methods are listed. The complete output of results from

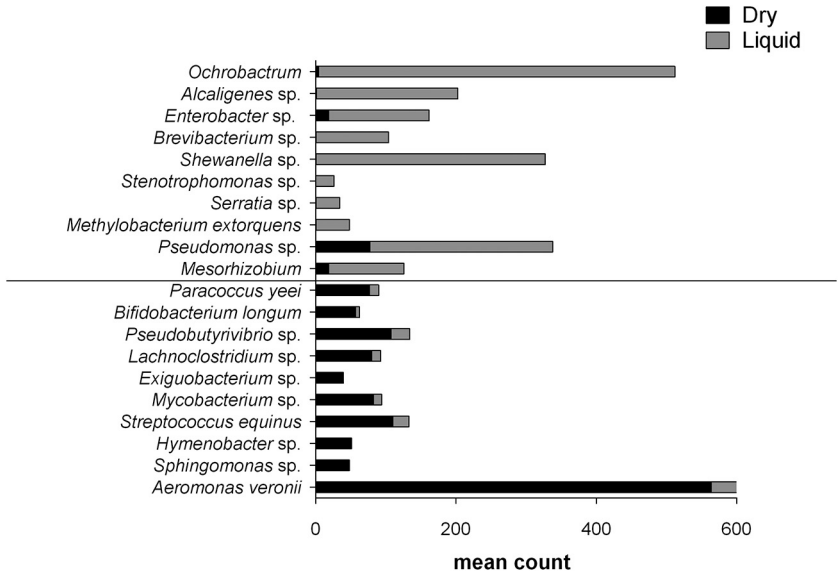

FIG 8 Bacterial OTUs with statistically significant differential abundances across the samples collected with a dry electrostatic sampler (SASS 3100) and two liquid cyclonic samplers (SASS 2300 and Coriolis) from four WWT plants.

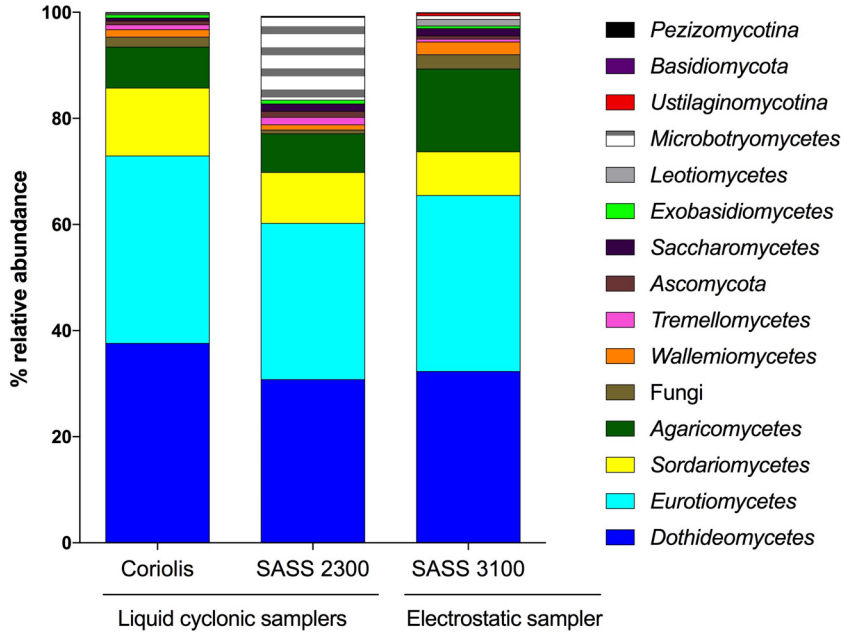

FIG 9 Relative abundances of the classes of fungi detected using three high-airflow-rate samplers at five dairy farms. Four classes of fungi represent the majority of the relative abundance of fungi in the three groups of samples. The class Microbotryomycetes was only detected using the SASS 2300 sampler. Leotiomycetes, Ustilaginomycotina, and Pezizomycotina were only detected using the SASS 3100 sampler.

the statistical G-test is presented in Data Sets S1 and S2 in the supplemental material. In DF, the *P* values of the first 10 OTUs with difference in abundances ranged from 0.004 to 0.0002 for the electrostatic dry sampler and from 0.0001 to 0.00003 for liquid cyclonic samplers. In WWT samples, these numbers ranged from 0.002 to 0.008 for the electrostatic dry sampler and 0.005 to 0.0003 for the liquid cyclonic samplers. For DF samples, the most striking example in the dry list is *Irpex hypnoides*, with a mean count of more than 500 sequences across the samples collected with the electrostatic dry sampler and fewer than 50 sequences in the samples collected with the liquid cyclonic samplers. Also, *Podospora pleispora* was present with a mean count of more than 300 sequences in the samples collected with the liquid cyclonic samplers and only a few sequences in the samples collected with the electrostatic dry sampler. As shown in Fig. 8, bacterial OTUs from the WWT plants had more extreme differential abundances between dry and liquid sampling than did fungal OTUs in dairy farms. For example, *Aeromonas veronii* had a mean count of 580 sequences across the group of samples collected with the electrostatic dry sampler and fewer than 50 sequences in the samples collected with the liquid cyclonic samplers. *Ochrobactrum* was detected with a mean count of more than 550 sequences in the samples collected with the electrostatic dry sampler and fewer than 10 sequences in the samples collected with the liquid cyclonic samplers.

