

Article

Investigation of Sources, Diversity, and Variability of Bacterial Aerosols in Athens, Greece: A Pilot Study

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Abstract: We characterized the composition, diversity, and potential bacterial aerosol sources in Athens' urban air by DNA barcoding (analysis of 16S rRNA genes) during three seasons in 2019. Air samples were collected using the recently developed Rutgers Electrostatic Passive Sampler (REPS). It is the first field application of REPS to study bacterial aerosol diversity. REPS samplers captured a sufficient amount of biological material to demonstrate the diversity of airborne bacteria and their variability over time. Overall, in the air of Athens, we detected 793 operational taxonomic units (OTUs), which were fully classified into the six distinct taxonomic categories (Phylum, Class, Order, etc.). These OTUs belonged to Phyla *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria*, and *Fusobacteria*. We found a complex community of bacterial aerosols with several opportunistic or potential pathogens in Athens' urban air. Referring to the available literature, we discuss the likely sources of observed airborne bacteria, including soil, plants, animals, and humans. Our results on bacterial diversity are comparable to earlier studies, even though the sampling sites are different or geographically distant. However, the exact functional and ecological role of bioaerosols and, even more importantly, their impact on public health and the ecosystem requires further air monitoring and analysis.

Keywords: bioaerosols; 16S rRNA gene; bacteria; health; REPS sampler; urban air; taxonomy; Greece



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1. Introduction

Bioaerosols are airborne particles of biological origin, including pathogenic and non-pathogenic, viable and nonviable, culturable and nonculturable microorganisms, and their products (e.g., toxins, cysts, or fragments), high molecular weight allergens, as well as pollen. The size of individual bioaerosol particles and the agglomerates containing them ranges from tens of nanometres to about a tenth of a millimetre [1–3]. Bioaerosols play a vital role in the Earth's ecosystem, particularly in the interactions between the atmosphere, biosphere, climate, and public health. Over the past few years, bioaerosol science has evolved into a multidisciplinary field with contributions and interest from aerosol science, biology, toxicology, ecology, plant and animal pathology, microbiology, air quality, public health, and meteorology.

Despite the importance of bioaerosols, their sources, abundance, diversity, and interactions within the atmospheric microbiome are not yet sufficiently well characterized and understood. Terrestrial, marine ecosystems, and biosphere are major sources of atmospheric bioaerosols. In general, bioaerosols are readily released from agricultural and waste facilities and various urban and industrial activities [4,5]. Humans and animals are also well-known sources of bioaerosols. Bioaerosol release and dispersion from environmental sources are often facilitated by meteorological processes, such as buoyant air masses created by solar heat or wind shear. Meteorological conditions (e.g., wind speed, relative humidity,

temperature, and solar radiation) and physicochemical factors affect the diversity and dispersion of airborne microbial communities [6–8]. For example, high relative humidity and rain have been negatively associated with bacterial diversity, since moisture intensifies their deposition by increasing particle sizes, and wet soil surfaces make aerosolization less likely [9].

Once airborne, bioaerosols can be transported over substantial distances, including across oceans, depending on particle size and prevailing air currents [10,11]. Such dispersal is conducive to spreading human diseases and agricultural pathogens across and even between countries [10]. Bioaerosol viability in the air, a key factor in disease spread, depends on microorganism biology, physiology, and ability to survive in challenging environmental conditions [2]. Overall, it is estimated that global emissions of bacteria attached to airborne particulate matter range from 0.7 to 28.1 Tg a⁻¹ [2,10]. Other studies suggested that the average number flux of bacterial and fungal spores over continental regions is $\sim 10^2$ m⁻² s⁻¹ [12], reflecting an intense and rapid exchange of biological matter and genetic information between the atmosphere and biosphere.

The bioaerosol source type, natural or anthropogenic, affects the composition of airborne microbial communities. Usually, individual airborne microorganisms are attached to larger particles [13]. Aggregation seems to favour cell survival, but this comes at the cost of airborne time and reduces the potential dispersion distance. Hence, aerial dissemination of bacteria is a compromise between the distance travelled (which decreases for large aggregates) and the chances of successful dissemination, which increases for large aggregates [14].

The role of bioaerosols in disease transmission has been recognized for years [1,2,15], and, as we have seen, it also plays a very important role in the current COVID-19 pandemic. At the same time, we increasingly recognize the importance of bioaerosols in atmospheric processes. Once released from the biosphere into the atmosphere, bioaerosols undergo various physical and chemical aging processes (e.g., coagulation, surface coating, reaction with photo-oxidants, etc.) and serve as cloud condensation and ice nuclei, leading to the formation of clouds and precipitation [10,14,16,17]. Bioaerosols are removed from the air via dry and wet deposition and might interact with terrestrial or aquatic ecosystems and the biosphere [2,10], triggering various biological processes.

Given the interest in bioaerosols due to their role in environmental and health processes, numerous studies have determined their concentration and species found in the atmosphere [18]. Until relatively recently, such air microbiome studies have been limited to the use of culture-based methods, which are known to determine only a fraction of airborne microorganisms; cultured bacteria were typically classified only as Gram-positive or Gram-negative, or one estimated number of colony-forming units (CFU) of airborne bacteria and fungi without providing high-level taxonomic identification [10]. Advances in next-generation sequencing tools were quickly adapted [19–23] to investigate the presence and species of bioaerosols, especially bacteria [2,7,8,24]. Such studies suggested that airborne bacteria often belong to groups associated with common soil bacteria, such as the Terrabacteria category, as well as human and animal commensals [18,25].

