INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Monitoring of airborne biological particles in outdoor atmosphere. Part 2: Metagenomics applied to urban environments

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Summary. The air we breathe contains microscopic biological particles such as viruses, bacteria, fungi and pollen, some of them with relevant clinic importance. These organisms and/or their propagules have been traditionally studied by different disciplines and diverse methodologies like culture and microscopy. These techniques require time, expertise and also have some important biases. As a consequence, our knowledge on the total diversity and the relationships between the different biological entities present in the air is far from being complete. Currently, metagenomics and next-generation sequencing (NGS) may resolve this shortage of information and have been recently applied to metropolitan areas. Although the procedures and methods are not totally standardized yet, the first studies from urban air samples confirm the previous results obtained by culture and microscopy regarding abundance and variation of these biological particles. However, DNA-sequence analyses call into question some preceding ideas and also provide new interesting insights into diversity and their spatial distribution inside the cities. Here, we review the procedures, results and perspectives of the recent works that apply NGS to study the main biological particles present in the air of urban environments. [**Int Microbiol** 19(2): 69-80 (2016)]

Keywords: airborne biological particles · metagenomics · next-generation sequencing (NGS) · air biomonitoring · urban aerobiology

Introduction

Worldwide population is coarsely concentrated in urban environments where people are exposed to allergens and pathogens transported by the air like pollen, fungi, bacteria and viruses. Pollen and fungal spores may come from distant natural locations surrounding the metropolitan areas, while airborne active pathogenic bacteria and viruses come likely from closer sources inside the cities. We scarcely know the

biodiversity present in urban areas and, strikingly, a reliable automatic system has not been developed yet in order to monitor the bioaerosols present in the air, partly because of the low concentration of the biological particles and sample collection difficulties [23]. Moreover, the different airborne biological particles are usually studied by different disciplines independently and, hence, we have a lack of information concerning the existence of relationships about their relative abundance or fluctuations to each other.

During the last years, metagenomics have increased significantly our knowledge on the biodiversity in every environment compared with conventional methods. Current technologies in sequencing are not restricted to bacteria, so any biological sample with a complex mixture of organisms can be analyzed. Thus, next-generation sequencing (NGS) offers an interesting alternative to study the metropolitan

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atmosphere and uncover the real diversity present in the air. Metagenomics and the technologies related have simplified the steps required to characterize a particular environment like the urban air (Fig. 1). Here, from sample collection to taxa characterization, we review the current knowledge about airborne biodiversity in urban environments from DNA sequencing-based studies.

Sampling procedures in NGS studies applied to urban atmosphere

Traditional studies in microbiology for airborne bacteria and fungi are usually culture-based methods, whose bias concerning to biodiversity is widely known. Although several commercial devices are available, they are usually designed to fulfill air control legislation indoors, with fixed times and/or volumes to sample. Sometimes it is the collection surface that determines these factors, like agar media, which tent to dehydration. Unfortunately, and according to our estimations, higher sampling volumes and times than those preselected in these devices may be required to study some biological particles present in the outdoor atmosphere by Next-Generation Sequencing (NGS) [55].

In addition to vacuum filtration used in outdoor urban and also non-urban areas [8,9,18,51,70], most metagenomic studies conducted in metropolitan environments employ Particulate Matter (PM) collectors, as those operating in air quality monitoring stations [7,10,14,25,56] (Table 1). The particles are harvested in fiber filters at flows >200 l/min and the DNA is subsequently extracted. Some samplers employed typically in aerobiological studies for visual fungi and pollen identification (Hirst-type spore trap, Fig. 2) have been also recently tested in NGS studies by some authors including us [27,43]. The particles are collected on an adhesive tape and the airflow rate is significantly lower than the formers (ca. 10 l/min), but closer to human inhalation rate.

Thus, solid surfaces seem preferable to collect airborne biological particles when DNA-sequencing technologies are applied in urban environments. Only a few works have employed liquid collection [20,74], likely because water-based buffers tend to evaporate quickly, restricting the airflow rate and sampling time. However, new devices as the Spin-Cyclon used by Yooseph et al. [77] look an interesting option, with an airflow rate of 450 l/min. Fahlgren et al. [17] performed a study comparing the results obtained from three different devices (a modified impactor, a liquid impinger, and a Teflon membrane filter), concluding that there are no significant
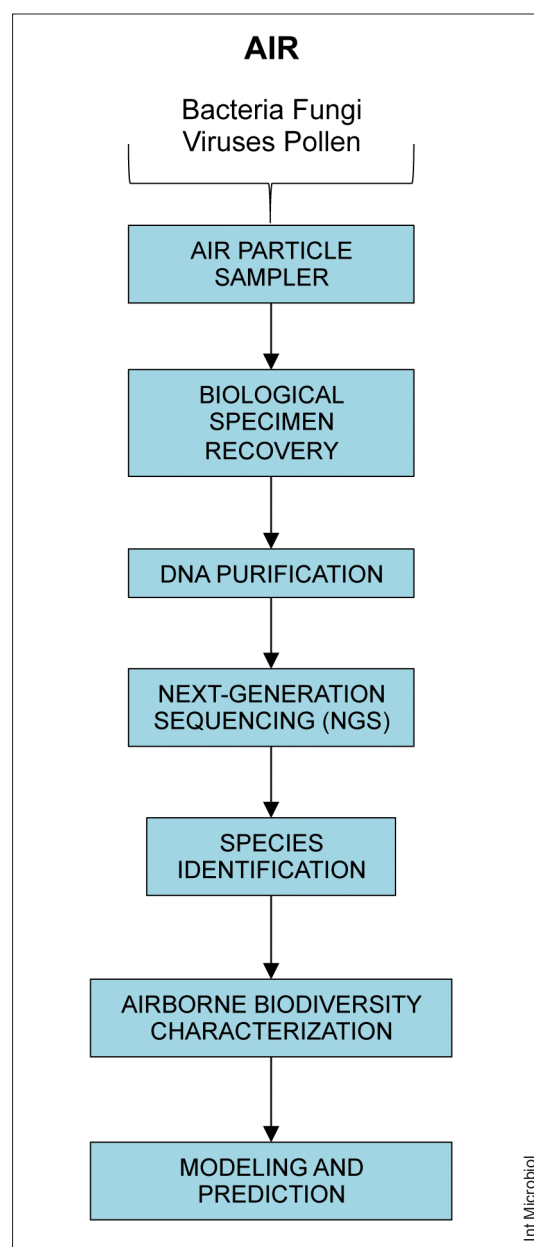
Fig. 1. Workflow scheme for metagenomic studies applied to air samples designed for the Program AIRBIOTA-CM.

differences on the bacterial diversity and dominant species based on 16S rRNA sequences analyses. However, Hoisington et al. [33] conducted a similar survey indoors employing and comparing diverse air samplers, finding significant disparities in the OTUs of bacteria and fungi detected by each device. Thus, more studies analyzing the concordance between the results of different sampler-types for NGS analyses and also for different biological particles should be performed to confirm and standardize the sampling methodology.