Taxonomic analyses. The taxonomy of OTUs assigned using the UNITE database was analyzed more carefully to establish a relative abundance profile for air samples collected using the three high-airflow-rate samplers at dairy farms and wastewater treatment plants. To accomplish this, air samples were divided into three groups based on the collection device, the Coriolis, SASS 2300, or SASS 3100. Figure 9 shows the taxonomic distribution according to the common features forming the classes of fungi in air from DF. The four most abundant classes had similar relative abundances across the three groups of samples. Those classes represented more than 80% of the taxonomic profile, except for SASS 2300 samples, where they represented 78%. Dothideomycetes, Eurotiomycetes, Sordariomycetes, and Agaricomycetes were represented

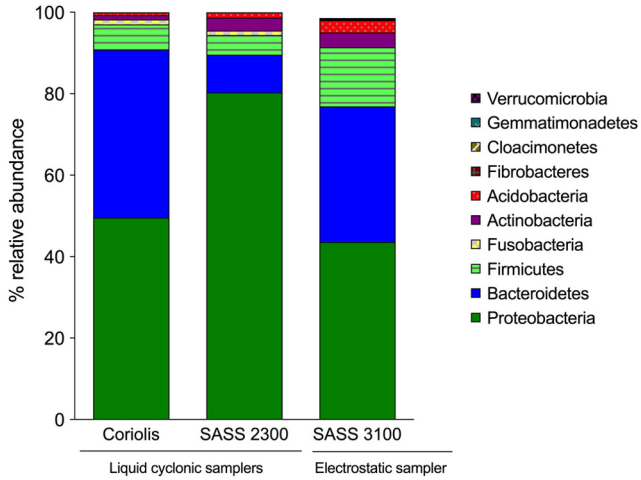

FIG 10 Relative abundances of the different bacterial phyla detected using three high-airflow-rate samplers at four wastewater treatment plants.

equally using each of the three samplers. Wallemiomycetes, Tremellomycetes, Saccharomycetes, and Exobasidiomycetes were also present with similar low relative abundances in the three groups of samples. Inversely, in air samples collected with the SASS 2300, Microbotryomycetes made up 15% of the relative abundance but were not detected at all in samples collected using the Coriolis or the SASS 3100 sampler. The SASS 3100 sampler had a more extensive taxonomic profile, which included Leotiomycetes, Ustilaginomycotina, and Pezizomycotina. These classes were not present in samples collected with the SASS 2300 or the Coriolis sampler.

In WWT plants, three phyla of bacteria represented more than 90% of the taxonomic profile for the three groups of samples, *Proteobacteria*, followed by *Bacteroidetes* and *Firmicutes*. The samples collected with the SASS 2300 exhibited an overrepresentation of *Proteobacteria*. *Fusobacteria* were only present in the samples collected with the two liquid cyclonic samplers (Coriolis and SASS 2300), while *Verrucomicrobia* were only present in the SASS 3100 group. *Acidobacteria* and *Actinobacteria* were represented with the same low relative abundances across samples collected with all three samplers (Fig. 10).

At the genus level, the taxonomic profiles showed greater differences in abundance for both fungi and bacteria. At dairy farms, these differences were most profound in the fungal groups. At wastewater treatment plants, these differences were greatest among the bacteria. Figures 11 (fungi) and 12 (bacteria) present the 20 most abundant taxa in samples collected with each of the samplers. In samples from DFs, 30% of the fungal taxa collected with the SASS 2300 sampler were different from those collected using the Coriolis and SASS 3100 samplers. These taxa consisted mostly of *Rhodotorula* and *Ascosphaera*. Some taxa were also shared between the SASS 2300 and SASS 3100 samplers but had very low relative abundances in samples from the Coriolis sampler. These included *Phanerochaete*, *Ganoderma*, *Candida*, *Chaetomiaceae*, and *Microascus*. For the three sample groups, *Aspergillus*, Myriangiales, *Penicillium*, *Fusarium*, and *Bipolaris* represented more than 50% of the 20 most abundant fungi collected from DF samples.

At WWT plants, 50% of the relative abundances of the 20 most abundant bacterial genera collected with the SASS 2300 sampler are different from those collected using the Coriolis and the SASS 3100 samplers. This difference is mainly due to *Stenotrophomonas*, *Ochrobactrum*, *Shewanella*, and *Serratia* spp., which made up 40% of the 20 most abundant genus taxonomic profiles of the SASS 2300 group. Moreover, *Pseudomonas* was overrepresented in the SASS 2300 group (25%) compared to the Coriolis (8%) and the SASS 3100 (5%). *Rhizobium*, *Brevibacterium*, *Macellibacteroides*, *Alcaligenes*, and *Mesorhizobium* were only present in the 20 most abundant genera of the SASS

