# Investigation of sources, diversity, and variability of bacterial aerosols in Athens, Greece: a pilot study

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# Abstract

This study is the first attempt to describe the composition, diversity, and potential sources of bacterial aerosols in the urban air of Athens by DNA barcoding (analysis of 16S rRNA genes). It is also the first field application of the recently developed Rutgers Electrostatic Passive Sampler (REPS) to study the microbial diversity of aerosols. Three sampling campaigns 6–10 days in duration were conducted in the summer and fall of 2019. The completely passive REPS 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, 793 OTUs were detected. Firmicutes, Proteobacteria, and Actinobacteria were the dominant Phyla, while the *Cyanobacteria, Bacteroidetes*, and *Fusobacteria* were the minor Phyla. The observed Phyla were further classified into 54 families. The families with high prevalence across our samples contained genera known to have pathogenic species, *e.g., Streptococcus*, *Corynebacterium*, *Gemella*, *Pseudomonas*, *Staphylococcus*, *Neisseria*; many species belonging to human or animal commensal microbiota were also detected. The paper discusses the likely sources of observed airborne bacteria, including soil, plants, animals, humans. Given the variability in bacterial composition over time, it is obvious that the contribution of those sources to airborne microbiota is dynamic. However, a more accurate linkage between the sources and airborne bacteria requires further study. Also, the exact functional and ecological role and, even more importantly, the impact of observed bacterial aerosols on public health and the ecosystem is still unknown and required further analysis.


# 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 bioaerosol particles ranges from tens of nanometers to about a tenth of a millimeter (Douwes et al. 2003; Fröhlich-Nowoisky et al. 2016; Lindsley et. al., 2017; Tang et al. 2018). 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 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. Wherever microbial sources are present, their perturbation (e.g., soil cultivation, crashing of waves, and similar actions) can lead to the release of biological particles into the air resulting in the presence of biological aerosols. Thus, bioaerosols are readily released from agricultural and waste facilities, as well as due to various urban and industrial activities (Jones and Harrison, 2004; Zhai et al. 2018). Humans and animals are also well-known sources of bioaerosols. Bioaerosol release from environmental sources is 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 (Bertolini et al. 2013; Zhen et al. 2017; Li et al. 2019). 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 (Uetake et al. 2019). Once airborne, bioaerosols could be transported through the air over substantial distances, including across oceans, depending on their size and prevailing air currents (Després et al. 2012; Smith et al. 2013). Such dispersal is conducive to spreading human diseases and agricultural pathogens across and even between countries (Després et al. 2012). Bioaerosol viability in the air, a key factor in disease spread, depends on microorganism biology, physiology, and ability to survive challenging environmental conditions (Fröhlich-Nowoisky et al. 2016). Overall, it is estimated that global emissions of bacteria attached to airborne particulate matter range from 0.7 to 28.1 Tg $a^{-1}$ (Fröhlich-Nowoisky et al. 2016; Després et al. 2012). Other studies suggested that the average number flux of bacteria and fungal spores over continental regions is $\sim 10^2$ $m^{-2}$

$s^{-1}$ (Burrows et al. 2009), reflecting an intense and rapid exchange of biological matter and genetic information between the atmosphere and biosphere.

The bioaerosol source type, e.g., natural or anthropogenic, affects not only the composition of airborne microbial communities but also the size of suspended particles, thereby determining their residence time in the air. Usually, individual microorganisms are attached to larger particles (Maki et al. 2013), and the resulting bioaerosol particles are typically larger than individual bacteria or fungal spores. Thus, aggregation of bacteria seems to favor their survival, but this comes "at the cost" of the airborne time and potential distance of dispersion. Hence, for bacteria, aerial dissemination is a compromise between the distance traveled (which decreases for large aggregates) and the chances of successful dissemination, which increases for large aggregates (Amato et al. 2015).

The role of bioaerosols in disease transmission has been recognized for years (Fröhlich-Nowoisky et al. 2016; Polymenakou et al. 2008; Douwes et al. 2003), 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 (Després et al. 2012; Amato et al.2015; Estillore et al. 2016; Urbano et al. 2011). Bioaerosols removed from the air via dry and wet deposition might interact with terrestrial or aquatic ecosystems and the biosphere (Fröhlich-Nowoisky et al. 2016; Després et al. 2012), triggering various biological processes.

Given the interest in bioaerosols due to their role in environmental and health processes, there have been numerous studies to determine their concentration and species found in the atmosphere (Maki et al. 2017; Šantl-Temkiv et al.2020; Mainelis, 1999; Franchitti et al. 2020; Ferguson et al. 2019). Until relatively recently, such studies have been limited to the use of culture-based methods, which is known to determine only a fraction of airborne microorganisms; cultured bacteria were classified only as Gram-positive or Gram-negative or one estimated the number of colony-forming units (CFU) of airborne bacteria and fungi without providing taxonomic identification at a higher level (Després et al. 2012). The advance of the next-generation sequencing tools was quickly adapted to investigate the presence and species of airborne microorganisms, especially bacteria (Fröhlich-Nowoisky et al. 2016; Zhen et al. 2018; Wei et al. 2019; Li et al. 2019). Such studies suggested that airborne bacteria often belong to groups associated with common soil bacteria, e.g., the Terrabacteria category, as well as human and animal commensals (Bowers et al. 2013; Ruiz-Gil et al. 2020).

While the investigation of airborne bacteria composition has been carried out in many countries (Ruiz-Gil et al. 2020; Fröhlich-Nowoisky et al. 2016; Després et al. 2012; Górny, 2020; Li et al. 2019), such studies have been limited in Greece and especially in Athens. Thus, the main goal of this project was to perform a pilot study to investigate the diversity of bacterial aerosols over Athens, Greece, using molecular analysis of their 16sRNA gene (DNA barcoding). This study is the first of such kind in Athens and the only second in Greece (Polymenakou et al. 2008), where 16sRNA was investigated during a dust storm in Crete. Another novel element of the study was the in-field application of Rutgers Electrostatic Passive Sampler (REPS) (Therkorn et al. 2017) to capture bacterial aerosols for their subsequent sequencing – a first such application of this device.

# 2. Materials And Methods

In the first study of its kind in Athens, Greece, 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 analyzed using next-generation sequencing. Local meteorological conditions such as temperature, relative humidity (RH), and solar radiation were recorded by IERSD during the three sampling periods at meteorological N.O.A. station of Thisseion (https://www.iersd.noa.gr/ENG/iersd_climatological.htm). Athens' air quality data is constantly monitored (http://84.205.254.113/airqualmap/leafletmap.html ) and could be easily accessed if needed.