Yet only a relatively limited number of studies on the composition of airborne bacteria have been carried out using Next Generation Sequencing [2,8,10,26]. Particularly, in Greece, to date, only a few such studies have been published: e.g., the study of the airborne microbiome during a dust event in island Crete [15], the first microbial characterization of PM10 in the Athens METRO underground railway system [27], or the variability of airborne bacteria in Thessaloniki [28]. Thus, the main goal of this project was to perform a pilot study to investigate the diversity of bacterial aerosols over the downtown of Athens, using a barcode amplicon sequencing method. Another novel element of the study was the field application of Rutgers Electrostatic Passive Sampler (REPS) [29] to passively capture bacterial aerosols over several days for their subsequent sequencing—the first such application of this device.

2. Materials and Methods

We studied the taxonomy and diversity of bacteria in the air of Athens. First, air samples were collected using a novel passive sampler, and the captured microorganisms were analysed using next-generation sequencing. Local meteorological conditions such as temperature, relative humidity, and solar radiation were recorded by the Institute of Environmental Research and Sustainable Development (I.E.R.S.D) during the three sampling periods by the meteorological station of the National Observatory of Athens at Thisseion (<https://www.iersd.noa.gr/>, accessed on 20 October 2020). In addition, the Ministry of Environment constantly monitors Athens' air quality (<https://ypen.gov.gr/perivallon/poiotita-tis-atmosfairas/dedomena-metriseon-atmosfairikis-rypansis/>, accessed on 20 October 2020), and the data could be easily accessed.

2.1. Sampling Method

Bioaerosols were collected using Rutgers Electrostatic Passive Sampler (REPS) [29]. This recently developed sampler is completely passive (i.e., there is no active air mover to provide airflow). It uses a specially configured and permanently polarized ferroelectric polymer film (e.g., polyvinylidene fluoride, or PVDF) to capture electrically charged airborne particles by the electrostatic field in addition to particle capture due to their gravitational settling. REPS uses a 70 mm × 130 mm strip of PVDF film wound into three evenly spaced layers held by a 3D-printed film holder (Figure 1a). This holder configuration creates the film spiral frame, where oppositely polarized film sides are spaced at 2.25 mm [29]. The resulting parallel film layers generate an electrostatic field between them (Figure 1b). Due to the field fringing effect, electrostatic field lines extend past the top of REPS and attract charged bioaerosol particles into the sampler. These particles are then deposited on the PVDF film and subsequently eluted for analysis. So far, the sampler has been used to measure the total and culturable bacteria and fungi in the air [29,30]. The sampling time could be as long as several days because the sampler does not require air movers and power supplies, which was taken advantage of in this project.

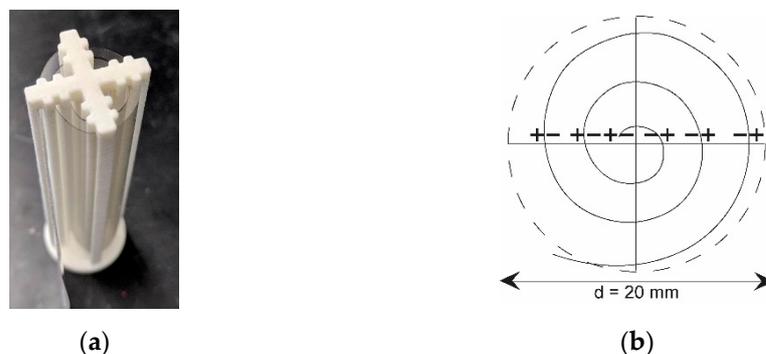


Figure 1. (a) REPS Sampler. (b) A top-view schematic showing the positioning of PVDF film inside the REPS film holder. The overlapping positive (+) and negative (−) film faces create an electrostatic field between the layers to attract and collect particles.

2.2. Sampling Location and the Number of Samples

Three continuous sampling campaigns of 6 to 10 days each, depending on weather conditions, were conducted in June, August, and October of 2019, in the vicinity of the Thisseion meteorological and environmental station of the National Observatory of Athens (N.O.A.: 37°58'24" N, 23°43'6" E). The samplers were placed on the roof of the N.O.A. building (3 m above ground level). In June and August, samples were collected for 10 consecutive days; in October, the sampling was stopped after 6 days to prevent the collected samples from being washed off by the oncoming rain. Five REPS samplers were used in each campaign, and they were separated by approximately 20 cm distance. In addition, we used two control REPS in each campaign: one brought into the field but not

used (internal control) and another REPS that was used to sample for 1 min to check for potential sample contamination during lab analysis and transfer of samplers.

2.3. Sample Preparation and Elution

The REPS were transferred to the laboratory immediately after completing the sampling. The captured particles were eluted into the liquid for DNA extraction and subsequent analysis. Because the amount of captured microorganisms varied between sampling campaigns and individual samplers in the same campaign, sample elution protocols were modified between campaigns to ensure efficient sample elution and sufficient DNA amount in the eluant.

For June samples, particles captured by REPS were extracted using an earlier published protocol [29]. Briefly, each REPS was inserted into a sterile 50 mL tube, and 35 mL of sterile PBS was added. The tubes were vortexed for 2 min and then sonicated for 10 min. The tubes were vigorously shaken, and the eluate with captured particles was removed for further analysis. However, since the volume of elution liquid (35 mL) for each REPS was large, we centrifuged the eluant from each sample and pooled the resulting five pellets into one sample (labelled “June”) to reach the average DNA concentration $> 0.25 \text{ ng}/\mu\text{L}$, i.e., above the threshold needed to proceed with NGS analysis. The final sample volume of the combined five June samples before DNA extraction was approximately 1.5 mL. The DNA concentration in both June controls was below the detection limit.