In addition to the variety in sampling devices, no consensus exists about the volume of air to analyze. Strikingly, works applying NGS to urban areas have employed air samples that range from 2.5 to 5000 m^3 (see Table 1). Fahlgren et al. [18] for bacteria, and Fierer et al. [20] for both bacteria and fungi, were able to performed microbial identification by cloning the DNA collected in air samples of only ≤3 m^3. Less than 10 m^3 are necessary to conduct high-throughput sequencing according to the data provided by Kraaijeveld et al. [43] and Woo et al. [75], who employed different samplers and analyzed airborne eukaryotic organisms. In regards to viruses, Whon and colleagues [74] were able to carry out a metagenomic study with 54 m^3 of air in the near-surface atmosphere. On the contrary, Yooseph et al. [77], despite the fact that they sampled large volumes of outdoor air (5900 m^3 at the 22nd floor air, 101 m above street level in Midtown Manhattan, New York City) did not obtain enough DNA, highlighting the influence of the altitude on airborne biological particles abundance.

Diverse sampling times have been also selected for each author, without any correlation with the final volume of air collected (see Table 1). Consequently, each work analyzes specimens from different devices, with different volume collected and different times sampled, what may lead the results between studies to differ, especially regarding the minor representatives of the airborne communities.

As we previously reviewed [55], biological particles abundance and diversity vary significantly across the year and they are also susceptible of quick changes (in days or even hours). Therefore, sampling volume and time are two variables to consider in order to obtain a representative sample of the "airbiota" in a certain area, and it may be necessary to modify these parameters throughout the time and depending on the purposes of the study.

DNA extraction

In addition to the different methodology for sampling, the DNA extraction procedure is something to take into account. Quality and high concentration are desirable for NGS analysis but difficult to obtain when samples come from poorly inhabited environments like the air. Some studies comparing different methods suggest that there are remarkable differences regarding yield and purity, which could bias the further analysis [30], although it has been also exposed that DNA yield does not always correlate with differences in microbial diversity after the analysis [78]. The debate is even more controversial regarding DNA extraction
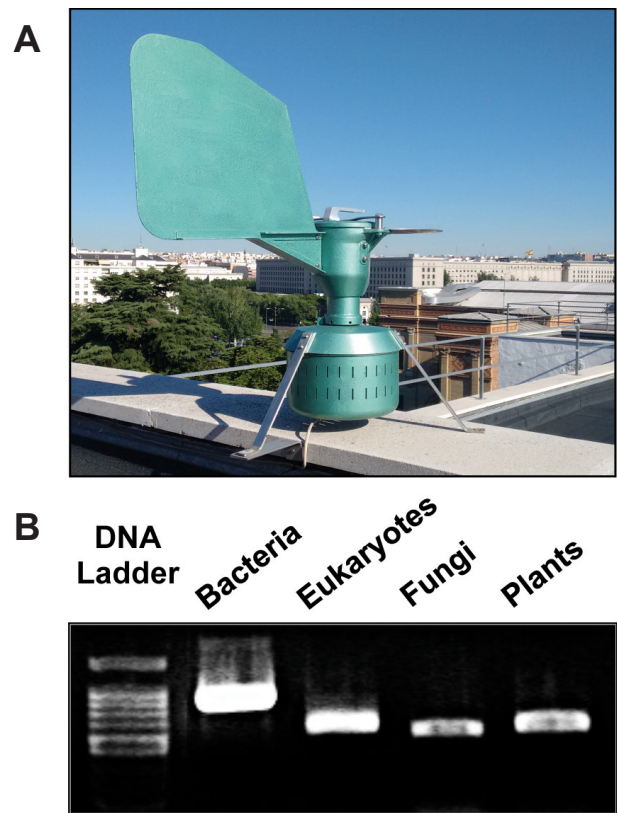
Fig. 2. (**A**) Volumetric Hirst-type spore trap for fungi and pollen collection employed in aerobiological studies placed on the roof of the Higher Technical School of Industrial Engineering, Technical University of Madrid (Madrid, Spain). (**B**) Agarose gel showing the results of the PCRs to detect the DNA from different groups of organisms obtained with such collector after sampling for 7 days (50 m^3) in April 2015 (unpublished data from the Program AIRBIOTA-CM).

for viral metagenomic studies because of the difficulty of the procedures for purifying these organisms [5,72].

While some traditional methods (phenol:chloroform extraction or CTAB procedure) are still in use to study airborne microorganisms [18,51,63,74], the tendency is to incorporate commercial DNA purification kits when NGS technology is applied [4,7,9,10,68,70,76] (see Table 1). Nowadays, commercial kits provide similar quality than handling methods, saving time and preventing manipulation issues. A common factor is to include mechanical disruption such as a bead-beating step in addition to chemical disruption [78]. Accordingly, Jiang et al. [36] have recently described a protocol for purifying DNA from particulate matter to identify airborne microorganisms by metagenomics, which includes the use of a commercial kit for the DNA extraction with some modifications.

Nonetheless, one remarkable conclusion from Hart et al.

[30] is that the method for DNA extraction must be amended for each case. This is particularly important when we require the characterization of different types of organisms (with their physical and chemical particularities) coming from a complex habitat like urban air.

Next-Generation Sequencing (NGS)

Clinical and environmental samples containing a complex combination of organisms (viruses, archaea, bacteria, fungi, animals and/or plants) can be currently analyzed at once thanks to metagenomics, saving time and avoiding the previous bias of culturing. Traditional studies in aerobiology can also take advantage of this new branch of genomics to identify fungi and plants that are hardly assigned to low taxonomic levels (genus or species). Moreover, this novel methodology is particularly interesting for those unculturable organisms or obligate-intracellular parasites such as viruses or zoonotic pathogens from the genera *Rickettsia*, *Coxiella* or *Chlamydia*.

Although some recent DNA-based identification studies still apply traditional molecular stages (cloning, Sanger sequencing of RFLPs, etc.), current high-throughput DNA sequencing technologies permit to skip these steps. 454 pyrosequencing (Life Science, Roche) was the pioneer platform for the so-called next-generation sequencing (NGS). While it is still in use, more recent platforms as Illumina (MiSeq/HiSeq), Ion Torrent/Proton (ThermoFisher Scientific), MinION (Oxford Nanopore Technologies) or PacBio RS system (Pacific Biosciences) have quickly replaced the former, improving the cost, timing and reliability [26].

Two main approaches exist in environmental NGS studies:
  • *Targeted Amplicon Sequencing (TAS)*. This strategy implies the selection of a target region of the DNA to analyze. Any region can be chosen but it is usually one present in all the organisms to study, especially in biological diversity surveys (16S rRNA gene for bacteria or 18S rRNA gene/ITS for eukaryotic organisms). The technique requires a previous PCR amplification step by using specific primers for the preferred region. "Universal primers" have been described for each group of organisms (archaea and bacteria [2], fungi [67,73], eukarya [35], plants [13,34]), and the use of degenerated oligonucleotides reduces the bias for sequence poly-morphisms. Unfortunately, there is always a fraction

of sequences that are amplified with less efficiency, so some groups of organisms can be underestimated or undetected while others are magnified, sloping the relative abundance. Furthermore, this methodology is limited for viral identification because these organisms do not share any common marker gene (such as 16S rRNA in bacteria or 18S rRNA in eukaryotes), so a *shotgun* approach is the only way to uncover viral communities [64,74]. Despite these limitations, targeted sequencing is currently widely adopted for biological diversity characterization.