### Sampling method

Bioaerosols were collected using Rutgers Electrostatic Passive Sampler (REPS) (Therkorn et al. 2017). This recently-developed sampler is completely passive (i.e., there is no active air mover to provide airflow). The sampler uses a specially configured and permanently polarized ferroelectric polymer film (e.g., poly(vinylidene fluoride), or PVDF) to capture electrically charged airborne particles by the electrostatic method in addition to particle capture due to their gravitational settling. REPS uses a 70 mm x 130 mm strip of PVDF film wound into three evenly spaced layers held by a 3D-printed film holder (Fig. 1a). This holder configuration creates the film spiral, where oppositely polarized film sides are spaced at 2.25 mm (Therkorn et al. 2017). The resulting parallel film layers generate an electrostatic field between them (Fig. 1b). Due to the field fringing effect, electrostatic field lines extend past the top of REPS and attract charged bioaerosol particles inside 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 (Therkorn et al. 2017; Manibusan and Mainelis, 2020). Because the sampler does not require air movers and power supplies, the sampling time could be extended to several days, which was taken advantage of in this project.

**Figure 1a** REPS Sampler

**Figure 1b** A view from the top of REPS shows the positioning of PVDF film inside REPS to create an electrostatic field between the layers

### Sampling location and the number of samples

Three sampling campaigns were conducted in June, August, and October of 2019, in the vicinity of the Thisseion meteorological and environmental station (37°58'24"N 23°43'6"E) of N.O.A (National Observatory of Athens). In June and August, samples were collected for ten consecutive days; in October, the sampling was stopped after 6 days to prevent the collected samples from being washed off by the oncoming rain. In each campaign, five REPS samplers were used. In addition, in each campaign, we used two control REPS: one brought into the field but not used (internal control) and another REPS that was used to sample for one minute to check for potential sample contamination during lab analysis and transfer of samplers.

### 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 campaigns and samplers, 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 (Therkorn et al. 2017). 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 minutes. The tubes were vigorously shaken, and the eluate with captured particles was removed for further analysis. However, the volume of elution liquid (35 ml) for each REPS was large. Consequently, we centrifuged each sample and pooled the resulting five pellets into one sample (labeled "June") to reach the average DNA concentration > 0.25 ng/μL, i.e., above the threshold needed to proceed with NGS analysis. The final sample volume 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, PVDF film from each REPS was cut into 12 pieces and put in 2 tubes (2 ml) for each sample in the

lab with autoclaved scissors. The samples were incubated at 56°C overnight in 1000 µL PowerSoil bead solution, 300 µL solution C1, and 100 µg proteinase K. The samples were then vortexed and added to the PowerBead tubes for extraction.

Still, five sampled from August and five from October were pooled to reach an average DNA concentration > 20 ng/µL per sample because of low DNA concentration. The resulting two samples are labeled "August" and "October," according to the month of sampling. Only one-minute sampling controls had detectable DNA concentrations, and they are labeled 415c for August and 417c for October. Thus, a total of three samples and two controls were analyzed by the NGS. The final DNA concentration range in samples was 0.25–50 ng/µL, and in controls, the DNA concentration was < 0.7 ng/µL.

### Contamination prevention

Given 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, pretreatment, and DNA extraction steps. When the REPS were prepared at Rutgers University, they have been thoroughly cleaned with 75% (vol/vol) ethanol inside a Class II Biosafety cabinet. The samplers were then placed in 50 mL tubes. All the tubes and tools used in sampling and analysis were sterilized (autoclaved, if possible) or cleaned with 75% (vol/vol) 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 per sampling campaign, as described above. Contamination prevention is a crucial study design aspect because any contamination of samples before field sampling 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 has to be considered in studies where DNA yields are expected to be low (Spring et al. 2018).

### DNA extraction and analysis

Genomic DNA from the eluate was extracted using the DNeasy PowerSoil (Qiagen, Valencia, CA), according to the manufacturer's protocol. It was then available for downstream processing via next-generation sequencing and PCR. The DNeasy PowerSoil Kit uses a humic substance/brown color 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, 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). Before sequencing, the bacterial 16S rRNA gene sequences were amplified by PCR with universal primer sets (515F bacterial + archeal). They were sequenced using Ion Torrent (Ion S5 XL), with a reading length of 300bp and nominal 15–20,000 reads/assay. Sequence data were processed using the company's proprietary analysis pipeline.

In summary, sequences were depleted of barcodes and primers, then sequenced < 150bp, and sequences with ambiguous base calls and with homopolymers, runs exceeding 6bp, were all removed. Next, sequences were de-noised, then 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) and NCBI (www.ncbi.nlm.nih.gov). For bacteria, the generally accepted levels of discrimination are 99% similarity for strains, 97–99% for species, and 95–97% for genera (Georgakopoulos et al. 2009).

First quality control step to control possible contamination and low taxonomical parameters of OTUs

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 counts in samples, this OTU was deleted.

At this phase, we selected 805 OTUs from all analyzed samples with high taxonomical parameters (identity id > 97%, bit score > 300, and e-value < $2.17e^{-79}$). There is potential for the 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* tool for further analysis.

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

**Second data quality control by MicrobiomeAnalyst (minimum counts, prevalence, normalization, and cut-off values)**

Visual exploration of our data (16sRNA barcoding) was made by MicrobiomeAnalyst software (Dhariwal et al. 2017). The observed abundance of OTUs was rarefied to exclude low-abundance features and improve the presentation of the data. Features (OTUs) with exceedingly small counts were likely due to sequencing errors, and they were removed using a minimum count cut-off of 4 (software default). A filter for 20% prevalence was applied, meaning that at least 20% of its values should contain at least four counts for any feature (OTU) to be further analyzed. OTUs having low count and low prevalence were removed during this step. Counts of the duplicates in each taxon level were merged in the final abundance catalog. No data rarefaction and transformation were performed, but we performed total sum normalization of data. After this phase, low abundance and low variance features were removed by the MicrobiomeAnalyst software. The number of features (OTUs) remaining after all the data filtration steps was 253, and their abundance was approximately 86000 counts.

# 3. Results

Meteorological conditions during sampling

The average daily values (average ± standard deviation) of meteorological and air quality conditions during sampling are shown in Table 1. In June, the highest daily total solar radiation (7241 $W/m^2$) was recorded, while in August, the highest daily temperature (average t= 28.8 $^oC$) and wind speed (3.9 m/s). Ozone concentration was highest in August (31.43 ppb), $SO_2$ (2.7 ppb) in October, and $NO_2$ (29.05 ppb) in June.

*Description of raw data*

We identified 793 OTUs based on taxonomical parameters after the first quality control phase. The OTUs were classified in 10 phyla with more than 100,000 counts of total actual abundance. They were further classified into 14 classes, 26 orders, 104 families, 229 genera, and 713 species. We analyzed the selected OTUs using SPSS software (IBM SPSS Statistics 27.0). The bacterial phyla in the descending order of abundance were *Proteobacteria* (39,8%), *Firmicutes* (35%), *Actinobacteria* (18%), *Bacteroidetes* (3,62%). Minor phyla (1-3%) were *Fusobacteria*, *Cyanobacteria*, and *Tenericutes*, while rare phyla (<0.1%) were *Deinococcus-thermus, Acidobacteria,* and *Spirochaetes*. This OTU table was uploaded to the MicrobiomeAnalyst tool for further analysis (second data quality control) as described above.

