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REVIEW ARTICLE

Perspectives on Sampling and New Generation Sequencing Methods for Low-Biomass Bioaerosols in Atmospheric Environments

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Abstract | Bioaerosols play essential roles in the atmospheric environment and can affect human health. With a few exceptions (e.g., farm or rainforest environments), bioaerosol samples from wide-ranging environments typically have a low biomass, including bioaerosols from indoor environments (e.g., residential homes, offices, or hospitals), outdoor environments (e.g., urban or rural air). Some specialized environments (e.g., clean rooms, the Earth's upper atmosphere, or the international space station) have an ultra-low-biomass. This review discusses the primary sources of bioaerosols and influencing factors, the recent advances in air sampling techniques and the new generation sequencing (NGS) methods used for the characterization of low-biomass bioaerosol communities, and challenges in terms of the bias introduced by different air samplers when samples are subjected to NGS analysis with a focus on ultra-low biomass. High-volume filter-based or liquid-based air samplers compatible with NGS analysis are required to improve the bioaerosol detection limits for microorganisms. A thorough understanding of the performance and outcomes of bioaerosol sampling using NGS methods and a robust protocol for aerosol sample treatment for NGS analysis are needed. Advances in NGS techniques and bioinformatic tools will contribute toward the precise high-throughput identification of the taxonomic profiles of bioaerosol communities and the determination of their functional and ecological attributes in the atmospheric environment. In particular, long-read amplicon sequencing, viability PCR, and meta-transcriptomics are promising techniques for discriminating and detecting pathogenic microorganisms that may be active and infectious in bioaerosols and, therefore, pose a threat to human health.

Keywords: *Bioaerosol, Low-biomass, Microorganism, New generation sequencing (NGS), High-volume air sampler*

1 Introduction

Bioaerosols, or biological aerosols, consist of airborne particulate matter of biological origin, such as bacteria, archaea, fungi, or viruses[1,2]. Bioaerosols play essential roles in the atmospheric environment, including cloud and ice cloud formations that influence climate[3–5]. Bioaerosols also facilitate the transfer of plant and animal pathogens, potentially damaging crops and affecting livestock production[6–8], and human exposure to these bioaerosols can result in asthma, allergies, and infectious diseases[9–12]. The recently emerged virus, SARS-CoV-2, is also known to be spread through indoor aerosols[12].

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It has been reported that bioaerosols may account for up to 30% of the aerosol mass in urban and rural air[3], and the concentration of microbial cells varies from ~ 10^2 to ~ 10^6 cells m^{-3} of air in the atmosphere[8]. Thus, collected bioaerosol samples typically have a low biomass in indoor environments (e.g., residential homes, offices, or hospitals) and outdoor environments (urban or rural air), with the exception of certain specialized environments, such as farm or rainforest environments[3,13,14]. This makes it challenging to analyze the target matter despite the increased attention on bioaerosols in some specialized environments showing ultra-low biomass, for example, operating rooms and intensive care units in hospitals, clean rooms in industrial or pharmaceutical manufacturing sites, or the international space station searching for life on other planets. Therefore, new methods need to be developed to collect a sufficient amount of target matter in aerosols or to characterize target matter accurately from a small biomass[15].

At present, culture-based methods are most widely employed for bioaerosol characterization[16]. These methods have the advantage of simple operation, not requiring a pump or air mover. The particles are collected on a Petri dish based on gravitational settling or an electrostatic capture mechanism[16,17]. However, culture-based techniques can significantly underestimate the number and diversity of organisms in the microbiota of bioaerosols because of the use of an unknown air volume, and they provide only qualitative information on bioaerosol presence and dynamics[2]. In addition, some viable airborne microorganisms cannot be cultivated because they are unable to grow under the culture conditions or the cells are damaged during the culturing process[18,19]. In this respect, new generation sequencing (NGS) analysis methods coupled with high flow rate air samplers have been attracting attention as an alternative to culture methods. Gene analysis techniques may include quantitative PCR (qPCR) or NGS analysis of the amplicons obtained from the 16S ribosomal RNA (rRNA) gene, 18S rRNA gene, and the ribosomal internal transcribed spacer (ITS) region. According to our survey that interrogated the ISI Web of Science database on November 24, 2022, there were over 2,000 publications on the topic of bioaerosols between 1989 and 2022, and 167 of these used NGS techniques (Fig. 1, Table S1). Bioaerosol research employing sequencing analysis methods was first reported in 2007 and has increased over the last five years (Fig. 1).