  • *Shotgun metagenomics* (see Sharpton [69] for a full review). The entire DNA present in the sample is sequenced without specific-region enrichment, reducing the bias introduced in the other modality. Although bioinformatics analyses after sequencing are challenging, the results from this strategy include taxonomic biodiversity (mainly from ribosomal DNA fragments) and biological functions (from DNA coding sequences). This approach, frequently used for whole genome sequencing (WGS), is especially interesting for assembling genomes, functional metagenomics, viral identification and global biome characterization, providing also absolute abundance information.

In regards to urban environments, far from exclusive, both approaches are complementary, offering critical knowledge for results interpretation. However, because of the cost, the difficulties to obtain enough DNA from some areas and the complexity of the bioinformatics analyses for *shotgun* analyses, TAS is the favored strategy to study microbial communities in urban airborne spaces (see Table 1).

Specific marker genes for sequencing-based analyses

Although 16S rRNA gene (SSU) is widely accepted as a target for bacterial identification in metagenomic studies, one issue that remains controversial is the region to sequence to acquire the best representation. Ideally, the complete gene should be sequenced to obtain accurate taxonomic assignments. Since the diverse hypervariable regions present in SSU show different variability they provide different level of taxonomic discrimination [50]. Several studies involving direct cloning of the 16S rRNA gene from airborne microorganisms were published between 2008-2013 [17,18,20,58,60,61]. Universal primers 27F-1492R encompassing V1-V8 regions of the 16S rRNA [45] were very suitable for these analyses, providing

the first results extracting the microbial DNA directly from the collection surface. However, the current NGS tendency is to analyze the shortest length necessary to obtain good taxonomic identification, reducing the cost for sample and time for further bioinformatic analyses [26,42].

Accordingly, fragments comprising one or several regions within V1–V5 hypervariable span have been successfully used for both environmental and clinical studies [42,47,50,52], discarding V6–V9 regions for providing less resolution. Chakravorty et al. [12] and Salipante et al. [66] have demonstrated that the V1–V3 regions are more suitable for clinical studies, distinguishing important pathogenic bacterial species from the genera *Staphylococcus*, *Mycobacterium*, *Streptococcus* or *Haemophilus*. In recent published works using NGS, the amplification of fragments covering total or partial V1–V4 regions is favored to study airborne bacterial communities in urban environments (Table 1).

Although the results from any of these regions may be acceptable within the same study, a global consensus about the specific hypervariable regions to analyze is required for comparing among different studies in order to extract correct conclusions.

Similar to bacteria, ribosomal RNA is the favorite region for fungal taxa identification. Three regions: LSU (25S-28S), SSU (18S) and internal transcribed spacers (ITS1 and ITS2), are the most popular options for DNA sequence-based studies, being the latter (ITS2) proposed as universal barcode marker by the Fungal Barcoding Consortium [67]. Only a handful of studies performed in urban environments have analyzed fungal communities by NGS technologies (Table 1). Some authors have used LSU regions, domains D1 and/or D2 [56]; and some others the18S and/or ITS fragments [14,70,75,76].

Again, this dichotomy in the selected region makes more difficult to compare among different studies and extract significant conclusions, since the results obtained from LSU and ITS may not match completely [49].

Defining a DNA barcode for plants is a challenging task and the debate is still on. Starting with the genomic DNA region ITS2 [13,73], latest reports are focused on plastid genes such as *rbcL*, *matK*, trnH-pbsA, *trnL* or a combination of two for obtaining an undisputable taxonomic identification at species level [6,34]. Although affordable for individual analyses, current NGS platforms cannot integrate two distant markers at the same time. Sequencing-based identification is an attractive approach for pollen identification since morphological characters are not always sensitive enough to distinguish genus or species by microscopy (the plant families *Poaceae* or *Chenopodiaceae*, for instance). Just a few works

have been recently published evaluating NGS technologies for plant identification using *trnL* as gene marker [43], ITS2 [40], or *rbcL* [24,31], being the former the only one that analyzes urban airborne samples and generates promising results for using DNA sequence-based strategy as an alternative to traditional methods for pollen identification.

As pointed above, viruses do not possess common sequences to use as a marker to design universal primers, so the *shotgun* approach must be adopted. In addition, the small genome size of viruses is another limiting factor for recovering enough DNA to carry out the analyses. Thus, previous random amplification procedures as Multiple Displacement Amplification (MDA) [46], or Sequence-Independent Single-Primer Amplification (SISPA) [38], are usually performed to increase the quantity of DNA or RNA, respectively. However, both techniques have some important biases [46,65]: MDA amplifies more efficiently circular DNA molecules than linear ones; and the SISPA method has a biased amplification that depends on the sequence of the primer used. Unfortunately, the best way to avoid such biases is, so far, to obtain sufficient viral biomass to sequence the sample directly. As a consequence, only Whon and colleagues [74] have confronted a complete airborne study of the viral communities in different locations in Korea using *shotgun* metagenomics, while two others surveys conducted in urban areas detected some viral sequences but not as a main goal [4,11].

Bioinformatics

Open-source programs and web-based tools for bioinformatics processing have been strikingly promoted by NGS technology. A complete review about the analytical tools applied in DNA sequence-base studies was published by Kim and colleagues [41]. MEGAN, RDP Classifier, Mothur, QIIME or Muscle are some examples of software suites frequently used to process high-throughput sequencing data in addition to BLAST. Some of them require an open-access database to assign taxonomic affiliations to the sequences. Greengenes for bacteria, UNITE for fungi, and Silva for bacteria and eukarya, are the most popular curated databases for stand-alone workflow, all relying on the sequences and annotations submitted to GenBank and regularly updated.

After the bioinformatics processing, DNA sequences are assigned to taxonomic classification, providing the information necessary to characterize the diversity of each biological community in our samples.

Table 1. Studies of airborne organisms in urban environments implying direct purification of the DNA and NGS analysis (studies about particular events such as dust storms have been omitted)