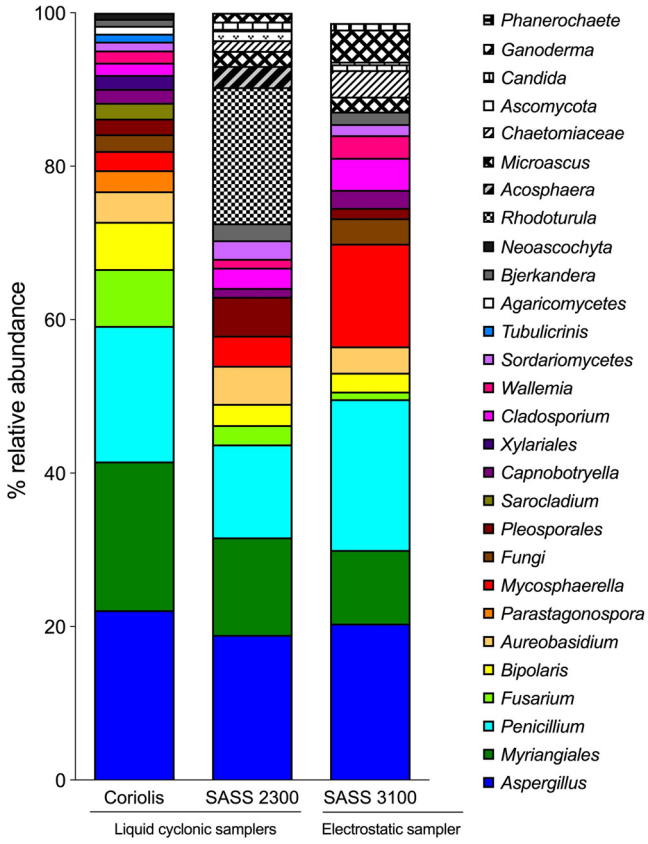

FIG 11 Relative abundances of fungal genera detected in air samples from five dairy farms using three high-airflow-rate samplers. The 20 most abundant genera from each group of samples were considered for the analysis.

2300 group. *Lachnospiraceae*, *Paracoccus*, *Sphingomonas*, *Coprostanoligenes*, *Ruminiclostridium*, and *Methylobacterium* were only present in the 20 most abundant genera of the SASS 3100 group. All the taxa in the 20 most abundant genera of the Coriolis group were covered by the SASS 3100 group. *Sediminibacterium*, *Acinetobacter*, *Delftia*, *Acidovorax*, and *Flavobacterium* spp. represented more than 75% of the top 20 taxa of the Coriolis and SASS 3100 groups.

Even though the differences observed in Fig. 11 and 12 are important, it should be noted that the analyses were performed in only the 20 most abundant genera in each group of samples. When all of the identified taxa were considered, the results from the SASS 3100 sampler included a large majority of the taxonomic groups that were not identified using the SASS 2300 and the Coriolis samplers.

DISCUSSION

The accurate quantification and identification of taxa present in bioaerosols depend heavily on the sampling equipment used. Air samplers have different particle-size-capture efficiencies (63). Various studies have been conducted comparing different air samplers using specific requirements and criteria. That work focused mainly on sampling efficiency associated with particle size and concentration, meteorological conditions, and/or culturability. Some studies focused on targeting specific microorganisms or on one type of air sampler (43, 47, 48, 64–70). Since the rise in popularity of sequencing techniques, bioinformatics analyses have allowed for a better understanding of the microbial composition and dynamics in several different environments. Applying these approaches to bioaerosol science enables a better characterization of human exposure to microbes in different environments (18, 19, 71, 72).

The intention of this study was to fill the gap in the current literature addressing the

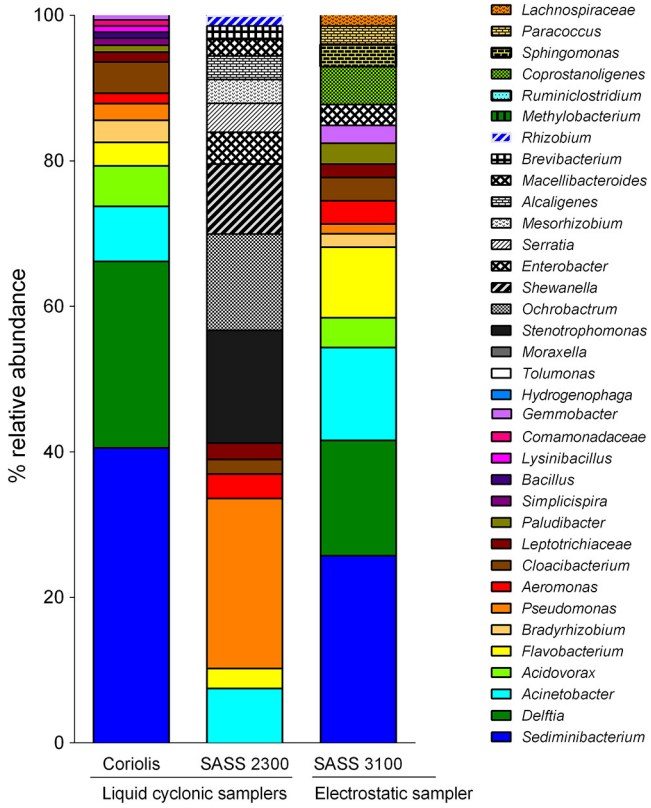

FIG 12 Relative abundances of bacterial genera detected in air samples from four wastewater treatment plants using three high-airflow-rate samplers. The 20 most abundant genera from each group of samples were considered for the analysis.