In response to the increased application of NGS technologies in bioaerosol studies, this review provides a brief overview of bioaerosol sources, aerosol sample collection methodologies, productive sample filtrations, and the efficiency of target DNA extraction from liquefied aerosol samples. Further, the advances in NGS techniques and bioinformatics and how these may lead to a more precise understanding of the active and infectious microorganisms in low-biomass bioaerosols are discussed, as are the potential implications for human health.

2 Sources of Bioaerosols

Atmospheric bioaerosols can originate from virtually all environments, including terrestrial and aquatic environments, and outer space[4,8]. It has been reported that bacteria can be released into the atmosphere from water, soil, and plant surfaces based on the theory of particle resuspension[20]. The aerosolized microorganisms have the potential to exist for long periods of time in the upper atmosphere because of their small size and low sedimentation rate, and they may travel long distances downwind[21]. It has been reported that viable microorganisms in the atmosphere may travel across continents and oceans[22–24]. However, microbial bioaerosols can be removed from the upper atmosphere by deposition on buildings, plants, water surfaces, the ground, and other surfaces in contact with the air, or by precipitation in the form of rain, snow, or hail[4,25].

Indoor bioaerosols are generally introduced via heating, ventilation, and air conditioning systems, water sprays, or via the entry of humans, pets, or plants into buildings[26]. For example, heating, ventilation, and air conditioning systems contain the dust that can be contaminated with fungal spores as a bioaerosol source[27]. Once these bioaerosols enter indoor environments, they are easily transported from one place to another and can remain in the indoor air for a long period of time, potentially affecting the respiratory health of the occupants within the building[6]. The number of occupants in a building and the ventilation rates are reportedly related, which suggested that human occupancy dominantly contributes to the concentration of indoor airborne bacteria[28], and ventilation had a demonstrated effect on indoor airborne community composition[29]. In addition, indoor bioaerosol concentration and diversity can rapidly fluctuate in response

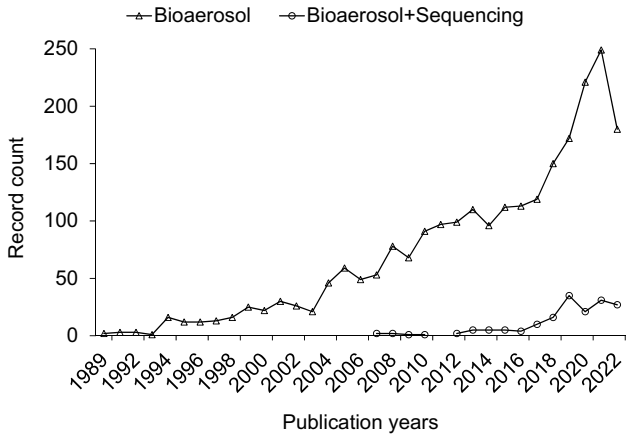

Figure 1: Distribution of published papers by year from 1989 to 2022 identified from the ISI Web of Science database with the search term "Bioaerosol", and refined with the search term "Sequencing". Record count: published papers.

to environmental factors, such as light, temperature, and humidity[30]. More specifically, over-humidification results in aerosols dominated by pathogenic bacteria such as *Pseudomonas* spp. and *Brevundimonas* spp.[31]. Toilet flushing spreads aerosolized bacteria such as *Clostridium difficile* and viruses such as norovirus and SARS-CoV-2[32–34]. Non-tuberculous mycobacteria, which are resistant to sterilization and detergents, can also cause infection through aerosolization in bathrooms[35]. Further, indoor plants or green spaces can enrich the indoor microbiota by increasing microbial diversity[36]. Indoor bioaerosols can be spread by coughing, sneezing, talking, bathing, using the toilet, or the resuspension of dust[31]. Therefore, all these factors must be considered to further our understanding of airborne microorganism dynamics in a given environment.