Ref.	Sampler	Flow rate	Time/Vol collected[a]	Collection surface	Extraction method	Org.	Region sequenced	Approach	Seq. technol	Database
[4]	Portable Sampling Unit (BioWatch Program)	ND	24 h x 7 days	Filters	UltraClean Soil DNA Isolation Kit[1]	All	NA	Shotgun	Illu	NCBI
[7]	High-volume aerosol sampler[b]	250 l/min	24 h x 10 days (360 m³)	Quartz fibre filter	Kit FastDNA Spin for Soil[2]	B	16S: V5-V6	TAS	Illu	RDP
[10]	High-volume aerosol sampler[b]	1,130 l/min	3 days (5,000 m³)	Quartz fibre filter	PowerSoil DNA isolation Kit[1]	B	16S: V2	TAS	454	RDP
[14]	High-volume aerosol sampler[b]	1,130 l/min	72 h (4881 m³)	Quartz fibre filter	PowerSoil DNA isolation Kit[1]	F	ITS: ITS1-2	TAS	454	NCBI
[21]	Low volume gravimetric sampler	38.33 l/min	24 h (55 m³)	PTFE filter	FastDNA Spin for Soil Kit[2]	B	16S: V3	TAS	454	RDP
[25]	High-volume aerosol sampler[b]	200 - 500 l/min	24 h (288 - 720 m³)	Quartz fibre filter	FastDNA Spin for Soil Kit[2]	B	16S: V5-V6	TAS	Illu	RDP
[43]	Burkard volumetric spore trap	10 l/min	24 h (7.2 m³)	Adhesive tape	QIAamp DNA Mini Kit[3]	P	plastid:TrnL	TAS	Ion	NCBI
[56]	High-volume aerosol sampler[b]	30 l/min	24 h (43 m³)	0.2 μm Polyester membrane filters	UltraClean Soil DNA Isolation Kit[1]	F	28S: D1-D2	TAS	454	RDP
[63]	8-stage non-viable impactor	28.3 l/min	4 days (163 m³)	Uncoated PCTE filters	phenol/chloroform protocol	B	16S: V3-V4	TAS	454	Greengenes, RDP
[70]	Vacuum filtration	24 l/min	10 h (28.8 m³)	0.45 μm cellulose ester filters	FastDNA Spin for Soil Kit[2]	B F	16S: V1-V3 ITS: ITS2	TAS	454	EzFungi, UNITE
[74]	Impinger	19 l/min	48 h (54 m³)	PBS	Chloroform, MiniElute Virus spin Kit[3]	All	NA	Shotgun	454	CAMERA, NCBI
[75]	Two-stage bioaerosol cyclone sampler	3.5 l/min	12 h (2.5 m³)	Filter	DNeasy Plant Mini Kit[3]	B E	16S: V3-V4 18S: V1-V3 ITS: ITS1-2	TAS	454	RDP Silva
[76]	Two-stage non-viable Andersen sampler	28.3 l/min	4 weeks (1,141 m³)	Glass fiber filter	PowerMax Soil DNA Isolation Kit[1]	F	ITS: 5.8S-ITS2	TAS	454	NCBI
[77]	SpinConHPAS 450-10A Wet Cyclone Portable Air Sampler	450 l/min	7 days (5,900 m³)	PBS	MO BIO PowerWater Kit[1]	All B	NA 16S: V3-V5	Shotgun TAS	454	NCBI

[1]: MoBio Laboratories, [2]: MP Biomedicals, [3]: Qiagen; NA: Not applicable; ND: Not described; TAS: Targeted amplicon-sequencing; NCBI: National Center for Biotechnology Information; RDP: Ribosomal Database Project .Organisms: B (Bacteria); F (Fungi); P (Plants); E (Eukaryotes). Sequencing Technology: Illu (Illumina); 454 (454-Roche); Ion (Ion Torrent). [a]: Estimated when not specified; [b]: Particulate matter collector

Lessons from DNA-sequencing studies in urban spaces

Abundance of microorganisms in the air. It is well characterized that some biological entities present in the air suffer important seasonal fluctuations as the noticeable variation in abundance and composition of the pollen grains throughout the year [28].

Likewise, culture-dependent studies performed in urban areas suggest that fungi and bacteria are, in general, more abundant in summer than in winter [19,37,54]. Recent culture-independent studies are in concordance with these results. Woo et al. [75] described that there is a peak of DNA concentration in the air in urban environments during summer season (June to September) independent on rural or urban environments. Consistently, some other authors have used different approaches as DAPI [10], *shotgun* sequencing [4] or qPCR [7] to show that airborne bacteria abundance is higher in summer, only outnumbered in spring by the group Plant-Fungi [4]. Moreover, both Dannemiller et al. [14] and Fröhlich-Nowoisky et al. [22] also agree on the fact that fungal richness is higher in spring, being reduced in winter.

However, Fierer et al. [20] showed that some airborne organisms are submitted to more rapid variations in metropolitan areas. In this study, they observed that the abundance of bacteria and fungi changes significantly within a period of 10 days, based on cloned sequences. Similar results were obtained by Shin et al. [70] and Oh et al. [56] using NGS. Furthermore, some culture-based studies have reported that daily precipitations can affect fungal abundance during the following days [32], and even diurnal oscillations for bacteria have been described [19,48,79]. Thus, some divergences between studies may be obtained depending on environmental factors as the weather or the time of day when the sampling is performed.

Relative abundance of different taxa. Contrary to culture-based studies in which spore-forming Gram-positive bacteria (e.g. *Bacillus, Micrococcus*) are usually the most abundant group identified outdoors [44,48,79], NGS has proved this is not necessarily real, showing an unexpected diversity of Gram-negative bacteria [18,25,75,77]. A general conclusion (independent on *shotgun* or TAS approaches) is that the phyla *Actinobacteria* and *Proteobacteria* are the most abundant in urban outdoor environments, followed by *Firmicutes* and *Bacteroidetes* [7,18,21,25,70,75].

Interestingly, controversial results using NGS have been found within the fungal kingdom. It is unclear which group is more abundant in metropolitan spaces. Several authors have reported that the abundance of *Ascomycetes* in metropolitan spaces is greater than *Basidiomycetes*, and they are at all seasons [14,56,75]. The opposite is shown by other authors like Shin et al. [70] and Pashley et al. [57], supported by morphological identification, and these contradictory results are independent on methodology (cloning, TAS, *shotgun*) or the DNA region considered (LSU, ITS or 18S). However, as underlined above, some works suggest that significant changes in fungal abundance may occur within short periods of time (days or even hours). Since most of NGS analyses only register a few isolated days, both conclusions may be plausible. To shed some light on the matter, some morphology and culture-based surveys performed systematically along the year concluded independently that *Ascomycetes* spores are the main group in urban areas, being *Cladosporium* the most abundant fungal spore [15,16].

Additionally, it is widely accepted that *Dothideomycetes* (in which *Cladosporium* is included) is the most represented taxa in *Ascomycetes*, as *Agaricomycetes* is within the *Basidiomycetes* group [14,70,76]. Moreover, a positive correlation has been observed between relative humidity and *Basidiomycetes* spores. Analyzing the LSU region of the fungi present in urban atmosphere, Pashley and colleagues [57] found an increment of these microorganisms during wet days, what partly could explain the differences among studies.

Regarding airborne viruses, Cao et al. [11] and Be et al. [4] have been able to detect viral sequences from urban airborne samples, mainly bacteriophages and some human-related viruses as herpesviruses and Adenovirus C. Additionally, most of the viruses identified by Whon and coworkers [74] in the city of Korea were related to geminivirus, circovirus, microvirus, nanovirus and bacteriophages (*Caudovirales*).

The influence of meteorological factors is a challenge to address in urban environments employing NGS technologies. While traditional studies agree temperature and wind positively correlate with an increment of bacterial abundance according to counting and culture-based results [29,53], some NGS studies found no correlation with meteorological parameters. Shin et al. [70] did not find a direct correspondence between the abundance of microbial taxa and temperature or relative humidity during the study in childcare facilities (indoors and outdoors), neither did Bowers et al. [9] in bacterial composition from different land-use sites. Seasonal differences in microbial communities have been also detected [10,18,25,74], however, several parameters change altogether throughout the year so it is difficult to analyze the influence of each one separately.