Because of the relatively large volume of liquid used to elute REPS samples in June and potential sample losses during centrifugation, we used a different sample processing technique in August and October to improve detection sensitivity. Here, after sampling, inside a clean containment, PVDF film from each REPS was cut into 12 pieces and put in two tubes (2 mL) for each sample in the lab with autoclaved scissors. Then, the samples were incubated at 56°C overnight in PowerSoil bead solution, lysis solution C1, and proteinase K prior to DNA extraction.

Still, because of potentially low DNA concentration, five samples from August and five from October were pooled to reach an average DNA concentration of $>25 \text{ ng}/\mu\text{L}$ per pooled sample. According to the month of sampling, the resulting two samples are labelled “August” and “October”. Among the control samples, only one-minute sampling controls had detectable DNA concentrations. They are labelled 415c for August and 417c for October. Thus, a total of three resulting samples (“June”, “August”, and “October”) and two controls (415c and 417c) were analysed by the NGS. The final DNA concentration range in samples was $0.25\text{--}50 \text{ ng}/\mu\text{L}$, and, in controls, the DNA concentration was $<0.7 \text{ ng}/\mu\text{L}$. The proportional contribution of individual samples to the pooled samples was adjusted to properly reflect the contribution of individual samples to the total abundance. On the other hand, the air volume from which particles were captured by REPS during each sampling campaign is unknown because of the nature of passive sampling. Additionally, since the sampling efficiency of REPS is affected by meteorological conditions (unpublished data of Manibusan S. and Mainelis G.), the amount of biological material captured between campaigns could be compared only qualitatively.

Contamination Prevention

Given the low biological content, samples were susceptible to bias due to potential laboratory and environmental contamination. Thus, we made an extensive effort to avoid contamination during sample collection, pre-treatment, and DNA extraction steps. When the REPS were prepared at Rutgers University, they were thoroughly cleaned with 75% (*v/v*) ethanol inside a Class II Biosafety cabinet. The samplers were then placed in 50 mL tubes while inside the cabinet. All the tubes and tools used in sampling and analysis were sterilized (autoclaved, if possible) or cleaned with 75% (*v/v*) ethanol before use. We used sterile surgical gloves and face masks in all steps of the analysis. The degree of potential contamination was assessed using two different negative controls, as described above. Contamination prevention is a crucial study design aspect because any contamination of

samples before field deployment might have an outsize effect on the results. Therefore, specific measures and precautions in all steps of sampling and analysis have to be taken. This issue is common across the entire microbial ecology field and is especially important in studies where DNA yields are expected to be low [31].

2.4. DNA Extraction and Analysis

Genomic DNA from the eluate was extracted using the DNeasy PowerSoil (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The DNeasy PowerSoil Kit uses a humic substance/brown colour removal procedure. Aliquots of the eluate were added to bead-beating tubes for rapid and thorough homogenization. Total genomic DNA was captured on a silica membrane in a spin column format. DNA was then washed and eluted from the membrane and quantified using a NanoDrop Spectrophotometer (Thermo, Wilmington, NC, USA). The isolated DNA in ultrapure water was then available for PCR analysis and other downstream applications. Bacterial and archaeal amplicon diversity of samples was characterized by a barcoded amplicon sequencing method under the trademark service bTEFAP[®] in a commercial laboratory (Molecular Research L.P., aka MrDNA, Shallowater, TX, USA). Before sequencing, the bacterial 16S rRNA gene sequences were amplified by PCR with universal primer sets (515F bacterial + archaeal). They were sequenced using Ion Torrent (Ion S5 XL), with a reading length of 300 bp and nominal 15–20,000 reads/assay. Sequencing data were processed using the company's proprietary analysis pipeline.

In summary, sequences were depleted of barcodes and primers, then sequenced <150 bp. First, sequences with ambiguous base calls and with homopolymers and runs exceeding 6 bp were removed. Next, sequences were de-noised, operational taxonomic units (OTUs) were generated, and chimeras were deleted. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a database derived from RDP (<http://rdp.cme.msu.edu>, accessed on 16 February 2020) and NCBI (www.ncbi.nlm.nih.gov, accessed on 16 February 2020). For bacteria, the generally accepted levels of discrimination are 99% similarity for strains, 97–99% for species, and 95–97% for genera [32].

Quality Control Steps to Avoid Possible Contamination and Low Taxonomical Parameters of OTUs

First quality control step. The microbial groups were identified through OTU analyses in tables where sequences were clustered together according to the sequence identity using a web database (NCBI BLAST). If an OTU was detected only in controls or its counts in a control sample were >10% of its total count in samples of the same campaign, this OTU was deleted. Next, the data of pooled samples were normalized and weighted. During this phase, we selected 805 OTUs from all analysed samples with high taxonomical parameters (identity id > 97%, bit score > 300, and e -value < $2.17e^{-79}$). There is the potential for primers to cross-react with non-target organisms. Thus, the bacterial assay can cross-react with some eukaryote plastids (chloroplast or mitochondria). This cross-reaction in our samples gave 8 fungal OTUs (phylum: 6 *Ascomycota*, 2 *Basidiomycota*); and 3 OTUs of *Viridiplantae* phylum *Streptophyta* with various counts across the samples. Of the 805 OTUs classified at the species level, 793 belonged to bacteria, and 365 were duplicates because several OTUs were classified as the same species but with different taxonomical parameters. In the last step, we uploaded our data as a key summary table containing only bacterial features (OTUs), abundance information across samples, along with sample metadata and taxon tables to the MicrobiomeAnalyst [33] tool for further analysis.