3 Sampling Methods of Low-Biomass Bioaerosols

Simple and passive sampling methods are generally used for culture-based detecting methods; however, it is not suitable for collecting sufficient amounts of target matter in aerosols and accurately characterizing the microorganisms from low-biomass environments[1]. Active sampling is used to overcome this issue, utilizing a pump to draw air at an adjusted flow rate and concentrate bioaerosols onto a collection filter/liquid medium, allowing for quantitative bioaerosol identification using NGS analysis[2,16]. The sampling devices suitable for this purpose are listed in Table 1.

3.1 Filtration-Based Samplers

The main advantage of using filters for bioaerosol collection is to elute the collected air samples into the liquid, which makes it possible to proceed with subsequent procedures such as DNA/RNA extraction. A concern with air sample filtration is that the filter materials (e.g., glass fibers, polycarbonate, cellulose ester, and polytetrafluoroethylene) can dry out the bioaerosol trapped in the filter, which can then decrease during sample recovery and affect the viability of target microorganisms. In this regard, nuclease-free gelatin membrane filters, which have a high collection efficiency for airborne particles between 0.5 and 3.0 μm in size[47], can preserve bioaerosol viability and have been developed to collect aerosol bacteria, fungi, and viruses for quantitative PCR analysis[40,48–51], although there are limited reports to date of their use for NGS[15]. Polyethersulfone membrane filters are another possible choice, especially for proceeding with DNA-based analysis because both gelatin and polyethersulfone filters can be directly dissolved in DNA extraction buffer and minimize sample loss during the elution step compared with fibrous filters[17,52–54]. In a recent report, electrostatic dry filters were used to collect pathogenic bacteria and spores[55], since they reportedly capture more bacteria and fungi compared with other filtration methods[56]. However, the stress associated with electrostatic dry filtration must be taken into consideration especially when RNA-based NGS analysis is subsequently performed on the filtered samples[46,54]. Factors other than the filtration material, such as the pore diameter size, porosity of the filter, and flow rate can also affect the bioaerosol sampling

Table 1: Representative commercially available samplers for collecting low-biomass bioaerosols for NGS analysis.

Sampling method	Sampler	Air flow-rate (L/min)	Characteristics	References
Filtration-base	High-volume air sampler (HV-500R) Sibata Scientific Technology, Tokyo, Japan	100–800	Size selectivity (PM2.5, PM 10); compatible with quartz fiber and polytetrafluoroethylene filters; collect particles both indoors and outdoors	Kawai et al.[37]
	SASS 3100 Dry Air Sampler Research International, Monroe, WA, USA	50–300	Operate with electret filter air samplers: support for sampler-specific semi-automated filter extraction (filter-to-liquid) to generate liquid samples for downstream analysis	Bøifot et al.[38]
	AirPort MD8 Air Sampler Sartorius, Gottingen, Germany	50	The gelatin membrane filters (3 µm pore size) can be used in combination with the Sartorius Airport MD8 sampler, which has high retention rate for microorganisms and viruses, and is easy to use and clean in the field	Lewandowski et al.[39]; Hasanthi et al.[40]
Liquid-base	BioSampler SKC Inc., Eighty Four, PA, USA	12.5	The classical impinger systems for liquid sample collection. It has been taken as a standard reference sampler for bioaerosol research because of lower microorganism stress	Tseng et al.[41]; Dybwad et al.[42]
	Coriolis µ Bertin Technologies, Montigny-le-Bretonneux, France	300	Take just 10 min to collect airborne particles using cyclonic technology paired with a high suction rate; compatible with rapid microbiological analysis methods	Kokubo et al.[43]; Carvalho et al.[44]
	SASS 2300 Wetted-wall Air Sampler Research International, Monroe, WA, USA	325	Extracts and transfers airborne pathogens, particulates, bacteria, and spores from sampled air to small water volume for analysis	Guo et al.[45]
	BioSpot-VIVAS™-BSS310 Aerosol Devices Inc. CO, USA	8	A laminar-flow water condensation particle growth technique substantially improves the collection of particles (<10 nm to 10 µm) sampling directly into a liquid medium, but it is complex to use, and it is not portable	Nieto-Caballero et al.[46]

performance. Thus, prior to selecting an aerosol filtration method, all these factors must be investigated to understand how they may affect the NGS results, such as amplicon sequence variants and diversity indices.