The attempts to detect pathogenic organisms and

allergens using high-throughput sequencing technologies deserve a special remark. During the study conducted by Woo et al. [75] using TAS approach, they were capable of identifying members of the genera *Legionella, Salmonella, Staphylococcus* (potentially dangerous), in addition to *Clostridium perfringens* and *Escherichia coli O157:H7* sequences. The same tactic was followed by Shin et al. [70] to detect fungal allergens such as *Cladosporium* and *Alternaria* both indoors and outdoors. However, the best strategy may be *shotgun* metagenomics, employed by Be et al. [4] and Cao et al. [11]. Thus, sequences from *Streptococcus pneumoniae*, *Klebsiella penumoniae*, *Staphylococcus epidermidis* and fungi *Alternaria, Cladosporium*, and *Aspergillus fumigatus* can be detected in the same analysis and additional information about absolute abundance is provided.

Differential distribution of microorganisms in urban spaces.
One interesting subject is whether particular places in the city have singular microbial communities and its correlation with anthropologic activities. Prussin et al. [62] studied virus-like and bacteria-like particles (VLPs and BLPs) concentration indoors and outdoors at different facilities, showing that the air composition outdoors (with higher VLPs counts) is the main source influencing the concentration of the indoor particles, both VLPs and BLPs. Similarly, Amend and coworkers [1] studied the fungal presence at different sites and countries, analyzing the ITS sequences extracted from dust samples. Besides a latitude connection, they concluded that fungal communities indoors are highly influenced by the outdoor environment, not finding differences among nearby buildings with diverse human activities. Some comparative studies performed at schools and childcare facilities support the idea that fungal populations are similar indoors and outdoors, while bacterial diversity differs due to human occupancy [63,70]. Thus, amplicon-sequencing results have shown common bacterial communities in both environments (indoors and outdoors) with species from *Rhodobacteria* and the genera *Sphingomonas* and *Pseudomonas*, but an increase in the relative abundance of human skin-associated bacteria indoors: *Staphylococcus*, *Corynebacteria* or *Propionibacteria* [18,63,70].

It has been also proposed that areas with high traffic density or sewage pollution have much higher concentrations of airborne bacteria [19,29], suggesting a strong influence of the antropogenic activities as a source of particular airborne biological communities. Accordingly, a metagenomic study conducted by Yooseph and colleagues [77] confirmed a common bacterial population in urban atmosphere with remarked abundance of some genera depending on the use of the building: *Klebsiella* and *Bordetella* in hospitals or marine-related *Plantomyces*, *Pirellula* and *Synechococcus* in piers. Alike, viral communities in the air of Korea can be distinguished based on the land use according to the study of Whon et al. [74].

Barberán and colleagues [3] performed a large-scale study collecting samples over a thousand houses in USA and evaluated dust-associated microorganisms by 16S and ITS analysis. Although they found that microbial communities compositions were highly variable across the United States (explained by climatic and soil variables), their results pointed out that microbial communities in urban air tend to homogenization, with less variability compared with rural areas (in accordance with Bowers et al. [9]). Additionally, Prussin et al. [62] observed that the concentration of VLPs and BLPs was similar between different city locations, supporting that the air is, in fact, a homogeneous fluid distributing microorganisms through all the spaces in the city and providing a common base of microbial diversity.

Nonetheless, despite its singularities as a microbial biome, urban environments are susceptible to external incomes from sporadic events like dust storms. Several studies have analyzed the biological diversity associated to these particles coming from long-distance locations as those conducted by Katra et al. [39] and Maki et al. [51]. Though performed in different regions, they agree the richness of DNA sequences in the air belong to prokaryotes and eukaryotes during these events is significantly increased with uncommon taxa that usually correlates with the soil and vegetation of the dust origin.

Taken all these studies as a whole, they clearly highlight the complexity of the urban airborne dynamics and the requirement to normalize the study procedures to reach a better comprehension of microbial communities in metropolitan environments. In perspective, these results remark the critical importance of the sampling organization (time, volume, synchronized sampling, etc.) and additional annotation regarding meteorological factors, human activity, microbial sources, etc. in order to obtain conclusive and comparable results.

Conclusions and final remarks

Recent studies have shown unexpected roles of the biological particles in the air, proving how important is to get a better knowledge of this habitat for environmental and clinical

reasons. The first challenge to face during the characterization of these airborne particles using NGS technologies is the low abundance of them and, as a result, low amount of DNA. Two approaches can be adopted to solve this problem: (i) to collect large volumes of air in a short time by using high volume cyclone samplers-type; (ii) to sample at lower flows but longer times. Assuming that the abundance and diversity of bioaerosols can shift very fast, the second option seems more appropriate to obtain a more representative sample. Even so, the first approach can be suitable for detecting specific pathogens or allergens in a particular site. Additionally, sampling at different weeks in a season can provide significant information to get a better characterization of the airborne biological diversity in a particular location. Either way, sampling methodology and devices should be adapted to the aims of the study.

Until *shotgun* metagenomics becomes economically more affordable, TAS is still a good proxy despite the loss of functional genes information and absolute abundance. V1-V4 regions within the 16S rRNA gene for bacteria and ITS2 for fungal propagules and spores are the most favored regions for identification and taxonomic assignation in airborne studies (see Table 1). In regards to pollen/plant identification, the discussion about the DNA barcode is still open. Up to now, Kraaijeveld and colleagues [43], using *trnL* as gene marker, have published encouraging results from metropolitan environments comparing with morphological determination. We have also tested the 18S rRNA gene to perform similar assays [27], although poor resolution at genus or species level can be obtained from this marker. More recently, we have evaluated the resolution of ITS2, confirming that this region is more suitable as a genomic marker and overcome the trouble with 18S gene (unpublished data).

Currently, Illumina is the preferred platform for high-throughput DNA sequencing in most researches. However, technical procedures are evolving and advancing strikingly fast. Ion Torrent, PacBio RS system or MinION are very promising platforms but they still need to prove their value in the environmental field.

The studies under reviewed suggest that microbial diversity of urban environments holds singular features, as a particular biome. Fungal, viral and majorly bacteria communities are under the influence of human presence and the building use [63,70,74,77]. Both culture-dependent and -independent studies indicate that the spring and summer seasons correlate with higher abundance and richness of microorganisms in the air and, as a result, an increase in DNA concentration. However, some apparent contradictions

can be found, especially when the abundance of organisms from different taxa are examined. The abundance of some biological entities in the air like bacteria or fungi can shift quickly [20,48,79]. Since most NGS studies are usually restricted to a few discrete days or even a few hours, some disagreements may be expected. Consequently, the application of combined techniques (sequence-based and traditional) to solve the discrepancies is the best strategy until sequencing becomes complete trustworthy, and standard procedures are established. In accordance, NGS yields an overwhelming amount of data, which is processed essentially by computers. Reviewing the results to confirm they are plausible is essential and a multidisciplinary collaboration among microbiologists, botanists and bioinformaticians is highly recommended to curate the NGS outcomes.