A second data quality control step was conducted by MicrobiomeAnalyst (e.g., minimum counts, prevalence, normalization, and cut-off values). The observed abundance of OTUs was rarefied to exclude low abundance (cut off = 4) features and improve the presentation of the data. Counts of the duplicates at each taxon level were merged in the final abundance catalogue. Features with very few counts were filtered based on their

abundance levels (minimum counts = 4) across samples (prevalence 20%). By default, features with zero counts across all the samples or only appearing in one sample were removed from further analysis. Taxa with counts less than 10 were merged into the “others” category, except for species (<100 counts) and genus taxa (<30 counts). After the second quality control step, we had 253 OTUs with approximately 86,000 counts across all samples.

The following sequences with metadata table were submitted (SUB8614146) to the NCBI SRA database. BioSample accessions numbers are: SAMN17098068, SAMN17098069, SAMN17098070, SAMN17098071, SAMN17098072.

3. Results

3.1. Meteorological Conditions during Sampling

The average daily values (average \pm standard deviation) of meteorological and air quality conditions during the days of sampling campaigns are shown in Table 1. In June, the highest daily total solar radiation (7241 W/m²) was recorded, while in August, the highest daily temperature (average $t = 28.8$ °C) and wind speed (3.9 m/s) were observed. Ozone concentration was highest in August (31.43 ppb), SO₂ concentration was highest in October (2.7 ppb), and NO₂ concentration was highest in June (29.05 ppb).

Table 1. Weather conditions and air pollutant levels during the sampling days of the three campaigns in Athens, 2019.

	Month	SOLTOT SUM Day, W/m ²	T, °C	RH, %	Wind Speed, m/s	O ₃ , ppb	SO ₂ , ppb	NO ₂ , ppb
Average	June	7240.9	28.6	46.0	2.5	9.39	1.37	29.05
St. deviation		1431.3	1.7	6.0	0.8	4.1	1.02	9.47
Average	August	7188.8	28.8	47.6	3.9	31.43	0.84	21.23
St. deviation		335.6	1.3	5.9	1.6	10.43	0.17	7
Average	October	3982.9	21.9	66.8	2.0	11.95	2.7	13.69
St. deviation		870.2	0.5	9.5	0.6	11.95	1.61	5.46

3.2. Description of Raw Data

We initially identified 793 OTUs based on taxonomical parameters after the first quality control step that were classified in 10 phyla with more than 100,000 counts for total actual abundance. After the second quality control step, we had 253 OTUs with approximately 86,000 counts across all samples.

3.2.1. The Abundance of Bacterial Aerosols at the Phylum Level

When actual abundance data of each of the three campaigns were converted into relative abundance % (fraction of the total abundance per campaign), the phyla *Firmicutes* comprised 0.75% of samples in June, 36.28% in August, and 59.65% in October. *Proteobacteria* were 98.55% in June, 29.89% in August, and 13.82% in October. *Actinobacteria* reached 0.32% in June, 27.76% in August, and 22.13% in October. *Cyanobacteria*, *Bacteroidetes*, and *Fusobacteria* were minor (~2%) in August and October and rare phyla (<1%) in June.

3.2.2. The Abundance of Bacterial Aerosols at the Class, and Order, and Family Level

The actual abundance at the Class level is shown in Figure 2. Blanks 415c and 417c (i.e., controls) are included for comparison, and it is obvious that the control samples had very low abundance and variability compared to the actual samples. Moreover, the relative abundance % of the main bacterial classes across the three campaigns was estimated. In June, *Gammaproteobacteria* (55%) and *Alphaproteobacteria* (20%) were the dominant classes. The abundance of *Bacilli*, *Clostridia*, *Betaproteobacteria*, *Cytophagia*, and *Actinobacteria* ranged in descending order between 8% and 3%. In August, *Bacilli* (26%) and *Actinobacteria* (13%) were the dominant classes. Abundant also were the classes of *Clostridia*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (~10%). In

October, *Actinobacteria* (26%) and *Clostridia* (22%) were the dominant classes, while *Bacilli*, *Gammaproteobacteria*, *Betaproteobacteria*, and *Alphaproteobacteria* reached more than (10%).

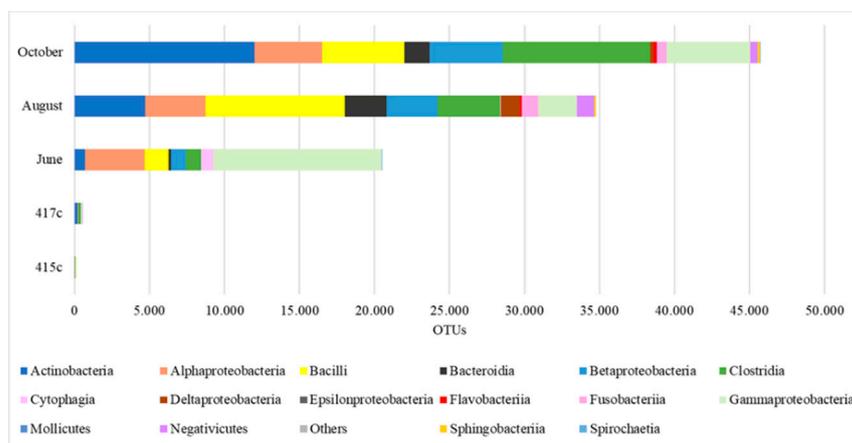


Figure 2. The actual abundance of bacterial aerosols in the urban air of Athens, at the Class level.

In August and October, the dominant bacterial orders were *Lactobacillales*, *Actinomycetales*, and highly abundant *Bacillales*, *Clostridiales*, *Pseudomonadales*. In June, the dominant order was *Rhizobiales* and abundant *Pseudomonadales*. In August the orders *Neiseriales*, *Rhodobacterales*, *Selenomonadales*, and *Bacteroidales* were abundant.