***Analysis of data by*** **MicrobiomeAnalyst tool**

After the second quality control step, we had 253 OTUs with approximately 86000 counts across all samples. The data were visualized by MicrobiomeAnalyst. We acknowledge that quantifying 16S rRNA sequences as a proxy for the total abundance of bacteria is subject to bias because some bacteria may carry more than one copy of the 16S rRNA gene; plus, DNA extraction and sequencing biases are inherent in air sampling methods. Therefore, this result should only be interpreted as the abundance of detected bacterial 16S rRNA genes (Spring et al. 2018).

MicrobiomeAnalyst software provides an option to view sample composition at various taxonomic levels (e.g., class, order, family, genus, and species) using stacked bar diagrams. Viewing composition at higher levels (e.g., class, order, family) provides a more informative visual overview of the data than at lower levels (genus, species) when the number of species in a community is large and diverse. Thus, we selected OTUs with a prevalence >20% and a count cut-off of 10 based on total counts across all samples for all taxa, except for species that had a cut-off of 30. Our selected cut-off of 30 improved the presentation of data at the species level. Overall, the phyla *Firmicutes (40%), Proteobacteria (36 %), and Actinobacteria (20%)* were dominant, while *Cyanobacteria, Bacteroidetes* and *Fusobacteria* were minor phyla (~2-3%).

**Fig. 2** The actual abundance of bacterial aerosols at the Class level

The actual abundance at the class level is shown in Fig. 2. Controls are included for comparison, and it is obvious that the control samples had very low abundance and variability compared to the actual samples. When actual abundance data of the three samples (controls not included) was converted into relative abundance (fraction of the total abundance), the relative abundance of main bacterial classes across the three samples in descending order were as follows: *Gammaproteobacteria, Bacilli, Actinobacteria, Alphaproteobacteria* and *Clostridia* with a relative abundance of 0.74, 0.46, 0.43, 0.41, respectively. In June, the major class of airborne bacteria in Athens was *γ- Proteobacteria*, in August, it was *Bacilli*, and in October, it was A*ctinobacteria*.

**Fig. 3** The relative abundance of bacterial aerosols at the Order level.

The major orders (Fig. 3) were *Rhizobiales, Lactobacillales, and Actinomycetales,* with a relative abundance of 0.8, 0.6, and 0.51, respectively, across the three samples. In June, the major orders of airborne bacteria were *Rhizobiales* (0.75) and *Pseudomonadales* (0.2). In August, the major orders were *Actinomycetales, Bacillales, Lactobacillales, and Clostridiales.* In October, the major orders were *Lactobacillales, Actinomycetales, Bacillales, and Clostridiales.* The orders *Actinomycetales, Bacillales, Lactobacillales, Pseudomonadales, Rhizobiales, Rhodobacterales, Sphingomonadales*, *Spirulinales Burkholderiales* and *Enterobacteriales* were found in samples from all months.

**Fig. 4** The relative abundance of bacterial aerosols at the Family level.

In total, 54 bacterial families (Fig. 4) were identified. Dominant families were *Rhizobiaceae* (0.63), *Corynebacteriaceae, Peptoniphilaceae, Streptococcaceae, Bacillales_Family_XI__Incertae_Sedis,* and *Pseudomonadaceae* (>0.2); *Micrococcaceae, Methylobacteriaceae,* and *Lactobacillacea*e (~0.1). In June, the major families were *Rhizobiaceae* (0.63) *and Pseudomonadaceae* (0.13). In August, the dominant families were *Corynebacteriaceae, Peptoniphilaceae, Bacillales_Family_XI__Incertae_Sedis* (>0.1). In October, the dominant families were *Streptococcaceae Peptoniphilaceae, Corynebacteriaceae, and Bacillales_Family_XI__Incertae_Sedis.* In addition, families *Rhizobiaceae, Burkholderiaceae, Moraxellaceae, Intrasporangiaceae, Corynebacteriaceae, Carnobacteriaceae, Brevibacteriaceae, Enterobacteriaceae, Oxalobacteraceae, Streptococcaceae*, *Micrococcaceae, Bacillaceae, Sphingomonadaceae, Spirulinaceae, Rhodobacteraceae, Lactobacillaceae, Bacillales_Family_XI__Incertae_Sedis, Bradyrhizobiaceae, Caulobacteraceae, Phyllobacteriaceae, Methylobacteriaceae,* and *Pseudomonadaceae* were found in samples from all three months.

**Fig. 5** The relative abundance of bacterial aerosols at the Genus level.

The dominant genera (Fig. 5) in descending order (18000 - 8000 OTUs) were *Streptococcus, Corynebacterium, and Agrobacterium* with a relative abundance of 0.6, 0.4, and 0.3, respectively. *Pseudomonas, Gemella, Methylobacterium, Anaerococcus,* and *Finegoldia* followed, with a relative abundance of 0.3, 0.2, and 0.1, respectively. Many of the detected genera (e.g., *Streptococcus*, *Corynebacterium*, *Gemella*, *Pseudomonas*, *Staphylococcus*, *Neisseria*, etc.) contain pathogenic species. In June, the main genera in descending order were *Agrobacterium, Pseudomonas, Methylobacterium, and Rhizobium* (0.66 - 0.6). In August, the main genera in descending order (0.13-0.03) were *Corynebacterium, Gemella, Actinomyces, Staphylococcus, Finegoldia, Streptococcus, Anaerococcus, Alloiococcus, Methylobacterium, Prevotella, Neisseria, Paracoccus, Veillonella, Halospirulina, Granulicatella,* and *Peptoniphilus.* Similarly, in October, the main genera (0.35-0.03) were *Streptococcus, Corynebacterium, Gemella, Lactobacillus, Anaerococcus, Finegoldia*, and *Rothia,* and *Acinetobacter.*

**Fig. 6** The relative abundance of the bacterial aerosols at the Species level

In June, the major species (Fig. 6) in descending order (0.5 - 0.02) were *Agrobacterium tumefaciens, Pseudomonas pseudoalcaligenes, Rhizobium sp, Methylobacterium tardum, Mesorhizobium* sp, *Stenotrophomonas maltophilia, Agrobacterium sp, and Methylobacterium sp*. In August, the main species in descending order (0.08-0.02) were *Sphingomonas sp, Corynebacterium diphtheriae, Finegoldia magna, Streptococcus salivarius, Corynebacterium matruchotii, Anaerococcus sp, Alloiococcus otitis, Methylobacterium komagatae, Staphylococcus pettenkoferi, Paracoccus marinus, Halospirulina sp, Veillonella dispar, Neisseria sp, Actinomyces orihominis, Knoellia sinensis, Campylobacter gracilis, Actinomyces viscosus, Granulicatella paradiacens, Neisseria elongate, Prevotella denticola,* and *Rothia mucilaginosa*.