3.2 Liquid-Based Samplers

Traditional impingers collect bioaerosols into a liquid using inertia to separate particles from the airstream and deposit them into a liquid collection medium[16]. The liquid is available

for a variety of culture-independent analysis techniques, especially PCR-based methods. The all-glass impinger (Ace Glass, Inc., Vineland, NJ, USA) and BioSampler (SKC Inc., Eighty Four, PA, USA) are the classical impinger systems for liquid sample collection, which can effectively prevent dehydration of bioaerosols[41]. In particular, the BioSampler has become the standard reference sampler because of its low-stress on microorganisms and its relatively low level of collection fluid loss resulting from its relatively gentle swirling motion[41,42,57–59]. However, both impinger systems require an external power source, and the disadvantage of these two impingers is that the instruments have a limited flow rate, and thus cannot be easily applied to field sampling and it takes time to collect enough bioaerosol. The wetted-wall cyclones, such as the Coriolis family of samplers (Bertin Technologies, Montigny-le-Bretonneux, France), are another alternative, operating at faster flow rates of 100 to 300 L/min and using centrifugal or cyclonic forces to trap the particles into 10–15 mL of liquid[44,60–62]. The Coriolis μ sampler has the advantage of collecting a large volume of air in just 10 min and can be used for long-term collection to monitor an area up to 6 h by adding a collection buffer during operation. Larger biomass sampling using a similar method was accomplished by the SASS 2300 Wetted-wall Air Sampler (Research International, Inc., Monroe, WA, USA), extending the sampling duration to several days by adding a greater liquid volume to the system. However, the larger aggregated particles collected in the liquid sample by these strong cyclones with high flow rates caused sampling variability in a previous study on the detection of airborne bacteriophages[62]. In addition, for collecting indoor fungal aerosols, the BioSampler showed better performance than the Coriolis sampler[59]. Another notable collection method involves condensation growth tube technology (Aerosol Devices Inc., CO, USA), marketed as the BioSpot-viable virus aerosol sampler (VIVAS). This was developed to capture bacterial and viral bioaerosols into genomic preservatives, which is considered to reduce the stress on bioaerosol samples experienced on air filters, impactors, and impingers[46]. Hence, the advantages and disadvantages of the different collection mechanisms for liquid-based sampling must be considered when selecting an appropriate method.

4 NGS Methods for Low-Biomass Bioaerosols

Once low-biomass microorganisms in aerosols are trapped in filtrations at sufficient volume, the samples can be subjected to DNA extraction, followed by NGS, which can provide new insight into the diversity of environmental microbiota in the bioaerosols. Table 2 lists some of the recent discoveries in bioaerosols based on the application of amplicon sequencing technologies. For example, aerosol studies incorporating amplicon technologies evidenced that installing good ventilation and air filters in a given building improved the air quality by altering the fungal and bacterial composition of the indoor aerosols[29,63,64]. Other studies evidenced the transportation of microorganisms in a bioaerosol from one place to another by determining the microbial community structures and compositions by amplicon-NGS analyses[15,22,65,66]. One study revealed that the variability in airborne bacterial communities is influenced by meteorological parameters, and long-range transportation of atmospheric bioaerosols occurred from the southern slopes of the Himalayas to the southern Tibetan Plateau and the Mongolian Plateau[24]. In another study, the bioaerosols in Earth's lower stratosphere were collected using an Aircraft Bioaerosol Collector (ABC) and characterized by amplicon sequencing, which revealed the homogeneous distribution of bacteria in the atmosphere up to 12 km[15]. As exemplified above, adopting NGS technologies provides valuable insight into our understanding of bioaerosols in low-biomass environments including ultra-low-biomass environments.