Moreover, the main Families of bacterial aerosols in June were *Rhizobiaceae* (62%) and *Pseudomonadaceae* (18%). In August, the main families were *Corynebacteriaceae* (14%), *Peptoniphilaceae* (11%), and *Bacillales* (10%), while in October, *Streptococcaceae* (35%) and *Corynebacteriaceae* (16%).

3.2.3. The Abundance of Bacterial Aerosols at the Genus Level

The diagram of the relative abundance (% , with RA > 1%) of airborne bacterial Genera during 2019 is shown in Figure 3; RA is shown per campaign. The main genera in June were *Agrobacterium* (57%), *Pseudomonas* (18%), *Methylobacterium* (7%), *Rhizobium* (6%), *Mesorhizobium*, and *Stenotrophomonas* both (~4%). In August, the main genera were *Corynebacterium* (13%) and *Gemella* (10%), and *Staphylococcus* (5%). In October, the main genera were *Streptococcus* (34%) and *Corynebacterium* (16%), *Gemella* (8%), and *Lactobacillus* (6%).

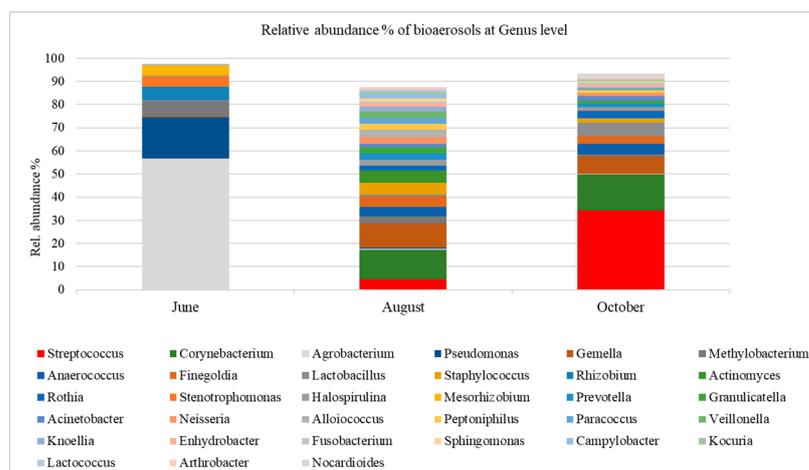


Figure 3. The relative abundance % of bacterial aerosols at the genus level in the urban air of Athens.

3.2.4. The Abundance of Bacterial Aerosols at the Species Level

The diagram of the relative abundance % (RA > 1%) of bacterial species of urban air of Athens during 2019 is shown in Figure 4, with independent scale for each column or campaign. In June, the main species were *Agrobacterium tumefaciens* (53%) and *Pseudomonas pseudoalcaligenes* (18%). The minor ones (5% – 4%) were *Rhizobium* sp., *Methylobacterium tardum*, *Mesorhizobium* sp., and *Stenotrophomonas maltophilia*. In August, the main species were *Sphingomonas* sp. (7%), *Corynebacterium diphtheriae* (5%), and less abundant are *Finegoldia magna* and *Corynebacterium matruchotii*. In October, the main species were *Streptococcus tigurinus* (12%), *Streptococcus sanguinis* (10%), *Corynebacterium tuberculo-stearicum*, *Streptococcus* sp. (5%). *Lactobacillus gallinarum*, *Streptococcus sinensis*, *Anaerococcus* sp. reached a relative abundance (~4%), while *Finegoldia magna*, *Streptococcus parasanguinis*, and *Gemella* sp. were less abundant.

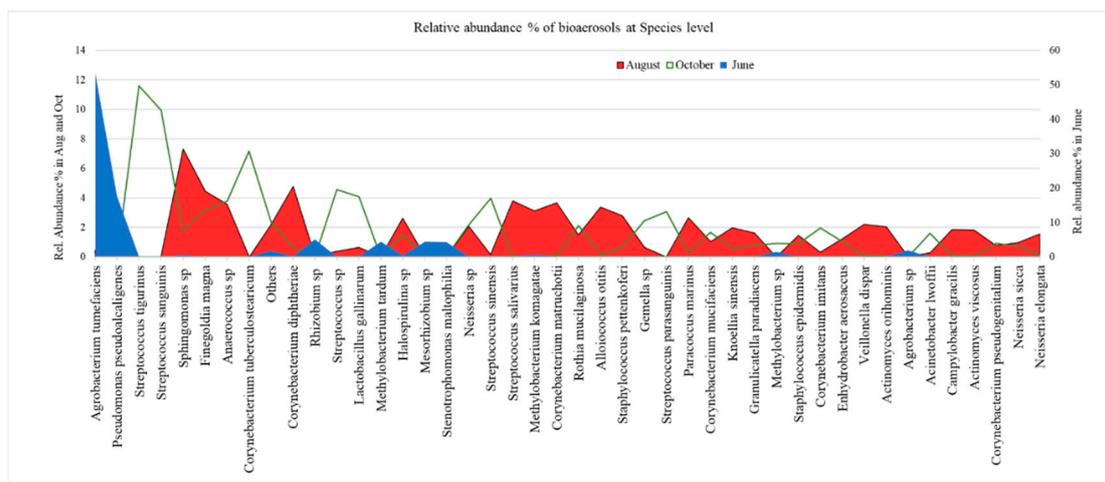


Figure 4. The relative abundance % of bacterial aerosols at the species level in the urban air of Athens.