In October, the main species in descending order (0.17 - 0.02) were *Streptococcus tigurinus*, *Streptococcus sanguinis, Corynebacterium tuberculostearicum, Streptococcus sp., Lactobacillus gallinarum, Streptococcus sinensis, Anaerococcus sp, Finegoldia magna, Streptococcus parasanguinis, Gemella sp, Neisseria sp, Rothia mucilaginosa, Corynebacterium imitans, Sphingomonas sp, Corynebacterium mucifaciens, Nocardioides sp, Acinetobacter lwoffii, Halospirulina sp,* and *Corynebacterium urealyticum.*

In total, the main species of bacterial aerosols in Athens in descending order (0.5 - 0.05) were *Agrobacterium tumefaciens, Pseudomonas pseudoalcaligenes, Streptococcus tigurinus, Streptococcus sanguinis, Sphingomonas sp, Finegoldia magna, Anaerococcus sp, Corynebacterium tuberculostearicum, Corynebacterium diphtheriae, Rhizobium sp, Streptococcus sp, Lactobacillus gallinarum, Methylobacterium tardum,* and *Halospirulina sp.*

# 4. Discussion

This pilot study is the first attempt to describe 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. Despite the pilot nature of the study and only three sampling campaigns 6-10 days long, REPS units 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*. Our decontamination procedures and additional data filtration to remove low-quality or likely contaminant features minimized errors in our analysis. Low OTU levels in controls demonstrate that the diversity and abundance found in samples are not due to contamination but reflect the presence of bacteria in the air.

Low diversity and abundance in June samples can be attributed to the used sample elution technique (high initial 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 a part of the captured bacteria. October is the richest month in diversity and actual abundance of the bacterial communities (Fig 2). 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 all the human activities in the city and its potential contribution to bioaerosol diversity. Remarkably, October's bacterial diversity was much higher than August's, although sampling that month was four days shorter than in August due to oncoming rain.

Air over different cities has different bioaerosol compositions and abundance, and there is no single, standardized description of urban airborne bacteria diversity (Depres et al. 2012). The influence of the bacterial sources and their seasonal dependence can be observed at each sampling site (Ruiz-Gil et al. 2020; Zhai et al. 2018). Diverse taxa of airborne bacteria can originate from various sources (e.g., soils, plants, animals, water bodies, human commensals, and agricultural or waste facilities (Ruiz-Gil et al. 2020; Zhai et al. 2018). Thisseion is located in the city center, 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 that describe the sources of airborne bacteria (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 (Zhai et al. 2018; Ruiz-Gil et al. 2020).

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 Jersey, USA) (40.48°N, 74.44°W) by Zhen et al. (2018). 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) developed a new Remote Airborne Microbial Passive (RAMP) sampling system to study bioaerosols at the height of 150 m in the atmosphere in Kalamazoo, Michigan, 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 (Ruiz-Gil et al. 2020; Zhen et al.2018; Spring et al.2018; Xia et al. 2015; Depres et al. 2012). Our results on bacterial diversity are similar to earlier studies, although the sites are different and geographically distant. 10 major bacterial phyla out of the total 92 named bacterial phyla have been detected in bioaerosols globally; the dominant phyla are *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes,* all of which have multiple sources (Table 2).

Table 2. The bacterial Phyla detected in the bioaerosols of Athens and their probable sources

**Potential sources of airborne bacteria**

Terrabacteria (https://www.ncbi.nlm.nih.gov/taxonomy) 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* (Després et al. 2012). Delgado-Baquerizo et al. (2018) 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 of these phylotypes included *AlphaProteobacteria* (e.g., *Bradyrhizobium sp., Sphingomonas sp., Devosia sp.), BetaProteobacteria, Actinobacteria, Acidobacteria,* and *Planctomycetes*. Out of 793 OTUs (after the first data filtration step) found in our samples near the N.O.A. Thiseion 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*.

**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*. Mescioglou et al. (2019) found that the most abundant airborne bacteria over the Mediterranean 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*. *Cyanobacteria* were detected in the aerosol over the N. Pacific Ocean (Xia et al. 2015).

**Human commensals.** Human skin microbiota bacteria mostly belong to *Actinobacteria* and *Firmicutes* phyla or *Proteobacteria* (Cosseau et al. 2015). The majority of the taxa found in the human oral microbiome are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, and *Fusobacteria* (Dewhirst et al. 2010). 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 potential pathogenic species for humans (*Actinomyces sp, Corynebacterium diphtheriae, Corynebacterium tuberculostearicum, Finegoldia magna, Haemophilus parainfluenzae, Sphingomonas sp, and Streptococcus sp*, etc.).

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* (Dewhirst et al. 2010) were detected in our samples as dominant, minor or rare species (Fig. 6). Also, we found the genera *Anaerococcus*, *Finegoldia*, Parvimonas, *Peptoniphilus*, and *Peptostreptococcus*, which are part of the commensal human microbiota and opportunistic pathogens (Murphy and Frick, 2013; Neumann et al. 2020)

A complex microbial community colonizes human skin. In our samples, we detected the following species of this community: *Staphylococci, Micrococci*, *Corynebacteria, Brevibacterium spp.*; 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).

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 (Bernard, 2012). In Athens, we detected several dominant species belonging to this genus: *Corynebacterium tuberculostearicum*, *Corynebacterium diphtheriae*, and with a lower abundance, the species *C. amycolatum*, *C. durum, C. imitans*, *C. matruchotii, C.mucifaciens, C. pseudogenitalium*, *C. urealyticum, etc.* (Fig. 6).

The genera *Streptococcus* and *Staphylococcus*, which contain medically important species (Baron, 1996; Kaci et al. 2014; Patterson, 1996), are also dominant or abundant in our samples. 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/). Bacteria in the genus *Staphylococcus* are 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/).

We detected three rare species of *Actinomyces: A. israelii, A.orihominis*, and *Actinomyces viscosus*. *Actinomyces* are often isolated with other normal human commensals such as *Capnocytophaga*, *Staphylococci, Streptococci*, or members of *Enterobacteriaceae* depending on the site of infection (Valour et al. 2014).

**Animal commensals**. *Nocardioides* and *Neisseria* (>1500 OTUs) were abundant genera in our October samples, whereas *Neisseria* was also found in August. *Nocardioides* is a genus of Gram-positive, aerobic irregular rod-shaped bacterial strains. Many members of *Nocardioides* have been isolated from various sources, including soil, marine sediment, plants, and animals. (Wang et al. 2018). 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 (Bowers et al. 2011). *Neisseria* is closely associated with humans, and it is an abundant, multi-habitat, diverse genus. Commensalism of *Neisseria* in dogs and cats has been recognized. *Neisseria*–mammal commensalism extends from pets to primates, non-primates, herbivores, marsupials even marine mammals (Liu et al. 2015). There have been sporadic reports of *Neisseria* species in the environment with no obvious association with a host (Liu et al. 2015; Tzeng et al. 2014). In Thisseion and Athens, the pet population is high, so animal commensals (*Corynebacterium, Streptococcus, Staphylococcus, Neisseria,* etc.) are expected to be found in aerosols (Figs. 5 and 6).

**Normal habitats in soil and water**

The genus *Methylobacterium* is also abundant in our samples. It can survive atmospheric stressors (due to desiccation tolerance, nitrogen-fixing activity, biofilm formation, facultative methylotrophy, and pigmentation), and it is often present on pollen, in soil, water, and air samples (Kato et al. 2008; Dourado et al. 2015; Kovaleva et a.,2014). 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 (Dourado et al. 2015). *Methylobacterium* also harbors 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 (Ruiz-Gil et al. 2020).