efficiency of bioaerosol sampling. This was accomplished using a next-generation sequencing approach to characterize the microbial diversity of bioaerosols in field environments using three different high-airflow-rate samplers, two liquid cyclonic samplers (Coriolis and SASS 2300) and one dry electrostatic sampler (SASS 3100). Sampling efficiency may be the most important factor in ensuring accurate quantification. It is also essential that collected samples have concentrations of microbes that are above the detection limit of the method used. The three high-airflow-rate samplers used for this study provided samples with adequate concentrations for use with Illumina MiSeq technology. Furthermore, the collection size range of the three samplers was comparable in the environments sampled. The aerodynamic diameter of particles in wastewater treatment plants is around 5 μm (73) and from 2 μm to 10 μm in agricultural settings (74). The particle size of aerosols is influenced by the source of aerosolization. As the sources of aerosols in wastewater treatment plants and dairy farms are not extremely varied from one location to the other, they were not measured in this study. At the airflow rate used in this study, the three samplers were at their highest sampling efficiency in terms of particle size mentioned above, when they were considered separately (it does not mean that they had equal collection efficiencies). Thus, for the size range of particles mentioned above, the three air samplers did not affect the diversity results. However, it is possible that samplers had different collection efficiencies for smaller particles, therefore affecting the diversity results. Appropriate references about the detailed overall physical collection efficiency of the samplers used in this study are provided herein (see references 47 and 48).

The MiSeq Illumina sequencing depth used was sufficient for obtaining true diversity coverage in bioaerosol samples in both environments examined. The first measures used to compare the three samplers were richness and diversity estimators. Shannon and Simpson indexes are widely used by microbial ecologists to describe the alpha

diversity of samples (75–79). The dry electrostatic sampler SASS 3100 was consistently more powerful in collecting more fungal and bacterial OTUs at wastewater treatment plants and dairy farms. Both the number of species and the proportion in which each species is represented in the aerosol samples were higher in the electret filter.

The multivariate analyses, PCoA, coupled with the PERMANOVA, provided a robust test for statistical significance of sample grouping using distance matrices. The fact that the two analyses resulted in similar sample clusters confirms their usefulness as tools to visualize and measure sample grouping. At dairy farms, the source of the bioaerosols seems to have more impact on the fungal communities of the samples than the type of sampler used to collect them. These results concur with those from a study where the fungal bioaerosols strongly correlated with the type of source present at the time of sample collection (18). At dairy farms, the main source of variation was associated with the type of cattle feed used. Dairy cattle are fed on a wide range of feedstuffs, from forage (grasses, legumes, hay, straw, grass silage, and corn silage) to concentrates (barley and maize). Biochemical changes in these products, like pH and water content, can affect their fungal composition (80, 81). Other factors, like the building characteristics, animal population density, milking activities, handling of feed, seed, and silage, and spreading of the bedding can affect the fungal content of the bioaerosols at dairy farms (82–85). However, groupings of air samples collected from the wastewater treatment plants did not correlate with the source (type of waste treated in each plant). The four plants visited did certainly treat the same type of municipal waste, making the hypothesis that air samples collected have the same bacterial communities. Surprisingly, the most significant sample grouping was associated with the type of sampler used. The fact that the samples collected with the SASS 2300 clustered separately from the SASS 3100 and the Coriolis samples may be explained by the technical complexity of the air sampling method used by the SASS 2300 sampler. This sampler has a larger internal surface area and larger tubing than the other samplers. The collection liquid may come in contact with and particles might adhere to those surfaces, causing the loss of some biological particles in the process. This loss may have been reflected in the sequencing results. Also, the complexity of the inner tubing may contribute to contamination even though rigorous cleansing was conducted before sample collection.

It is important to note that other factors may have affected the multivariate analyses. Relatively few samples were collected at dairy farms, and sample grouping analyses are known to perform better with a large number of samples. Larger sample sizes collected with the three samplers from dairy farms or any other agricultural environment would likely support the findings of these analyses. Likewise, fewer samples were collected with the SASS 2300 sampler than with the SASS 3100 and Coriolis samplers. This is because the SASS 2300 sampler was acquired while the sampling campaign at the wastewater treatment plants was already under way. Distance matrices and PCoA plots may have been affected by a larger number of samples collected with the SASS 2300 sampler. Overall, PCoA combined with statistical testing of sample variation should not be used as a unique approach to compare the microbial ecology of bioaerosols. While PCoA provides initial insight about the variables that affect sample grouping, these variables are often hard to define. A set of chosen explanatory variables does not guarantee that they have true explanatory power. It is always possible that a covariate is the real causal influence on the microbial ecology of the samples. These results should undergo further examination and critical observation (86).