4. Discussion

This pilot study analyses and describes the composition, diversity, and potential sources of bacterial aerosols observed in Athens' urban air at Thisseion N.O.A.'s station. In addition, this is the first field application of a new passive sampler REPS to study the microbial diversity of aerosols by NGS. Despite the pilot nature of the study and only three sampling campaigns 6–10 days long, REPS samplers were able to capture a sufficient amount of biological material to demonstrate the diversity of airborne bacteria and their variability over time. In June, we found 32 different bacterial species, in August—117, and in October—126, with an abundance of more than 10 OTUs for each species. Our controls had very low OTU levels compared to the samples: some OTUS were detected only in October control, belonging mainly to the genus of *Streptococcus*. We acknowledge that quantifying 16S rRNA sequences is a proxy for the total abundance, as it is subject to bias because some bacteria carry more than one copy of the 16S rRNA gene and DNA extraction and sequencing biases are inherent to these techniques (e.g., NGS). Therefore, this result should only be interpreted as the abundance of detected bacterial 16S rRNA genes [31]. The number of copies of the 16S rRNA gene, commonly used as a measure of microbial biomass, is not directly related to bacterial cell number due to the presence of multiple ribosomal operons across bacterial genomes and is a limitation as a result of differences in cell DNA content and DNA extraction biases [34].

Our decontamination procedures and additional data filtration steps to remove low-quality or likely contaminant features minimized errors in data analysis. Low OTU levels in controls demonstrate that the diversity and abundance found in air samples were not due to contamination but reflect the presence of bacteria in the air.

Low diversity and abundance in June samples could be attributed to the used sample elution technique (high initial eluate volume followed by centrifugation), possible losses during DNA extraction, as well as a sudden drizzle in the middle of this sampling period (with 4 days left), which could have washed off part of the captured bacteria. October is the richest month in terms of the diversity of the bacterial communities (Figures 2–4). However, August sampling was conducted in the middle of the month, during a very hot, dry period. August is also the main season for summer vacations, which minimizes human activities in the city and their potential contribution to bioaerosol presence and diversity. Additionally, a sampling of >7 days under dry and hot conditions might have resulted in extreme stress to the cells captured by the sampler, thus affecting the quality of their DNA.

According to existing studies, bioaerosol composition and abundance varies site-to-site, and there is no single, standardized description of urban airborne bacteria diversity [10,18]. However, the influence of the bacterial sources and their seasonal dependence can be observed at each sampling site [5,18]. Diverse taxa of airborne bacteria can originate from various and multiple sources, e.g., soils, plants, animals, water bodies, human commensals, and agricultural or waste facilities [5,10,18]. Thisseion is located in the city centre, in an archaeological park with intense anthropogenic activity. The area has vegetation, animals, and it is not far from Saronic bay. Based on existing studies describing airborne bacteria sources (Table 2), we infer that the main sources of airborne bacteria in our study are the plant leaf surfaces and soil, followed by human or animal commensals. Another possible source is the coastal environment, where bacteria present in the top water layer are aerosolized by breaking waves or strong winds [5,18].

Our air samples contained phyla that were observed in other air studies as well. For example, paired 16S rRNA gene and 16S rRNA sequences were examined in outdoor air samples at the Rutgers University campus (New Brunswick, NJ, USA; 40.48° N, 74.44° W) by [5]. Here, the *Proteobacteria* was the most abundant bacterial phylum on average, and it accounted for 21.7%, 5.8%, and 10.2% of all reads for α -, β - and γ -*Proteobacteria* subgroups, respectively. Other dominant phyla included *Actinobacteria* (17.6%), *Bacteroidetes* (11.6%), *Cyanobacteria* (9.1%) and *Firmicutes* (9.8%). Spring et al. (2018) [31] developed a new Remote Airborne Microbial Passive (RAMP) sampling system to study bioaerosols at the height of 150 m in the atmosphere in Kalamazoo, MI, USA. They found that predominant bacterial phyla in the community were also *Firmicutes* (70%), *Proteobacteria* (17%), *Bacteroidetes* (7%), and *Actinobacteria* (5%). To summarize, previous studies in urban areas detected *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Fusobacteria*; in suburban areas, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* were detected; and in coastal sites, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Cyanobacteria* were found [7,10,18,28,31,35]. Our results on bacterial diversity are similar to earlier studies, even if the sites are different and geographically distant. Ten major bacterial phyla out of the total 92 named bacterial phyla have been detected in our samples; the dominant phyla are *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*, all of which have multiple sources (Table 2).

Terrabacteria (<https://www.ncbi.nlm.nih.gov/taxonomy>, accessed on 2 February 2021) possess resistance to environmental stressors (e.g., desiccation, ultraviolet radiation, and high salinity). Previous studies showed that many airborne bacteria belong to Terrabacteria, including phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* [10,27,28]. Delgado-Baquerizo et al. (2018) [36] analyzed soils from 237 locations across six continents and 18 countries. They found that only 2% of bacterial phylotypes (~500) consistently accounted for almost half of the soil bacterial communities worldwide. The most abundant and ubiquitous phylotypes included *Alpha Proteobacteria* (e.g., *Bradyrhizobium* sp., *Sphingomonas* sp., *Devosia* sp.), *Beta Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Planctomycetes*. Out of the initial 793 OTUs found in our samples near the N.O.A. Thisseion station, 439 belonged to the Terrabacteria group. The majority were Gram-positive bacteria classified in phyla *Firmicutes* (277 OTUs) and *Actinobacteria* (151 OTUs). The other Terrabacteria found in Thisseion belonged to phyla *Cyanobacteria*, *Tenericutes*, and *Deinococcus thermus*.

Table 2. The bacterial Phyla detected in bioaerosols of Athens and their probable sources based on literature.