Because the methodology used in each study can affect the observed diversity of bioaerosols, comparisons 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 (barcoding), regardless of the used air sampling devices. We do recognize that selecting a particular sampling methodology could introduce a basis in captured microorganism diversity (Mainelis 2019).

Microorganisms are omnipresent and essential to all other life forms via the feeding pyramid and the transmission of virulent factors (Fröhlich-Nowoisky et al. 2016; Zhai et al. 2018; Ruiz-Gil et al. 2020; Li et al. 2020). Microorganisms like *Neisseria, Corynebacterium, Sphingomonas, etc.,* 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 (Ruiz-Gil et al. 2020; Murphy and Frick, 2013).

This study investigated the airborne bacterial diversity in Athens, the first such study in Athens using novel passive air sampler and barcoding DNA analysis. As a result, we can present a full taxonomy of bacterial aerosols collected at different months. Moreover, our study is the first, as far as we know, that fully classified the bacterial aerosols into the eight distinct taxonomic categories, which is useful for the next comparative studies of bioaerosol diversity and variability over time and space. Due to the use of a passive sampling method, samples were integrated over 6-10 days. We found a complex community of bacterial aerosols with several opportunistic or potential pathogens in Athens' urban air. Their exact functional and ecological role and, even more importantly, the impact on our health and ecosystem is still unknown and required further analysis. Nonetheless, our observations will help understand the diversity of bacterial aerosols and the potential role of various bioaerosol sources. This pilot study will serve as a launch platform for a more detailed and extensive study of biological aerosols in Greece and their role in the environment and public health.

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## Declarations

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## Tables

**Table 1** Weather Conditions and levels of pollutants during the pilot study in Athens. 2019

| | Month | SOLTOT SUM day ($W/M^2$) | T ($^oC$) | RH (%) | Precipitation (mm) | Speed (M/s) | Sector | O3 ppb | SO2 ppb | NO2 ppb |
|---|---|---|---|---|---|---|---|---|---|---|
| average | June | 7240.9 | 28.6 | 46.0 | 0.2 | 2.5 | 7.5 | 9.39 | 1.37 | 29.05 |
| S.d. | | 1431.3 | 1.7 | 6.0 | 0.4 | 0.8 | 4.0 | 4.1 | 1.02 | 9.47 |
| average | August | 7188.8 | 28.8 | 47.6 | 0.0 | 3.9 | 5.5 | 31.43 | 0.84 | 21.23 |
| S.d. | | 335.6 | 1.3 | 5.9 | 0.0 | 1.6 | 4.7 | 10.43 | 0.17 | 7 |
| average | October | 3982.9 | 21.9 | 66.8 | 0.0 | 2.0 | 5.5 | 11.95 | 2.7 | 13.69 |
| *S.d.* | | *870.2* | *0.5* | *9.5* | *0.0* | *0.6* | *4.0* | *11.95* | *1.61* | *5.46* |

**ble 2** Phylla detected in the urban air of Athens and their possible sources based on previous studies

| hyla | Terra bacteria | | Marine/ coastal air | Human Skin | Hunan Oral | Outdoor air | Indoor air |
|---|---|---|---|---|---|---|---|
| *eria* | 1 | 3,6,7,8,11,13,16 | | 4 | 5 | 9,10,11,13,14 | 12,14 |
| | 1,2 | | 3,6,7,8,16 | 4 | 5 | 9,10,11,13,14 | 12,14 |
| *eria* | 1,2 | | 11,6,8,16 | 4 | 5 | 9,10,11,13,14 | 12,14 |
| *tes* | 3,11,6 | 4 | 5 | | 1,9,10,13,14 | 12,14 | |
| *ia* | ** | 5 | | 11 | 12 | | |
| *ria* | 1 | | 8,11 | 1,9,13,14 | 14 | | |
| *s* | 15 | | * | * | | | |
| *s_thermus* | 6 | 14 | | 12,14 | | | |
| *ria* | 1,13 | | 4 | 1,11,13,14 | 12,14 | | |
| *es* | 5 | | | | | | |
| | | *ocean, gut of marine animals (15) | *human gut (15) | | | | |

1.Depres et al., 2012; 2. Delgado Baquerizo et al.,2018; 3 Shaffer and Ligthhart, 1997; 4.Cosseau et al., 201 5 Dewhirst et al., 2010; 6.Mescioglou et al.2019; 7. Georgakopoulos et al., 2009;8 Xia et al., 2015; 9 Zhen et a 2018;10 Spring et al.,2018;11 Ruiz-Gil et al.,2020;12. Rintala et al., 2008; 13. Brodie et al.,2007; 14 Shin al.,2015; 15 Wang et al.,2019; 16 Urbano et al., 2011