Comparing OTU frequencies across sample groups is another way to study the differential abundance of the microbial community in bioaerosols. To integrate the biological information of bioaerosols with the physical processes associated with sampling strategies, samples were grouped into either liquid cyclonic samples or electrostatic dry samples. The G-test used for differential abundance in this study is a likelihood-ratio statistical test. It compares the ratio of the OTU frequencies in the sample groups to an extrinsic hypothesis about the desired distribution. The extrinsic hypothesis used in this case is that all sample groups have equal OTU frequencies. The test compares the ratio of the observed OTU frequencies in the sample groups to the

expected frequencies based on the extrinsic hypothesis (87). Substantial differences in OTU abundances between liquid and dry sampling were revealed. These differences in both fungal and bacterial OTU frequencies between dry and liquid sampling may be linked to the collection method of each type of sampler. The liquid collected in the liquid cyclonic impactors may be subject to evaporation, depending on the environment and the relative humidity. This liquid loss may cause an imbalance in the microbial diversity represented in bioaerosol samples. Hydrophobic microorganisms or hydrophobic particles carrying microorganisms that are more easily subject to evaporation may contribute to the dysbiosis observed between liquid and dry sampling groups. Furthermore, a phenomenon known as preferential aerosolization may also contribute to the loss that occurred during liquid sampling. This hypothesis suggests that some microorganisms may be preferentially aerosolized compared to others. Parker et al. introduced the idea that respiratory pathogens, such as *Mycobacterium* spp. and *Legionella* spp., could be enriched in aerosols from a source (88). Additionally, Moletta et al. confirmed the preferential aerosolization of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and TM7 phyla in biogas and sludge (89). Similar preferential aerosolization behavior was observed for *Actinobacteria* during the handling of compost (90). The potential for microorganisms to become aerosolized does not appear to be a random phenomenon but could depend on their morphological (form, structure, size, etc.) and biochemical characteristics, such as hydrophobicity, which can differ significantly between species. This preferential aerosolization may have played a role in the differential abundances of fungal and bacterial OTUs observed between liquid and dry samplings.

One main concern associated with filter sampling is the possible drop in pressure due to filter clogging that can cause resistance in the air flowing through the filter. The electret filter used with the SASS 3100 sampler has a larger pressure drop (5%) than do more commonly used filters (Research International, Monroe, WA, USA). However, during field sampling, no technical issues occurred because of filter blockage. Another main concern associated with dry sampling is the extraction efficiency. According to results obtained by the manufacturer of the SASS 3100 sampler, extraction efficiency is essentially constant as long as the extraction liquid exceeds 4 ml. The use of liquid versus solid particles also affects sampling efficiency, as filters seem to be more effective at capturing solid particles (91). This selectivity may also have contributed to the dysbiosis between liquid and dry sampling methods because of the different capture rates of microorganisms by solid and liquid particles.

The taxonomic analyses confirmed the results obtained by PCoA and PERMANOVA, as the SASS 2300 taxonomic profile different the most from those from the Coriolis and SASS 3100 samplers. Though the relative abundances of fungal classes and bacterial phyla were comparable between samples, taxonomic profiles at the genus level were completely different. The majority of the diversity revealed by dry electrostatic sampling was not identified using the cyclonic liquid impactors. For bacteria from wastewater treatment plants, close to 200 OTUs were detected only by the SASS 3100 sampler. For fungi from dairy farms, more than 200 OTUs were detected only by the SASS 3100 sampler. These findings suggest that the choice of a bioaerosol sampler should include information about the efficiency and ability of samplers to cover microbial diversity. Although these results suggest that electrostatic filters result in better coverage of the microbial diversity among the tested air samplers, further studies are needed in order to confirm this hypothesis.

Cyclonic liquid sampling is practical when viability measurements are required, as it can preserve the integrity of microbial cells compared to electrostatic sampling. For applicability matters and from a practical point of view, viability gives direct information on the dissemination and transmission of diseases. However, using molecular methods on nonviable DNA containing particles not amenable to collection by liquid impingement enables us to have a larger view of the microbial content of an aerosol sample. Thus, a direct link between aerosol content and the surrounding sources leads to a better evaluation of air contamination. For example, the detection of high

TABLE 1 Description of sampling sites and parameters affecting sampling environments in the dairy farms visited[a]

Dairy farm site	Type of milking	Animal space	Cattle feed	Ventilation	Temp (°C)	Time of sampling
DF1	Manual	Confined	Forage	Natural	22	6 a.m.–9 a.m.
DF2	Automatic	Confined	Forage	Mechanical	21	7 a.m.–10 a.m.
DF3	Manual	Confined	Concentrates	Natural	19	7 a.m.–10 a.m.
DF4	Automatic	Confined	Concentrates and forage	Mechanical	20	6 a.m.–9 a.m.
DF5	Manual	Semiconfined	Forage	Mechanical	23	11 a.m.–2 p.m.

[a]More information may be found in reference 72.

concentrations of *Aspergillus fumigatus* in a composting environment using qPCR is a good indicator that workers might be at great risk of developing health problems, regardless of the viability of *Aspergillus fumigatus* in our samples. Thus, using a next-generation sequencing approach can give an indication about the most abundant microorganisms in an environment. Then, qPCR can be applied to calculate the concentration per cubic meter of air of a specific genus. Finally, the role of this genus in the transmission and dissemination of diseases can be investigated. Therefore, molecular methods, specifically next-generation sequencing, act as a powerful exploratory tool.