On the other hand, culture-independent studies have proved to be useful to detect allergens and airborne pathogens [4,11,70,75]. However, one intrinsic weakness of DNA sequencing methods is that it cannot be distinguished between alive and dead, or complete and fragmented biological particles. For pollen and fungal allergens, even fragments can induce clinical symptoms when a threshold is overpassed [71]. In contrast, pathogenic fungi, viruses, bacteria or resistant spores usually need to be metabolically active to induce any disease. In both cases, any approach for quantification may be quite helpful. The results obtained from TAS permit to calculate relative abundance but we must take into consideration that it is based on several prior PCR amplifications. Although the number of cycles of these reactions is kept at minimum to keep the proportional ratio, the conclusions on relative abundance must be carefully considered. Moreover, the values of abundance can be biased for the number of copies of the selected DNA region. Several fungal genera (e.g. *Alternaria, Leptosphaeria, Stemphylium, Pleospora*) produce multicellular spores, so they contain several copies of genomic DNA, magnifying their representation. A similar effect exists in pollen grains from polyploid species when sequences from genomic DNA are chosen (SSU, LSU or ITS). Likewise, plastid sequences are controversial because of the number of chloroplasts that can be found in pollen grains or if they are even present in the pollen of all species [6,24,43]. As a result, DNA-based scores compared with morphology surveys might differ, so novel and unexpected results must be supported by other methods.

Some attempts to infer absolute abundance from the sequencing outcome have been proposed by some authors [14,59], and recently the RDP Classifier have implemented an algorithm with a gene-copy-number adjustment for bacteria

and fungi to make the analyses more quantitative. Yet, more studies evaluating the effectiveness of these approaches are needed.

Overall, high-throughput sequencing is an outstanding technology with an extraordinary potential to come. Although some adjustments are needed to apply this methodology to metropolitan environments, its qualities for easy identification of any type of organism at once, flexibility to adapt to specific goals and its potential for pathogens and allergens identification make NGS a promising tool for real-time bioaerosols monitoring and revealing the air genome.

References

1. Amend AS, Seifert KA, Samson R, Bruns TD (2010) Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics. Proc Natl Acad Sci USA 107:13748-13753 doi:10.1073/pnas.1000454107

2. Baker GC, Smith JJ, Cowan DA (2003) Review and re-analysis of domain-specific 16S primers. J Microbiol Methods 55:541-555 doi:10.1016/j.mimet.2003.08.009

3. Barberán A, Ladau J, Leff JW, Pollard KS, Menninger HL, Dunn RR, Fierer N (2015) Continental-scale distributions of dust-associated bacteria and fungi. Proc Natl Acad Sci USA 112:5756-5761 doi:10.1073/pnas.1420815112

4. Be NA, Thissen JB, Fofanov VY, Allen JE, Rojas M, Golovko G, Fofanov Y, Koshinsky H, Jain CJ (2015) Metagenomic analysis of the airborne environment in urban spaces. Microb Ecol 69:346-355 doi:10.1007/s00248-014-0517-z

5. Behzad H, Gojobori T, Mineta K (2015) Challenges and opportunities of airborne metagenomics. Genome Biol Evol 7:1216-1226 doi:10.1093/gbe/evv064

6. Bell KL, Burgess KS, Okamoto KC, Aranda R, Brosi BJ (2016) Review and future prospects for DNA barcoding methods in forensic palynology. Forensic Sci Int: Genet 21:110-116 doi:10.1016/j.fsigen.2015.12.010

7. Bertolini V, Gandolfi I, Ambrosini R, Bestetti G, Innocente E, Rampazzo G, Franzetti A (2013) Temporal variability and effect of environmental variables on airborne bacterial communities in an urban area of Northen Italy. Appl Microbiol Biotechnol 97:6561-6570 doi:10.1007/s00253-012-4450-0

8. Bowers RM, McCubbin IB, Hallar AG, Fierer N (2012) Seasonal variability in airborne bacterial communities at a high-elevation site. Atmos Environ 50:41-49 doi:10.1016/j.atmosenv.2012.01.005

9. Bowers RM, McLetchie S, Knight R, Fierer N (2011) Spatial variability in airborne bacterial communities across land-use types and their relationship to the bacterial communities of potential source environments. ISME J 5:601-612 doi:10.1038/ismej.2010.167

10. Bowers RM, Sullivan AP, Costello EK, Collett JL, Knight R, Fierer N (2011) Sources of bacteria in outdoor air across cities in the midwestern United States. Appl Environ Microbiol 77:6350-6356 doi:10.1128/AEM.05498-11

11. Cao C, Jiang WJ, Wang BY, Fang JH, Lang JD, Tian G, Jiang JK, Zhu

12. Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007) A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69:330-339 doi:10.1016/j.mimet.2007.02.005

13. Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, et al. (2010) Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS One 5:e8613 doi:10.1371/journal.pone.0008613

14. Dannemiller KC, Lang-Yona N, Yamamoto N, Rudich Y, Peccia J (2014) Combining real-time PCR and next-generation DNA sequencing to provide quantitative comparisons of fungal aerosol populations. Atmos Environ 84:113-121 doi:10.1016/j.atmosenv.2013.11.036

15. De Antoni Zoppas BC, Valencia-Barrera RM, Vergamini Duso SM, Fernández-González D (2006) Fungal spores prevalent in the aerosol of the city of Caxias do Sul, Rio Grande do Sul, Brazil, over a 2-year period (2001–2002). Aerobiologia 22:119-126 doi:10.1007/s10453-006-9022-2

16. Díez Herrero A, Sabariego Ruiz S, Gutiérrez Bustillo M, Cervigón Morales P (2006) Study of airborne fungal spores in Madrid, Spain. Aerobiologia 22:135-142 doi:10.1007/s10453-006-9025-z

17. Fahlgren C, Bratbak G, Sandaa RA, Thyrhaug R, Zweifel UL (2011) Diversity of airborne bacteria in samples collected using different devices for aerosol collection. Aerobiologia 27:107-120 doi:10.1007/s10453-010-9181-z

18. Fahlgren C, Hagström A, Nilsson D, Zweifel UL (2010) Annual variations in the diversity, viability, and origin of airborne bacteria. Appl Environ Microbiol 76:3015-3025 doi:10.1128/AEM.02092-09

19. Fang Z, Ouyang Z, Zheng H, Wang X, Hu L (2007) Culturable airborne bacteria in outdoor environments in Beijing, China. Microb Ecol 54:487-496 doi:10.1007/s00248-007-9216-3

20. Fierer N, Liu ZZ, Rodriguez-Hernandez M, Knight R, Henn M, Hernandez MT (2008) Short-term temporal variability in airborne bacterial and fungal populations. Appl Environ Microbiol 74:200-207 doi:10.1128/AEM.01467-07

21. Franzetti A, Gandolfi I, Gaspari E, Ambrosini R, Bestetti G (2011) Seasonal variability of bacteria in fine and coarse urban air particulate matter. Appl Microbiol Biotechnol 90:745-753 doi:10.1007/s00253-010-3048-7

22. Fröhlich-Nowoisky J, Pickersgill DA, Després VR, Pöschl U (2009) High diversity of fungi in air particulate matter. Proc Natl Acad Sci USA 106:12814-12819 doi:10.1073/pnas.0811003106

23. Fronczek CF, Yoon JY (2015) Biosensors for monitoring airborne pathogens. J Lab Automat 20:390-410 doi:10.1177/2211068215580935