## Figures

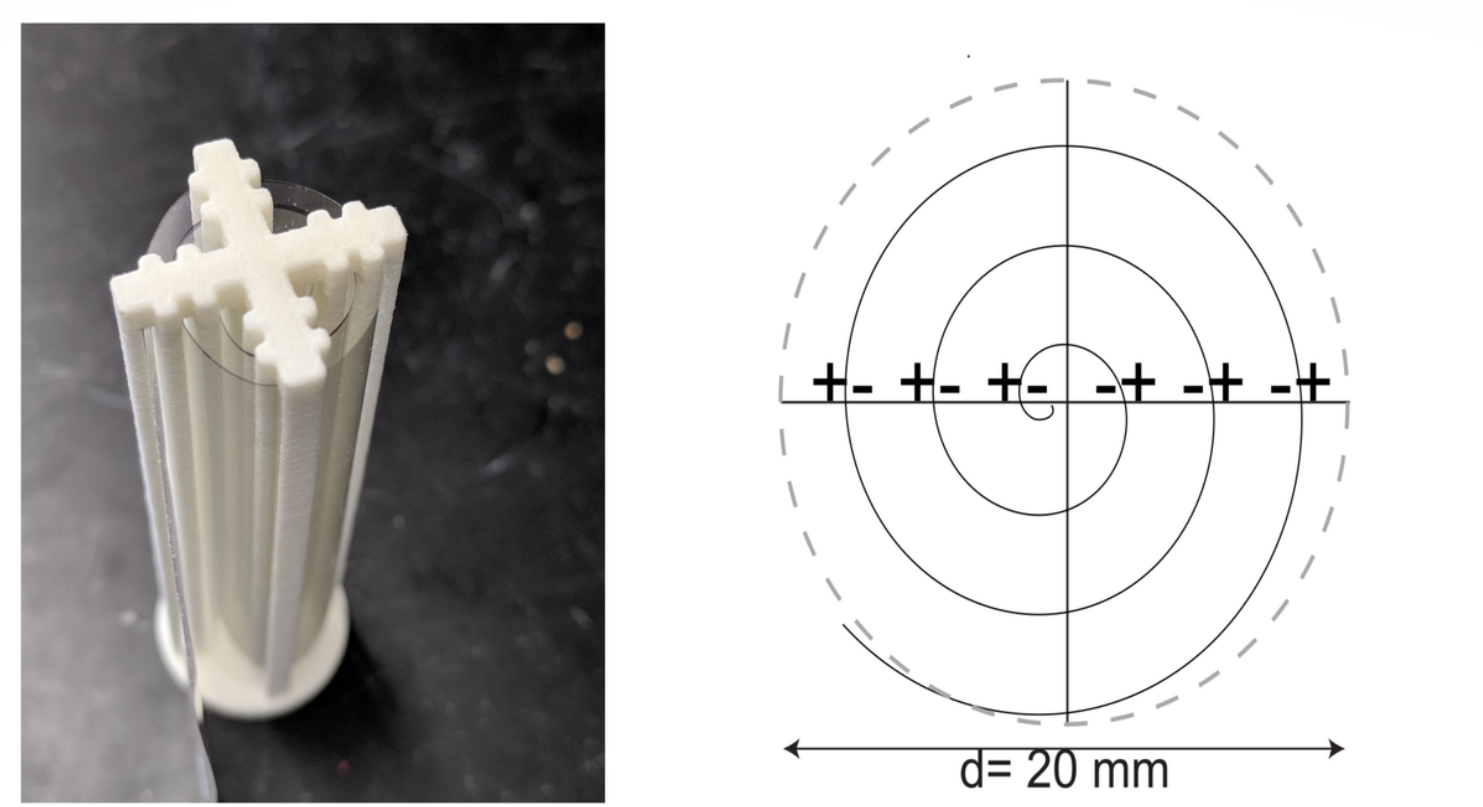



**Figure 1**

a. REPS Sampler. b. A view from the top of REPS shows the positioning of PVDF film inside REPS to create an electrostatic field between the layers.

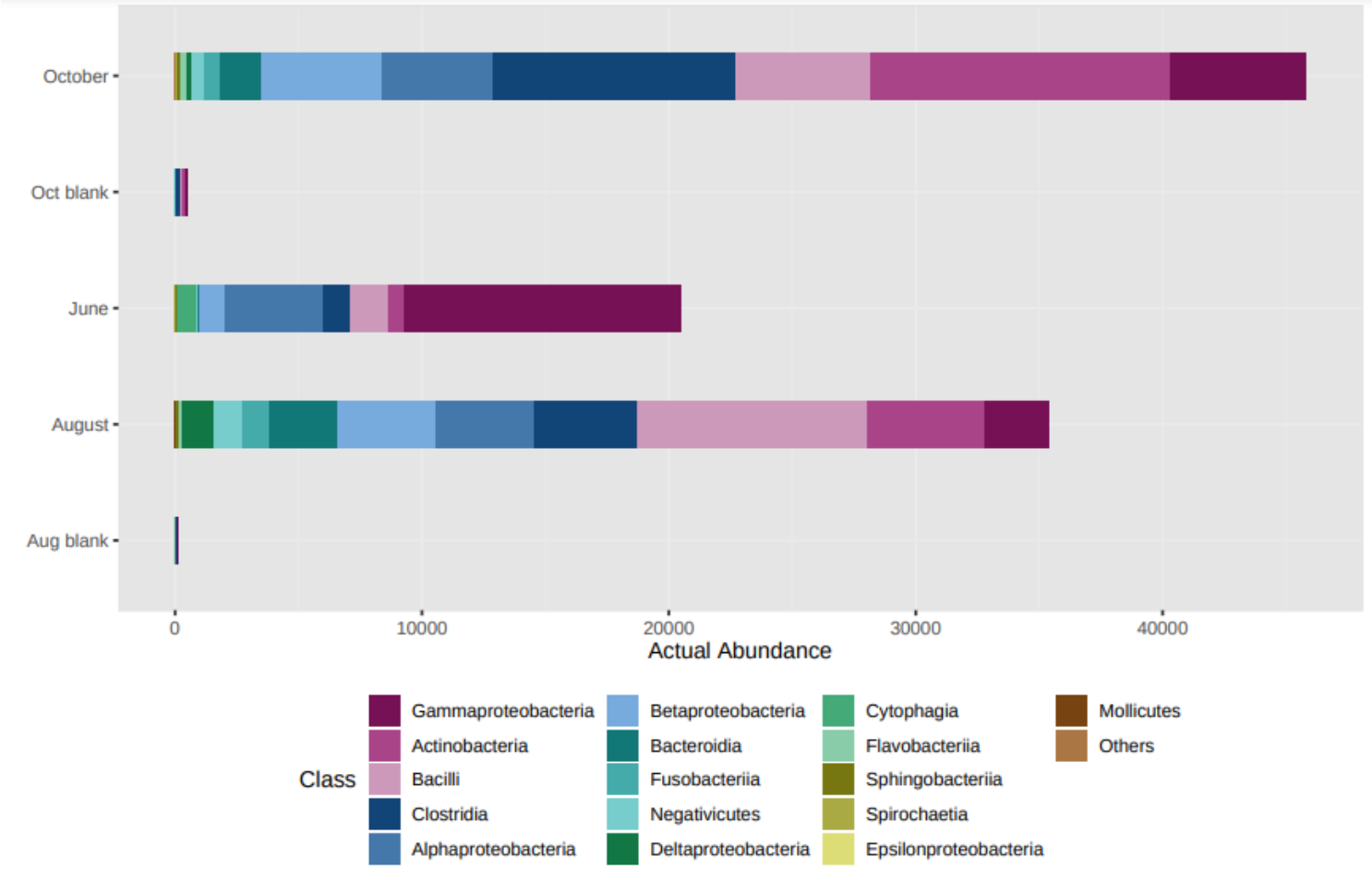
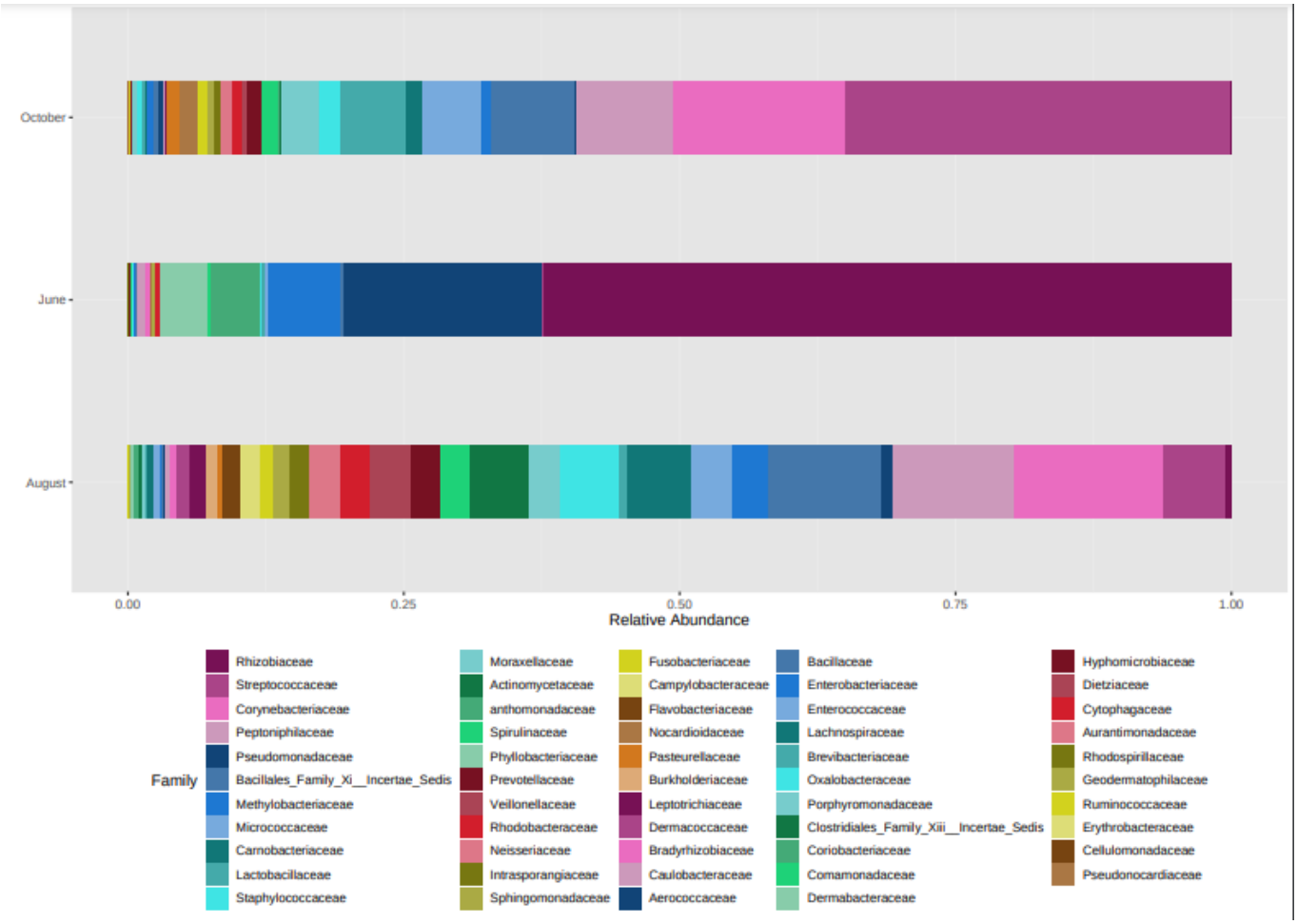