The impact of choosing either one of the samplers could lead to a selective microbial diversity vision, especially in the case of studies looking for some specific taxa. Acknowledging this bias is a first step toward a remedy to the problem. Future work should include the characterization of the flora lost during cyclonic liquid sampling and the selective capture of solid particles during dry electrostatic sampling. In addition, well-defined crafted samples could be used in parallel to unknown environmental samples to evaluate the sampling efficiencies of different samplers and compare their ability to retrieve the microbial content of bioaerosols.

MATERIALS AND METHODS

Sampling sites. Samples were collected from two different environments, dairy farms (DFs) and wastewater treatment plants (WWT plants). Those two types of facilities were chosen because they represent two different bioaerosol types, mostly bacteria (WWT) and mostly fungi (DF). The visited facilities were all located in the province of Quebec (Canada). Tables 1 and 2 present a description of the sampling sites in each environment.

Five dairy farms were visited during the summer (May to June). In each farm, indoor sampling sites were chosen based on where activities that generated the most bioaerosols were located. The specific location of each site was also influenced by its distance from the nearest ventilation system. Samples were collected concurrently with stall cleaning, which is when workers are the most exposed to bioaerosols. The types of activities that occurred during sampling were the handling of feed and silage and the spreading of bedding. Two types of food were given to animals, forage (grasses, legumes, hay,

TABLE 2 Description of sampling sites and parameters affecting sampling environments in wastewater treatment plants visited during winter and summer

Wastewater treatment plant site	Stage(s) of treatment	Temp (°C)	Time of sampling
WWT1	Screening	19/21	9 a.m.–12 p.m.
	Degritting/degreasing	12.5/22	9 a.m.–12 p.m.
	Biofiltration	13/23	9 a.m.–12 p.m.
WWT2	Screening	19/16	9 a.m.–12 p.m.
	Degritting/degreasing	14.5/18	9 a.m.–12 p.m.
	Biofiltration	9.5/19	9 a.m.–12 p.m.
WWT3	Primary screening	24/15.5	9 a.m.–12 p.m.
	Secondary screening	19/18	9 a.m.–12 p.m.
WWT4	Screening	17/19.5	9 a.m.–12 p.m.
	Degritting/degreasing	19.5/19.5	9 a.m.–12 p.m.
	Primary settling	16/18	9 a.m.–12 p.m./12 p.m.–2 p.m.

TABLE 3 Description of air sampling equipment compared in this study and parameters used for sampling

Air sampler	Manufacturer	Collection principle	Airflow rate (liters per min)	Collection liquid	Collection vol (ml)	Extra equipment
Coriolis μ	Bertin Technologies, Montigny-le-Bretonneux, France	Liquid cyclonic impactor	300	PBS	15	Coriolis cones
SASS 3100	Research International, Monroe, WA, USA	Filtration (electrostatic fields)	300	SASS 3010 extraction fluid	5	SASS 3010 extractor unit
SASS 2300	Research International	Liquid impingement (wetted-wall cyclone)	300	PBS	5	None

straw, grass silage, and corn silage) and/or concentrates (barley and maize). As the name suggests, natural ventilation occurs through air filtration and open doors and windows. Mechanical ventilation is a process where the rate of air exchange is controlled and monitored. At the dairy farms visited, all animals were in enclosed buildings. The term "confined space" refers to milking parlors where each cow is in a single row. "Semiconfined spaces" (observed only in DF5) are enclosed spaces where animals are free (Table 1).

Four wastewater treatment plants were visited during the summer and winter. Indoor samples were collected during the different stages of the wastewater treatment, with each stage representing one sampling site. Two samples were collected for each site, one during summer and one during winter. As shown in Table 2, WWT1 and WWT2 have three stages consisting of screening, degretting/degreasing, and biofiltration. WWT3 has two stages of screening (primary and secondary). In the primary screening stage, large objects are removed, while smaller waste is screened during the secondary stage. Like WWT1 and WWT2, WWT4 has three stages of wastewater treatment. However, the third stage is a settling step that uses flocculation agents for sedimentation instead of biofiltration.

Air sampling. The following three high-airflow samplers were compared in this study: a liquid cyclonic impactor, Coriolis μ; a liquid impingement/wetted-wall sampler, SASS 2300 (Research International, Monroe, WA, USA); and a dry/electrostatic filter, SASS 3100 (Research International). A description of the air sampling equipment, the air sampling conditions, and the technical features of each sampler are presented in Table 3. In both environments and at all of the sampling sites, the three samplers were placed within 1 to 2 m of the source and at a defined distance from each other so that the air output of one did not affect the air input of the other.

When using the liquid cyclonic impactor Coriolis μ (Bertin Technologies, Montigny-le-Bretonneux, France), air is aspirated and drawn into the cone, forming a vortex where particles are impacted into the liquid in the cone. The Coriolis μ sampler has a cutoff size of 0.5 μm for an operation flow of 300 liters/min (LPM), meaning that 0.5-μm particles are sampled at 50% efficiency and larger particles are sampled at a higher efficiency. Fifteen milliliters of sterile 50 mM phosphate-buffered saline (PBS) (pH 7.4) was placed in the sampling cone of the Coriolis μ sampler and used as the collection liquid. All samples collected with the Coriolis μ sampler were collected in triplicate for each site at a flow rate of 300 LPM, for a total of 10 min. Each replicate represents three cubic meters of air collected. Thus, at each site, a total of nine cubic meters was recovered with this sampler.