	Terra Bacteria	Marine/ Coastal Air	Human Skin	Hunan Oral	Outdoor Air	Indoor Air
<i>Proteobacteria</i>	1	3, 6, 7, 8, 11, 13, 16	4	5	9, 10, 11, 13, 14, 18	12, 14, 17 *
<i>Firmicutes</i>	1, 2	3, 6, 7, 8, 16	4	5	9, 10, 11, 13, 14, 18	12, 14, 17 *
<i>Actinobacteria</i>	1, 2	11, 6, 8, 16	4	5	9, 10, 11, 13, 14, 18	12, 14, 17 *
<i>Bacteroidetes</i>		3, 11, 6	4	5	1, 9, 10, 13, 14, 18	12, 14, 17 *
<i>Fusobacteria</i>				5	11	12
<i>Cyanobacteria</i>	1	8, 11			1, 9, 13, 14, 18	14
<i>Tenericutes</i>	15	15	15 *			
<i>Deinococcus_thermus</i>		6			14, 18	12, 14
<i>Acidobacteria</i>	1, 13		4		1, 11, 13, 14	12, 14
<i>Spirochaetes</i>				5		
		15	human gut (15 *)			17 METRO station

Data from: 1. Depres et al., 2012 [10]; 2. Delgado Baquerizo et al., 2018 [36]; 3. Shaffer and Lighthart, 1997 [37]; 4. Cosseau et al., 2015 [38]; 5. Dewhirst et al., 2010 [39]; 6. Mescioglou et al., 2019 [40]; 7. Georgakopoulos et al., 2009 [32]; 8. Xia et al., 2015 [35]; 9. Zhen et al., 2018 [7]; 10. Spring et al., 2018 [31]; 11. Ruiz-Gil et al., 2020 [18]; 12. Rintala et al., 2008 [41]; 13. Brodie et al., 2007 [42]; 14. Shin et al., 2015 [43]; 15. Wang et al., 2019 [44]; 16. Urbano et al., 2011 [17]; 17. Grydaki et al., 2021 [27]; 18. Genitsaris et al., 2017 [28]; * a new human source. Potential Sources of Airborne Bacteria.

Coastal/Marine environment. In culture-independent analyses, it has also been shown that bacteria at coastal and marine sites primarily stem from phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. For example, [28,40] found that the most abundant airborne bacteria in the Mediterranean air represented phyla *Firmicutes* (*Bacilli* and *Clostridia*) and *Proteobacteria* (α , β , and γ subdivisions *Proteobacteria*). Similarly, over the Pacific Ocean and the Norwegian Sea, the most abundant airborne bacterial phyla were *Firmicutes* (49.66% of total sequences) and *Proteobacteria* (48.17%) of the classes α , β , and γ -*Proteobacteria*. In addition, *cyanobacteria* were detected in the aerosol over the North Pacific Ocean [28,35].

Human commensals. Bacteria from human skin microbiota mostly belong to *Actinobacteria* and *Firmicutes* phyla or *Proteobacteria* [38]. The majority of the taxa found in the human oral microbiome are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, and *Fusobacteria* [39]. The families with high prevalence in our samples include many families with pathogenic species for humans or animals and many species common in human or animal commensal microbiota. Besides, several Terrabacteria are also detected in human or animal microbiomes. Thus, a complex network of sources for bioaerosols in Athens should be assumed, and there is likely no one source predominantly yielding airborne bacteria.

At the species level, 18.6% of OTUs in our samples belong to pathogenic or potentially pathogenic species for humans (*Actinomyces* sp., *Corynebacterium diphtheriae*, *Corynebacterium tuberculostearicum*, *Fingoldia magna*, *Haemophilus parainfluenzae*, *Sphingomonas* sp., *Staphylococcus* sp., and *Streptococcus* sp.).

Particularly, the Genera of the oral microbiome *Rothia*, *Leptotrichia*, *Actinomyces*, *Streptococcus*, *Haemophilus*, *Neisseria*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Gemella*, *Leptotrichia* *Kocuria*, *Propionibacterium*, *Dietzia*, *Turicella*, *Capnocytophaga*, *Bacteroides*, *Bifidobacterium*, *campylobacter*, *Corynebacterium*, *Veillonella*, and *Granulicatella* [39] were detected in our samples as dominant, minor or rare species (Figures 3 and 4). Additionally, we found

genera *Anaerococcus*, *Finegoldia*, *Parvimonas*, *Peptoniphilus*, and *Peptostreptococcus*, which are part of the commensal human microbiota and opportunistic pathogens [35,45,46].

A complex microbial community colonizes human skin. In our samples (Figure 4), we detected the following species of this community: *Staphylococci*, *Micrococci*, *Corynebacteria*, *Brevibacterium* sp.; the members of the genus *Acinetobacter* are the most frequently encountered in human long-term resident skin microbiota. Furthermore, we found *Sphingomonas* sp., *Neisseria*, *Haemophilus parainfluenzae*, *Paracoccus*, *Agrobacterium tumefaciens*, *Kokuria*, *Corynebacterium* sp., *Rothia*, *Streptococci*, etc. which belong to the skin microbiome according to Cosseau et al. (2015) [38].

The genus *Corynebacterium* is a cause of occasional infections in humans or is transmitted to humans by zoonotic contact. Many species of this genus had been recovered from animals or birds, the environment, water, foodstuffs, or synthetic materials. However, this group's most significant pathogen remains *Corynebacterium diphtheriae*, the primary cause of the severe disease diphtheria [47]. In Athens, we detected several abundant species (Figure 4) belonging to this genus. For example, the *Corynebacterium tuberculo-stearicum*, *Corynebacterium diphtheriae*, and, with a lower abundance, the species *C. amycolatum*, *C. durum*, *C. imitans*, *C. matruchotii*, *C. mucifaciens*, *C. pseudogenitalium*, *C. urealyticum*.