**Figure 2**

The actual abundance of bacterial aerosols at the Class level




## Figure 3

The relative abundance of bacterial aerosols at the Order level.

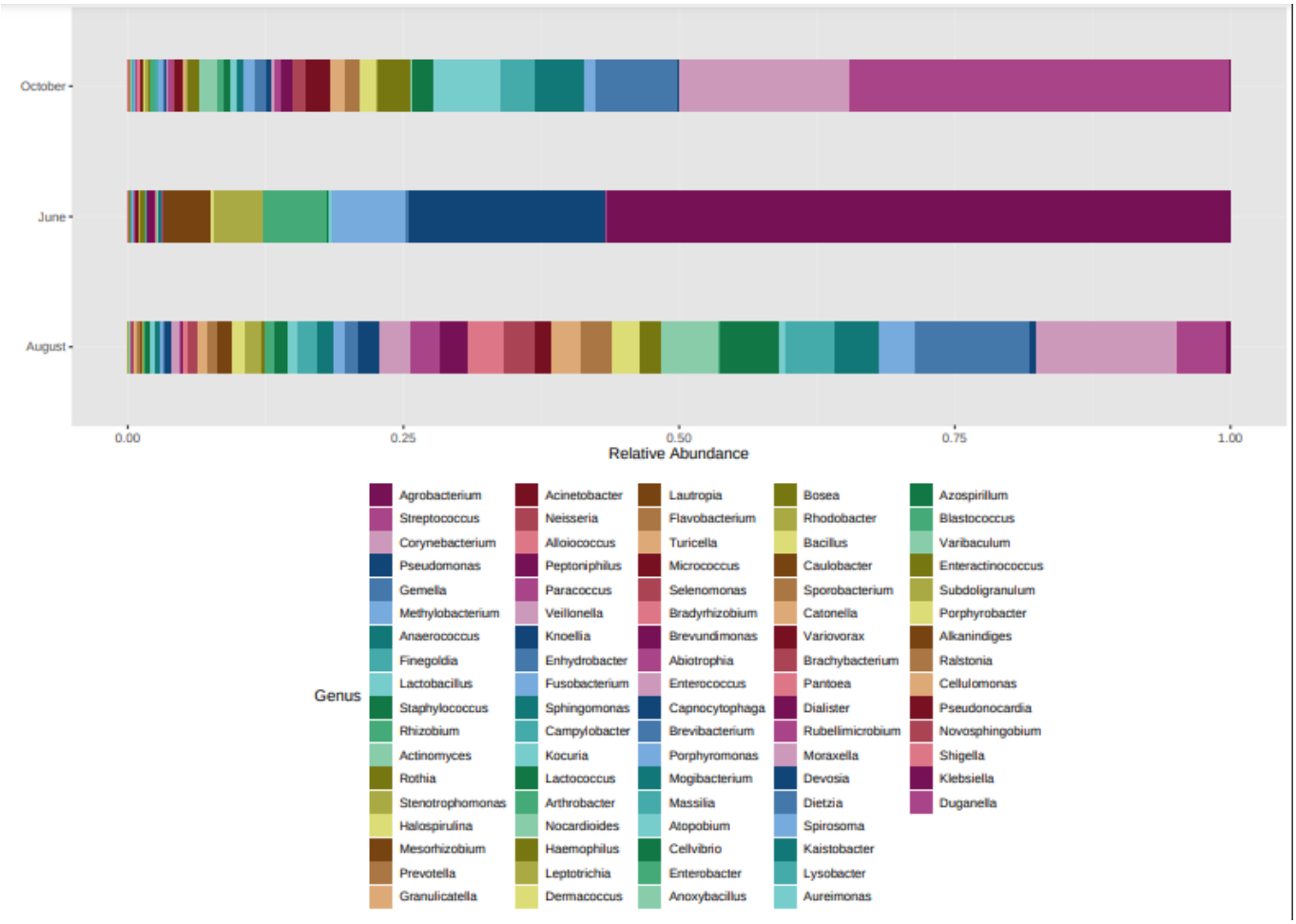
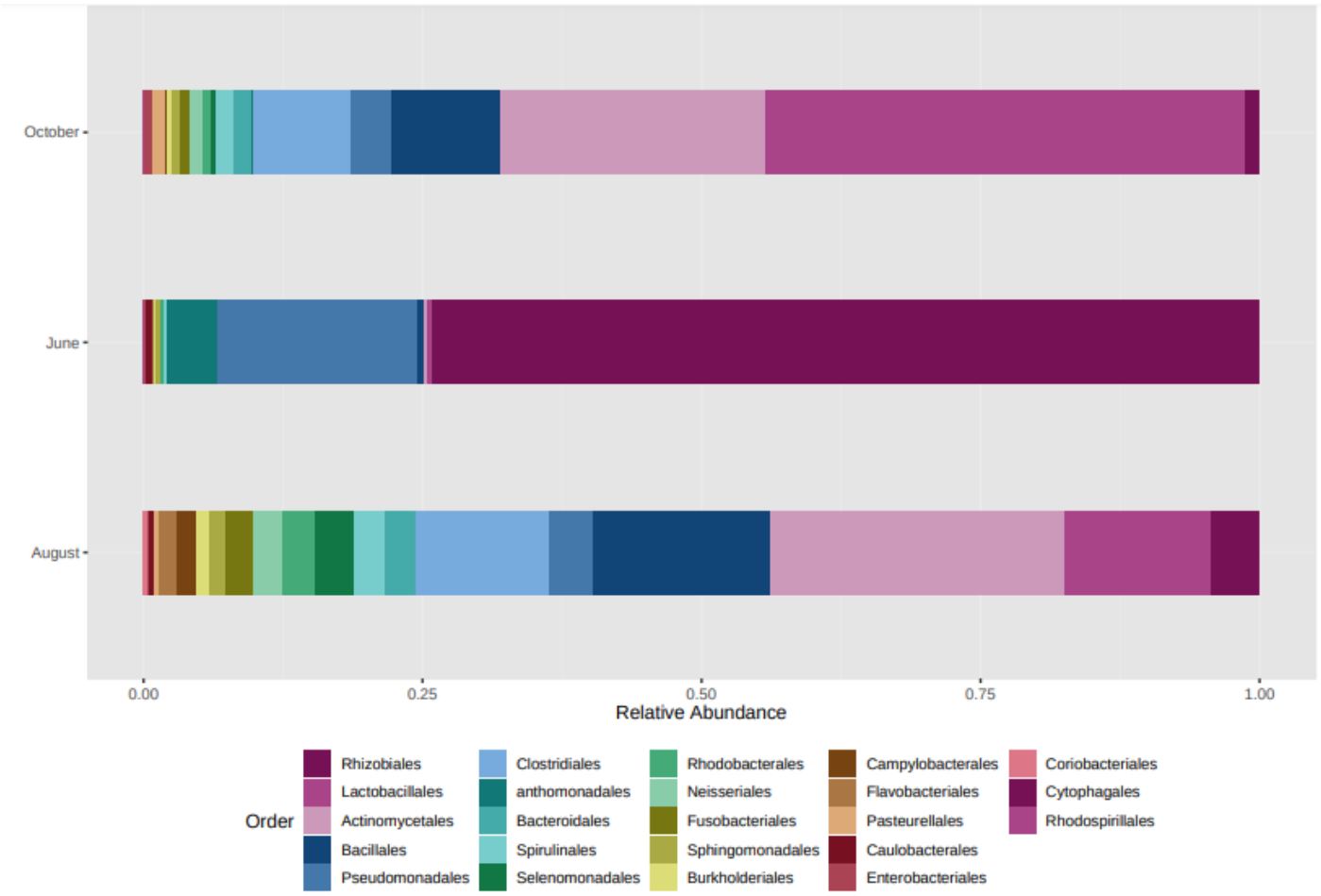