Using the liquid impingement/wetted-wall SASS 2300 sampler (Research International, Monroe, WA, USA), air is drawn into a threaded adapter on the back of the sampler, where a peristaltic pump transfers liquid samples to a sample vial-filling station. The incoming air is mixed, and then particles are collected via the recirculation liquid collector. Particles are extracted from the sampled air and collected in a small volume of liquid made up of a PBS solution. The cutoff size of the SASS 2300 is 0.9 μm for an operation flow of 300 LPM. Five milliliters of the liquid sample is dispensed from the filling station into a sample vial. In this study, samples collected with the SASS 2300 were taken at a flow rate of 300 LPM over 33.3 min. Each sample represents 10 cubic meters of air.

The dry/electrostatic filter SASS 3100 (Research International, Monroe, WA, USA) contains a 44-mm-diameter capture electret filter. The collection efficiency for 0.5- to 5-μm particles is approximately 92%, at an airflow rate of 120 LPM. At the maximum airflow rate (310 LPM), the collection efficiency for the same particle size range is 80%. However, the overall capture rate is much higher at a higher airflow. After particle collection on the electret filter, an SASS 3010 particle extractor (Research International, Monroe, WA, USA) is required to elute the captured particles in a buffer (SASS 3010 extraction fluid). In this study, the SASS 3100 sampler was used at 300 LPM for 33.3 min in order to collect 10 cubic meters of air at each site sampled.

Concentration of the biological content of aerosol samples prior to DNA extraction. (i) Fungi in dairy farms. The protocol used for fungal cell concentration is based on a recent study that focused on the optimization of fungal cell recovery from air samples (our unpublished data). At each farm, the 15 ml obtained from the three Coriolis replicates at each site was pooled to form 45-ml samples. For the SASS 3100 and SASS 2300 samplers, each sample was represented by the 5 ml of extraction and collection liquid, respectively. The liquid suspension from each sampler was filtered through a 2.5-cm polycarbonate membrane (0.2-mm pore size; Millipore) using a vacuum filtration unit. The filters were submerged with 750 μl of extraction buffer (bead solution) from a Mo Bio PowerLyzer PowerSoil DNA isolation kit (Carlsbad, CA, USA) and placed in a 1.5-ml Eppendorf tube with a tungsten bead of 0.3 cm in diameter. The filters were flash-frozen by placing the Eppendorf tube in a 99% ethanol solution and dry ice. Next, the frozen filters were pulverized using a bead-beating machine (Mixer Mill MM301; Retsch,

TABLE 4 Primers used for MiSeq amplification of the ITS1 gene in fungi

Primer by PCR round	Sequence (5′ to 3′)[a]
First-round PCR	
ITS1Fngs	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTCATTTAGAGGAAGTAA
ITS2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGCGTTCTTATCGATGC
Second-round PCR	
Generic forward	AATGATACGGCGACCACCGAGATCTACAC[index1]ACACTCTTTCCCTACACGAC
Generic reverse	CAAGCAGAAGACGGCATACGAGAT[index2]GTGACYGGAGTTCAGACGTGT

[a]The two indices (index1 and index2) represent base indices (Illumina).

Düsseldorf, Germany) at a frequency of 20 revolutions per second 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.

(ii) Bacteria in wastewater treatment plants. Two replicates for each site sampled with the Coriolis sampler were pooled (the third one was kept for other analyses). From those 30 ml, a 1.5-ml aliquot was used for further analyses. Similarly, a 1.5-ml aliquot was taken from the 5 ml of extraction fluid from the SASS 3100 sampler and from the collection liquid from the SASS 2300 sampler. The aliquots were centrifuged for 10 min at 14,000 × *g*. The supernatant was discarded, and the pellets were kept at −20°C until the DNA extraction.

DNA extraction. The same extraction protocol was applied for samples collected from the two environments. A Mo Bio PowerLyzer PowerSoil DNA isolation kit (Carlsbad, CA) was used to extract the total genomic DNA from the samples, according to the manufacturer's instructions. After the DNA elution, samples were stored at −20°C until subsequent analyses.

MiSeq Illumina sequencing. Amplification of the targeted genes, equimolar pooling, and sequencing were performed at the Plateforme d'Analyses Génomiques (IBIS, Université Laval, Quebec City, Canada).

ITS1 gene target for fungal diversity at dairy farms. Amplification of the ITS1 region was performed using the sequence-specific regions described by Tedersoo et al. (92), 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 dinucleoside triphosphate (dNTP), 1 U of Q5 high-fidelity DNA polymerase (NEB), and 1 μl of template cDNA. Initial denaturation started 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 for 30 s, and a final extension at 72°C for 2 min. The PCR product was purified using the Axygen PCR cleanup kit. The quality of the purified PCR product was checked on a 1% agarose gel. A 50- to 100-fold dilution of this purified product was used for a second PCR step for barcode addition (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. PCR mixtures were purified as described above, checked for quality on a DNA7500 Bioanalyzer chip (Agilent), and then quantified with the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Barcoded amplicons were pooled in equimolar concentration for sequencing on the Illumina MiSeq machine. The oligonucleotide sequences that were used for amplification are presented in Table 4.