24. Galimberti A, De Mattia F, Bruni I, Scaccabarozzi D, Sandionigi A, Barbuto M, Casiraghi M, Labra M (2014) A DNA barcoding approach to characterize pollen collected by honeybees. PLoS One 9:e109363 doi:10.1371/journal.pone.0109363

25. Gandolfi I, Bertolini V, Bestetti G, Ambrosini R, Innocente E, Rampazzo G, Papacchini M, Franzetti A (2015) Spatio-temporal variability of airborne bacterial communities and their correlation with particulate matter chemical composition across two urban areas. Appl Microbiol Biotechnol 99:4867-4877 doi:10.1007/s00253-014-6348-5

26. Glenn TC (2011) Field guide to next-generation DNA sequencers. Mol Ecol Resour 11:759-769 doi:10.1111/j.1755-0998.2011.03024.x

27. Gutiérrez-Bustillo AM, Ferencova Z, Núñez A, Alcamí A, Campoy P, Guantes R, Moreno DA (2016) Análisis por técnicas morfológicas y secuenciación de ADN del polen atmosférico de la Comunidad de Madrid: estudios preliminares. Rev Salud Ambient 16:71-77 http://ojs.diffundit.com/index.php/rsa/article/view/804 [In Spanish]

28. Gutiérrez-Bustillo AM, Sáenz C, Aránguez E, Ordóñez JM (2001) Polen

TF (2014) Inhalable microorganisms in Beijing's PM2.5 and PM10 pollutants during a severe smog event. Environ Sci Technol 48:1499-1507 doi:10.1021/es4048472

atmosférico en la Comunidad de Madrid. Documento Técnico de Salud Pública No. 70. Consejería de Sanidad, Comunidad de Madrid, 204 pp. [In Spanish]

29. Harrison RM, Jones AM, Biggins PDE, Pomeroy N, Cox CS, Kidd SP, Hobman JL, Brown NL, Beswick A (2005) Climate factors influencing bacterial count in background air samples. Int J Biometeorol 49:167-178 doi:10.1007/s00484-004-0225-3

30. Hart ML, Meyer A, Johnson PJ, Ericsson AC (2015) Comparative evaluation of DNA extraction methods from feces of multiple host species for downstream next-generation sequencing. PLoS One 10:e0143334 doi:10.1371/journal.pone.0143334

31. Hawkins J, de Vere N, Griffith A, Ford CR, Allainguillaume J, Hegarty MJ, Baillie L, Adams-Groom B (2015) Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences. PLoS One 10:e0134735 doi:10.1371/journal.pone.0134735

32. Hjelmroos M (1993) Relationship between airborne fungal spore presence and weather variables: *Cladosporium* and *Alternaria*. Grana 32:40-47 doi:10.1080/00173139309436418

33. Hoisington AJ, Maestre JP, King MD, Siegel JA, Kinney KA (2014) Impact of sampler selection on the characterization of the indoor microbiome via high-throughput sequencing. Build Environ 80:274-282 doi:10.1016/j.buildenv.2014.04.021

34. Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, van der Bank M, Chase MW, Cowan RS, et al. (2009) A DNA barcode for land plants. Proc Natl Acad Sci USA 106:12794-12797 doi:10.1073/pnas.0905845106

35. Hugerth LW, Muller EEL, Hu YOO, Lebrun LAM, Roume H, Lundin D, Wilmes P, Andersson AF (2014) Systematic design of 18S rRNA gene primers for determining eukaryotic diversity in microbial consortia. PLoS One 9:e95567 doi:10.1371/journal.pone.0095567

36. Jiang WJ, Liang P, Wang BY, Fang JH, Lang JD, Tian G, Jiang JK, Zhu TF (2015) Optimized DNA extraction and metagenomic sequencing of airborne microbial communities. Nat Protoc 10:768-779 doi:10.1038/nprot.2015.046

37. Kaarakainen P, Meklin T, Rintala H, Hyvärinen A, Kärkkäinen P, Vepsäläinen A, Hirvonen MR, Nevalainen A (2008) Seasonal variation in airborne microbial concentrations and diversity at landfill, urban and rural sites. Clean-Soil, Air, Water 36:556-563 doi:10.1002/clen.200700179

38. Karlsson OE, Belák S, Granberg F (2013) The effect of preprocessing by sequence-independent, single-primer amplification (SISPA) on metagenomic detection of viruses. Biosecur Bioterror 11(Suppl 1): S227-S234 http://online.liebertpub.com/doi/pdf/10.1089/bsp.2013.0008

39. Katra I, Arotsker L, Krasnov H, Zaritsky A, Kushmaro A, Ben-Dov E (2014). Richness and diversity in dust stormborne biomes at the southeast Mediterranean. Sci Rep 4:5265 doi:10.1038/srep05265

40. Keller A, Danner N, Grimmer G, Ankenbrand M, von der Ohe K, von der Ohe W, Rost S, Härtel S, Steffan-Dewenter I (2015) Evaluating multiplexed next-generation sequencing as a method in palynology for mixed pollen samples. Plant Biol 17:558-566 doi:10.1111/plb.12251

41. Kim M, Lee KH, Yoon SW, Kim BS, Chun J, Yi H (2013) Analytical tools and databases for metagenomics in the next-generation sequencing era. Genom Inform 11:102-113 doi:10.5808/GI.2013.11.3.102

42. Kim M, Morrison M, Yu Z (2011) Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. J Microbiol Methods 84:81-87 doi:10.1016/j.mimet.2010.10.020

43. Kraaijeveld K, de Weger LA, Ventayol García M, Buermans H, Frank J, Hiemstra PS, den Dunnen JT (2015) Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. Mol Ecol Resour 15:8-16 doi:10.1111/1755-0998.12288

44. Kumar B, Gupta GP, Singh S, Kulshrestha UC (2013) Study of abundance and characterization of culturable bioaerosol at Delhi, India. Int J Environ Res Manag 4:219-226 http://www.ripublication.com/ijeem_spl/ijeemv4n3_10.pdf

45. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics, pp 115-147

46. Lasken RS (2009) Genomic DNA amplification by the multiple displacement amplification (MDA) method. Biochem Soc Trans 37:450-453 doi:10.1042/BST0370450

47. Li H, Zhang Y, Li DS, Xu H, Chen GX, Zhang CG (2009) Comparisons of different hypervariable regions of rrs genes for fingerprinting of microbial communities in paddy soils. Soil Biol Biochem 41:954-968 doi:10.1016/j.soilbio.2008.10.030

48. Lighthart B (1997) The ecology of bacteria in the alfresco atmosphere. FEMS Microbiol Ecol 23:263-274 doi:10.1111/j.1574-6941.1997.tb00408.x

49. Liu J, Yu Y, Cai Z, Bartlam M, Wang Y (2015) Comparison of ITS and 18S rDNA for estimating fungal diversity using PCR-DGGE. World J Microbiol Biotechnol 31:1387-1395 doi:10.1007/s11274-015-1890-6

50. Liu Z, DeSantis TZ, Andersen GL, Knight R (2008) Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. Nucleic Acids Res 36:e120 doi:10.1093/nar/gkn491

51. Maki T, Puspitasari F, Hara K, Yamada M, Kobayashi F, Hasegawa H, Iwasaka Y (2014) Variations in the structure of airborne bacterial communities in a downwind area during an Asian dust (Kosa) event. Sci Total Environ 488-489:75-84 doi:10.1016/j.scitotenv.2014.04.044