It should be noted, however, that the recovery of bioaerosol genetic material and NGS analysis results depend on the sampling techniques applied[16,67]. Currently, there is no universal bioaerosol sampler, which makes it difficult to compare data both within and between laboratories. Several studies have compared results obtained by different sampling methods for a given aerosol sample (filter/liquid-based sampler), and bacterial and fungal diversity, and amplicon sequence variants varied greatly between the methodologies[46,54,56,68]. Jiang et al.[69] conducted a meta-analysis of 16S rRNA gene amplicon sequencing on 3226 airborne samples, and the results indicated that the significant effect of PCR primer bias on the air bacterial community was higher than environmental variations such as sampling seasons. Furthermore, it has been recognized that NGS is sensitive to environmental DNA contamination during the sample preparation, in particular when treating samples with

Table 2: Recent advances in the study of bioaerosols by NGS.

Sampling method	Main findings	References
Drawing air through open-face filters with analytic filter cups of 47 mm diameter and 0.2 μm pore size cellulose nitrate membrane	Occupants may not exert a strong influence on bioaerosol microbial composition in a space that, like many offices, is well-ventilated with air that is moderately filtered and moderately occupied	Adams et al.[63]
Aircraft Bioaerosol Collector with gelatinous filter membranes	Homogeneous distribution of bacteria in the atmosphere up to 12 km, but the influence of aircraft-associated bacterial contaminants could not be fully eliminated	Smith et al.[15]
A liquid cyclonic impactor (Coriolis μ), a liquid impingement/wetted-wall sampler (SASS 2300), and one electrostatic filter dry sampler (SASS 3100)	The choice of a bioaerosol sampler could lead to a selective microbial diversity and taxonomic profiles at the genus level	Mbareche et al.[56]
Particle size separation sampler using polycarbonate filters with a pore size of 0.2 μm	The variability in airborne bacterial communities is determined by meteorological parameters and long-range transportation in extreme environments, such as desert and alpine areas	Qi et al.[24]
Particulate matter with an aerodynamic size of 10 μm was sampled using high-volume samplers (PM_{10}) (GMW Model 1200, VFC $HVPM_{10}$: Sierra Andersen, Smyrna, GA, USA)	Airborne bacterial phyla associated with particulate matter are representative of those found in the atmosphere of urban areas and present local and seasonal differences	Calderón-Ezquerro et al.[78]
Particles of different sizes were collected via Anderson samplers (Qingdo Junray Intelligent Instrument Co., Ltd, Qingdao, China) with nylon filter membranes (0.22 μm) at different particle stages	Temperature, air quality index (AQI), and humidity were the key factors resulting in the change of air bacterial composition with seasons	Cai et al.[79]

low microbial biomass[70]. Therefore, a thorough understanding of the performance and outcomes of bioaerosol sampling by NGS analysis methods, and a robust universal protocol for aerosol sample treatment for NGS analysis are needed.

Development of sequencing platforms such as data processing techniques, and bioinformatics tools make it possible to improve the comparability and reproducibility of bioaerosol research[69]. One suggestion is to consider applying sample-intrinsic microbial DNA found by tagging and sequencing (SIFT-seq) to bioaerosol NGS analysis. This technique was developed bioinformatically to remove DNA contamination during sample preparation procedures[70], thereby reducing bias in the DNA sequence data of bioaerosol samples with low microbial biomass, because the contaminating DNA can easily overwhelm a sample with a low-biomass microbial DNA for NGS[71–73]. Moreover, rather than conventional short-read (e.g., Illumina MiSeq) amplicon sequencing, long-read (e.g., Pacific Biosciences, and Oxford Nanopore Technologies) amplicon sequencing is a possible alternative to obtain microorganism information for bioaerosols at the species level, which has better resolution for discriminating pathogenic bacteria or fungi related to human health[74], such as *Clostridium difficile*[32], *Mycobacterium avium*[35], or *Aspergillus fumigatus*[75]. The identification of viable and active, but non-culturable, microorganisms is of great importance when investigating bioaerosols in terms of public health. However, amplicon sequencing of 16S, 18S, and ITS rRNA genes cannot distinguish viable from dead microorganisms, but a broader view of the microbial diversity could be obtained compared with culture-dependent methods. Viability assays combining PCR with chemicals that crosslink DNA, such as propidium monoazide (PMA-PCR), have been applied to detect infectious microorganisms in bioaerosols[41], and, specifically, to characterize viable bacterial and fungal communities associated with surfaces on the international space station[76]. Amplicon sequencing of reverse-transcribed RNA is allowing us to discover microorganisms that are active in the atmospheric environment[77]. To the best of our knowledge, no study has combined amplicon sequencing with PMA-PCR or applied meta-transcriptomics for characterizing bioaerosol samples. These advances in techniques are promising in terms of visualizing microorganisms in situ, and thus may provide a further understanding of the taxonomic profiles of bioaerosol communities and their functional and ecological attributes (Fig. 2).