16S rRNA gene target for bacterial diversity in wastewater treatment plants. Amplification of the 16S V6-V8 region was performed using the sequence-specific regions described in Comeau et al. (93) using a two-step dual-indexed PCR approach specifically designed for Illumina instruments by the IBIS team. During the first step, the gene-specific sequence was fused to the Illumina TruSeq sequencing primers, and PCR was performed. Each reaction mixture (total volume, 25 μl) consisted of 1× Q5 buffer (NEB), 0.25 μM each primer, 200 μM each dNTP, 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 for 30 s, and a final extension at 72°C for 2 min. The PCR product was purified using an Axygen PCR cleanup kit. The quality of the purified PCR product was checked on a 1% agarose gel. A 50- to 100-fold dilution of this purified product was used as the template for a second PCR step with the goal of adding barcodes (dual-indexed) and missing sequence required for Illumina sequencing. Cycling for the second PCR was identical to that of the first PCR but with 12 cycles. PCR mixtures were purified as described above, checked for quality on a DNA7500 Bioanalyzer chip (Agilent), and then quantified spectrophotometrically with the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Barcoded amplicons were pooled in equimolar concentrations for sequencing on the Illumina MiSeq machine. The oligonucleotide sequences that were used for amplification are presented in Table 5.

Please note that primers used in this work contain Illumina specific sequences protected by intellectual property (Oligonucleotide sequences © 2007–2013 Illumina, Inc.) All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.

Bioinformatics workflow for data processing. After demultiplexing the raw FASTQ files, the reads generated from the paired-end sequencing were combined using the *make.contigs* script from mothur (94). The quality filtering was also performed with mothur, discarding homopolymers, reads with ambiguous sequences, and reads with suspicious length (the ones that did not assemble) using the

TABLE 5 Primers used for MiSeq amplification of the 16S rRNA gene in bacteria

Primer by PCR round	Sequence (5′ to 3′)[a]
First-round PCR	
Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCGHNRACCTTACC
Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGGGCRGTGWGTRCA
Second-round PCR	
Forward	AATGATACGGCGACCACCGATCTACA[index1]ACACTCTTTCCCTACACGAC
Reverse	CAAGCAGAAGACGGCATACGAGAT[index2]GTGACTGGAGTTCAGACGTGT

[a]The two indices (index1 and index2) represent base indices (Illumina).

screen.seqs script. Similar sequences were gathered together to reduce the computational burden, and the number of copies of the same sequence was displayed to keep track of the abundance of each sequence. This dereplication step was performed with VSEARCH (95). For ITS1, the selected region of fungal origin was extracted from the sequences with ITSx, which uses HMMER3 (96) to compare input sequences against a set of models built from a number of different ITS regions found in various organisms. Only the sequences belonging to fungi were kept for further analyses. As ITS1 regions are different lengths, VSEARCH was used to equalize the lengths of the sequences using the *fastx_filter* script. For bacteria, the 16S rRNA sequences were aligned with the bacterial reference SILVA core alignment using QIIME (97). Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using the UPARSE method implemented in VSEARCH. UCHIME was used to identify and remove chimeric sequences (98). QIIME 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. For 16S rRNA sequences, the SILVA database was used to assign taxonomy to OTUs. Two metadata files were produced, with one dairy farm OTU table and one wastewater treatment OTU table. The microbial diversity analyses, including statistical analyses conducted in this study, were achieved using QIIME plugins in version 1.9.0 described in the QIIME scripts (http://qiime.org/scripts/).

Statistical analysis. Descriptive statistics were used on sequencing data to highlight significant differences in the diversity measures shown with boxplots. 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 tests were performed to highlight significant differences showing a P value of less than 0.05. The results were analyzed using the software GraphPad Prism 5.03 (GraphPad Software, Inc.).

To determine the statistical significance of the variation observed with the PCoA, a PERMANOVA was performed on the Bray-Curtis dissimilarity matrix. The compare categories, the QIIME script was used for the differential abundance analyses. The G-test was used to ascertain whether or not differences in OTU abundance are statistically significant between the samplers.

Conclusion. Living organisms in the environment have the potential to cause diseases in humans. Bioaerosol studies aim to describe and understand the microbial content and the aerosolization processes linked to these diseases. Air samplers are used to collect, identify, and quantify biological aerosols. Some of the important characteristics of aerosol samplers are size, weight, airflow rate, sampling efficiency, power requirements, sampling mechanisms, output volume, etc. Selecting the right air sampling device to study bioaerosols is a critical step in every bioaerosol study. This study evaluates three air samplers and describes new considerations that have not been previously discussed in the scientific literature concerning the choice of air sampling equipment. The results of this investigation highlight the importance of choosing the appropriate sampling device and its effect on the description of the biodiversity found in bioaerosols. While it is difficult to determine a single universally optimal air sampler, this work provides an in-depth look at some of the considerations that are essential when choosing an air sampler for studying the microbial ecology of bioaerosols.

Data availability. Raw sequence reads of every sample used in this study and that support its findings have been deposited in the National Center for Biotechnology Information (NCBI) under BioProject PRJNA473493 and BioProject PRJNA474662.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01589-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

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