52. Methé BA, Nelson KE, Pop M, Creasy HH, Giglio MG, Huttenhower C, Gevers D, Petrosino JF, et al. (2012) A framework for human microbiome research. Nature 486:215-221 doi:10.1038/nature11209

53. Mouli PC, Mohan SV, Reddy SJ (2005) Assessment of microbial (bacteria) concentrations of ambient air at semi-arid urban region: Influence of meteorological factors. Appl Ecol Environ Res 3:139-149

54. Naruka K, Gaur J (2014) Distribution pattern of airborne bacteria and fungi at market area. Am-Eurasian J Sci Res 9:186-194 doi:10.5829/idosi.aejsr.2014.9.6.86254

55. Núñez A, Amo de Paz G, Rastrojo A, García AM, Alcamí A, Gutiérrez-Bustillo AM, Moreno DA (2016) Monitoring of the airborne biological particles in outdoor atmosphere. Part 1: Importance, variability and ratios. Int Microbiol 19:1-13 doi:10.2436/20.1501.01.258

56. Oh SY, Fong JJ, Park MS, Chang L, Lim YM (2014) Identifying airborne fungi in Seoul, Korea using metagenomics. J Microbiol 52:465-472 doi:10.1007/s12275-014-3550-1

57. Pashley CH, Fairs A, Free RC, Wardlaw AJ (2012) DNA analysis of outdoor air reveals a high degree of fungal diversity, temporal variability, and genera not seen by spore morphology. Fungal Biol 116:214-224 doi:10.1016/j.funbio.2011.11.004

58. Pearce DA, Hughes KA, Lachlan-Cope T, Harangozo SA, Jones AE (2010) Biodiversity of air-borne microorganisms at Halley station, Antarctica. Extremophiles 14:145-159 doi:10.1007/s00792-009-0293-8

59. Pitkäranta M, Meklin T, Hyvärinen A, Paulin L, Auvinen P, Nevalainen A, Rintala H (2008) Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Appl Environ Microbiol 74:233-244 doi:10.1128/AEM.00692-07

60. Polymenakou PN, Mandalakis, M (2013) Assessing the short-term variability of bacterial composition in background aerosols of the eastern Mediterranean during a rapid change of meteorological conditions. Aerobiologia 29:429-441 doi:10.1007/s10453-013-9295-1

61. Polymenakou PN, Mandalakis M, Stephanou EG, Tselepides A (2008) Particle size distribution of airborne microorganisms and pathogens during an intense African dust event in the eastern Mediterranean. Environ Health Persp 116:292-296 doi:10.1289/ehp.10684

62. Prussin AJ, Garcia EB, Marr LC (2015) Total concentrations of virus and bacteria in indoor and outdoor air. Environ Sci Technol Lett 2:84-88 doi:10.1021/acs.estlett.5b00050

63. Qian J, Hospodsky D, Yamamoto N, Nazaroff WW, Peccia J (2012) Size-resolved emission rates of airborne bacteria and fungi in an occupied classroom. Indoor Air 22:339-351 doi:10.1111/j.1600-0668.2012.00769.x

64. Rosario K, Nilsson C, Lim YW, Ruan Y, Breitbart M (2009) Metagenomic analysis of viruses in reclaimed water. Environ Microbiol 11:2806-2820 doi:10.1111/j.1462-2920.2009.01964.x

65. Rosseel T, Van Borm S, Vandenbussche F, Hoffmann B, van den Berg T, Beer M, Höper D (2013) The origin of biased sequence depth in sequence-independent nucleic acid amplification and optimization for efficient massive parallel sequencing. PLoS One 8:e76144 doi:10.1371/journal.pone.0076144

66. Salipante SJ, Sengupta DJ, Rosenthal C, Costa G, Spangler J, Sims EH, Jacobs MA, Miller SI, et al. (2013) Rapid 16S rRNA next-generation sequencing of polymicrobial clinical samples for diagnosis of complex bacterial infections. PLoS One 8:e65226 doi:10.1371/journal.pone.0065226

67. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W; Fungal Barcoding Consortium Author List (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA 109:6241-6246 doi:10.1073/pnas.1117018109

68. Seifried JS, Wichels A, Gerdts G (2015) Spatial distribution of marine airborne bacterial communities. MicrobiologyOpen 4:475-490 doi:10.1002/mbo3.253

69. Sharpton TJ (2014) An introduction to the analysis of shotgun metagenomic data. Front Plant Sci 5:209 doi:10.3389/fpls.2014.00209

70. Shin SK, Kim J, Ha SM, Oh HS, Chun J, Sohn J, Yi H (2015) Metagenomic insights into the bioaerosols in the indoor and outdoor environments of childcare facilities. PLoS One 10:e0126960 doi:10.1371/journal.pone.0126960

71. Taylor PE, Flagan RC, Miguel AG, Valenta R, Glovsky MM (2004) Birch pollen rupture and the release of aerosols of respirable allergens. Clin Exp Allergy 34:1591-1596 doi:10.1111/j.1365-2222.2004.02078.x

72. Thurber RV, Haynes M, Breitbart M, Wegley L, Rohwer F (2009) Laboratory procedures to generate viral metagenomes. Nat Protoc 4:470-483 doi:10.1038/nprot.2009.10

73. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal rna genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). PCR protocols: a guide to methods and applications. Academic Press, New York, pp. 315-322

74. Whon TW, Kim MS, Roh SW, Shin NR, Lee HW, Bae JW (2012) Metagenomic characterization of airborne viral DNA diversity in the near-surface atmosphere. J Virol 86:8221-8231 doi:10.1128/JVI.00293-12

75. Woo AC, Brar MS, Chan YK, Lau MCY, Leung FCC, Scott, JA, Vrijmoed LLP, Zawar-Reza P, Pointing SB (2013) Temporal variation in airborne microbial populations and microbially-derived allergens in a tropical urban landscape. Atmos Environ 74:291-300 doi:10.1016/j.atmosenv.2013.03.047

76. Yamamoto N, Bibby K, Qian J, Hospodsky D, Rismani-Yazdi H, Nazaroff WW, Peccia J (2012) Particle-size distributions and seasonal diversity of allergenic and pathogenic fungi in outdoor air. ISME J 6:1801-1811 doi:10.1038/ismej.2012.30

77. Yooseph S, Andrews-Pfannkoch C, Tenney A, McQuaid J, Williamson S, Thiagarajan M, Brami D, Zeigler-Allen L, Hoffman J, Goll JB, Fadrosh D, Glass J, Adams MD, Friedman R, Venter JC (2013) A metagenomic framework for the study of airborne microbial communities. PLoS One 8:e81862 doi:10.1371/journal.pone.0081862

78. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ (2012) Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One 7:e33865 doi:10.1371/journal.pone.0033865

79. Zhu H, Phelan PE, Duan T, Raupp GB, Fernando HJS, Che F (2003) Experimental study of indoor and outdoor airborne bacterial concentrations in Tempe, Arizona, USA. Aerobiologia 19:201-211 doi:10.1023/B:AERO.0000006571.23160.8a