The genera *Streptococcus* and *Staphylococcus*, which contain medically important species [48–50], are also dominant or abundant in our samples (Figure 3). Various streptococci are important ecologically as part of the normal microbial flora of animals and humans; some can also cause diseases (<https://www.ncbi.nlm.nih.gov/books/NBK7611/>, accessed on 10 October 2020). Bacteria in the genus *Staphylococcus* are among the main pathogens for people and other mammals. Some species of staphylococci are infrequent human or animal commensals (<https://www.ncbi.nlm.nih.gov/books/NBK8448/>, accessed on 10 October 2020).

We detected three rare species of *Actinomyces*: *A. israelii*, *A. orihominis*, and *Actinomyces viscosus*. Actinomycetes are often isolated with other normal human commensals such as *Capnocytophaga*, *Staphylococci*, *Streptococci*, or members of *Enterobacteriaceae* depending on the site of infection [51].

Animal commensals. *Nocardioide*s and *Neisseria* (>1500 OTUs) genera were detected in our October samples, whereas *Neisseria* was also found in August. *Nocardioide*s is a genus of Gram-positive, aerobic irregular rod-shaped bacterial strains (Figure 3). Many members of *Nocardioide*s have been isolated from various sources, including soil, marine sediment, plants, and animals. [52]. Companion animals are also a source of bacteria in urban areas, and pathogenic obligate anaerobes such as *Fusobacterium* were found to be the characteristic taxa following the aerosolization of dog feces [53]. *Neisseria* is closely associated with humans, and it is an abundant, multi-habitat, diverse genus. Commensalism of *Neisseria* in dogs and cats has also been recognized. *Neisseria*–mammal commensalism extends from pets to primates, non-primates, herbivores, marsupials even marine mammals [54]. There have been sporadic reports of *Neisseria* species in the environment with no obvious association with a host [54,55]. In Thiseion and Athens, the pet population is high, so animal commensals (*Corynebacterium*, *Streptococcus*, *Staphylococcus*, *Neisseria*, etc.) are expected to be found in aerosols (Figure 3).

Normal habitats in soil and water. The genus *Methylobacterium* is also abundant in our samples (Figure 3). It can survive atmospheric stressors (due to desiccation tolerance, nitrogen-fixing activity, biofilm formation, facultative methylotrophy, and pigmentation). Therefore, it is often present in pollen, soil, water, and air samples [56–58]. In Athens, we found *M. komagatae* (>1200 OTUs) and *M. tardum* (~1000 OTUs) as major species in samples but none in the controls. The *Methylobacterium* genus can be used to reduce environmental contamination due to its ability to degrade toxic compounds, tolerate high heavy-metal concentrations, and increase plant tolerance to these compounds [57]. *Methylobacterium* also harbours genes related to plant-bacteria interactions that may be important for developing strains that promote plant growth and protection against phytopathogens, showing its importance in agriculture and phytoremediation [18].

Because the methodology used in each study can affect the observed diversity of bioaerosols, a comparison of taxa of airborne bacteria detected by various methods in different studies should be made with care. We focused our comparison on studies with molecular analysis of 16sRNA gene (DNA amplicon barcoding), regardless of the used air sampling devices. We recognize that selecting a particular sampling methodology could introduce a bias for capturing microorganism diversity [59]. Despite the different methodology between studies, in Athens (this study), in Thessaloniki's urban air [28], and in the subway station of Metro of Athens [27], several common taxa of airborne bacteria were found. In the Metro station [27], genera *Paracoccus*, *Sphingomonas*, *Kocuria*, *Arthrobacter*, *Acinetobacter*, *Corynebacterium*, and *Staphylococcus* were detected, among others, and the less abundant taxa *Dietzia*, *Streptococcus*, *Enterobacter*, *Enterococcus*, *Anaerococcus*, *Pseudomonas* were also detected, all of which are also detected in Thisseion, except *Pseudomonas* as an abundant taxon (Figure 3). In the study [28], the medically important genera *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Streptococcus*, and *Corynebacterium* were also detected in Thessaloniki.

Microorganisms are omnipresent and essential to all other life forms via the feeding pyramid and the transmission of virulent factors [2,18,27,60]. Microorganisms such as *Neisseria*, *Corynebacterium*, *Sphingomonas* show the multiple and overlapping roles they can play as normal commensal or virulent factors or free-living bacteria in the environment. Along with all other animals and plants, the human condition is deeply affected by microbes. Microorganisms are essential to human immunity, health, and disease, and hence their abundance and diversity in the air are very important and should be monitored [18,45].

This study investigated the airborne bacterial diversity in Athens, using a novel passive air sampler outdoors in conjunction with Next Generation Sequencing. As a result, we can present a full taxonomy of bacterial aerosols collected during different months. We also used the same method to study fungal aerosols in Athens during 2019 and 2020 [60]. Passive sampling methods have been widely used for bioaerosol collection. They are less damaging to bioaerosols but are not able to quantitate the sampled air volume. Furthermore, due to different meteorological conditions during sampling days and the unknown total air volume, it is difficult to compare the quantity (mass or concentration) of bioaerosols captured during each sampling campaign. On the other hand, passive sampling provides an opportunity to continuously collect airborne microorganisms for several days, which is typically hard to achieve with traditional active samplers.

Moreover, our study fully classified the bacterial aerosols into distinct taxonomic categories, which is useful for future comparative studies of bioaerosol diversity and variability over time and space. We found a complex community of bacterial aerosols with several opportunistic or potential pathogens in Athens' urban air. The exact functional and ecological role of bioaerosol and, even more importantly, their impact on public health and the ecosystem is an issue that requires further analysis and monitoring. Nonetheless, our observations will help understand the diversity of bacterial aerosols in the urban air and the potential role of various bioaerosol sources in their diversity and variability.

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