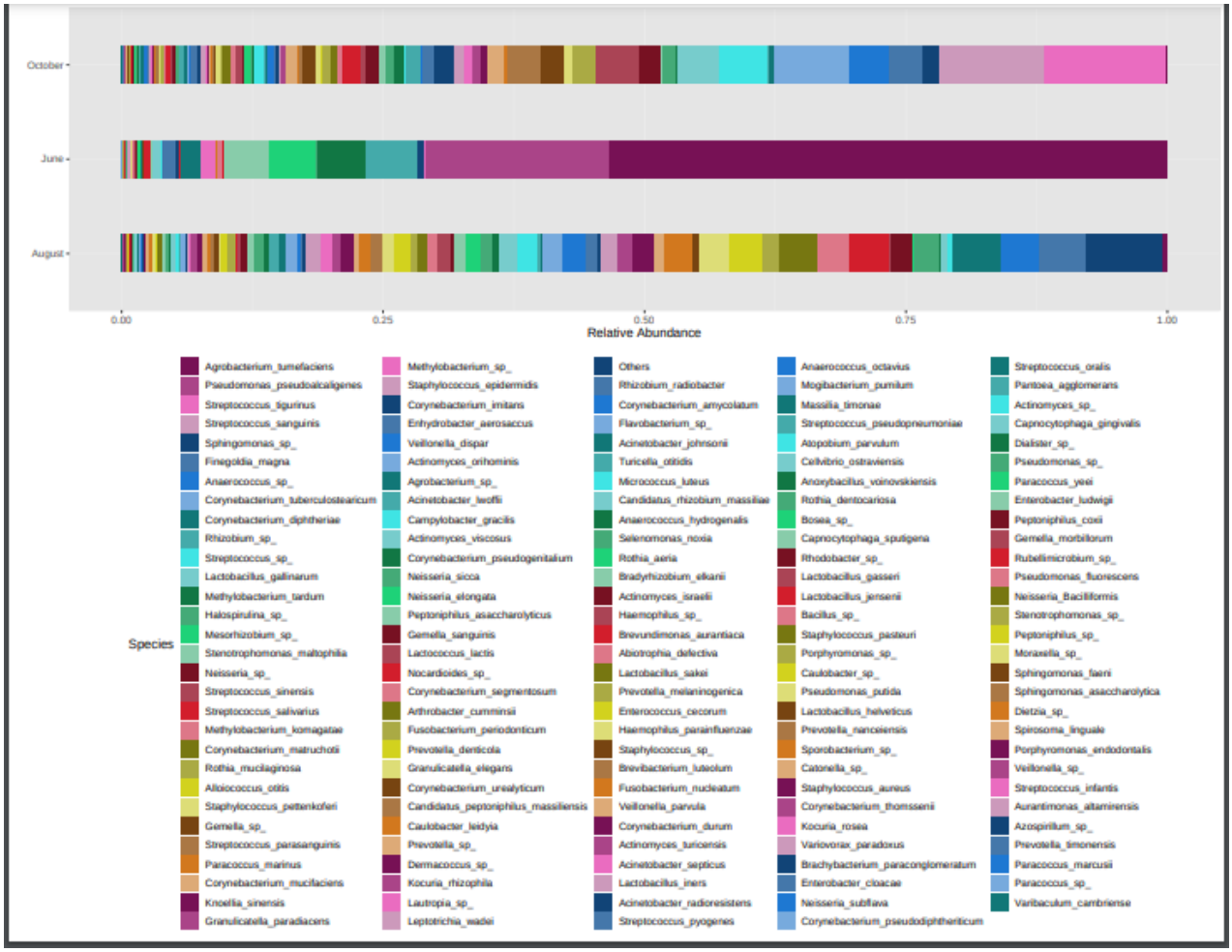

**Figure 4**

The relative abundance of bacterial aerosols at the Family level.




## Figure 5

The relative abundance of bacterial aerosols at the Genus level.




## Figure 6

The relative abundance of the bacterial aerosols at the Species level