Interested atmospheric environments with low-biomass bioaerosols

- Indoors environments: residential homes, offices, or hospitals
- Outdoors environments: urban or rural air
- Specialized environments: clean rooms, the Earth's upper atmosphere, or the international space station

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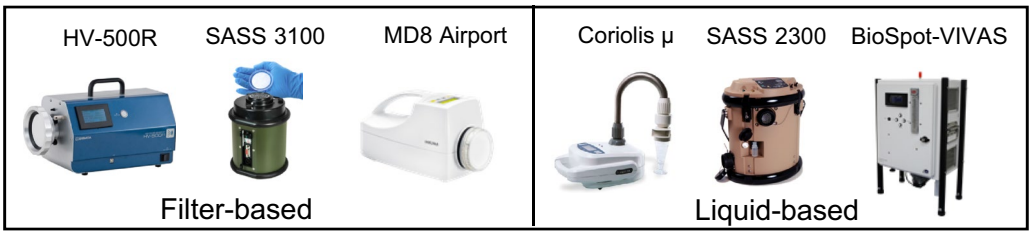
Available commercial air samplers for low-biomass bioaerosols



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Promising NGS analysis methods

- Long-read amplicon sequencing for **discriminating pathogenic bacteria or fungi related to human health**: Pacific Biosciences, Oxford Nanopore Technologies for taxonomic profiles at species levels
- NGS with viability PCR or reverse transcribed RNA for **detecting active and infectious microorganisms**: PMA-PCR or Meta-transcriptomics
- Bioinformatics analysis for **precise identification**: SIFT-seq, LEfSe, SourceTracker, etc.

↓

Clarify the taxonomic profiles of bioaerosol communities and their functional and ecological attributes in atmospheric environments

Figure 2: A summary of the findings of this review. Bioinformatics tools: SIFT-seq (Sample-Intrinsic microbial DNA found by tagging and sequencing), a technique to identify and remove DNA contamination; linear discriminant analysis effect size (LEfSe), a technique to determine the features (organisms, clades, operational taxonomic units, genes, or functions) most likely to explain differences between classes[80]; SourceTracker, a Bayesian estimator of the microbial sources that can help classify microbial samples according to the environment of origin[81].

5 Take-Home Message and Future Directions

In this review, high-volume filter-based or liquid-based air samplers compatible with NGS analysis methods are discussed that may improve the bioaerosol detection limits for low-biomass microorganisms in atmospheric environments such as indoor environments (e.g., residential homes, offices, or hospitals), outdoor environments (urban or rural air), and specialized ultra-low-biomass environments (e.g., clean rooms, Earth's upper atmosphere, or the international space station). Although the bias that is introduced by different air samplers may be emphasized by NGS analysis remains to be fully defined, advances in NGS techniques and bioinformatics tools enable a further step toward precise high-throughput identification of the taxonomic profiles of bioaerosol communities and their functional and ecological attributes in atmospheric environments. In particular, long-read amplicon sequencing, viability PCR, and meta-transcriptomics are promising techniques for detecting and discriminating

pathogenic microorganisms that may be active and infectious in bioaerosols and are therefore relevant to human health (Fig. 2).

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Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Conflict of interest

The authors report no conflicts of interest.

Ethical approval

Not applicable.

Supplementary Information

Below is the link to the electronic supplementary material.Supplementary file1 (DOCX 1104 KB)

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