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METAGENOMIC ANALYSIS OF MICROBIAL DIVERSITY IN THE URBAN AIR OF SOFIA (BULGARIA) DURING SPRING AND WINTER

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Abstract

The main scientific interest for decades remained focused primarily on physical and chemical air pollutants, which severely limited our knowledge of microbial diversity in the atmosphere. The ecology of airborne microorganisms is poorly understood, even though they are a constant element of the air we breathe. Their exploration is very important for understanding health and ecological impacts, mainly in the highly urbanized cities. The main purpose of this study is to explore the biodiversity of microorganisms in the atmosphere over the densely populated centre of Sofia (Bulgaria) during spring and winter by Next-generation sequencing. The results showed that the most prevailing bacteria in the urban bioaerosols were representatives of phyla *Firmicutes*, *Proteobacteria*, *Bacteroides*, *Actinobacteria*. Many of the identified species were soil and leaf surface dwellers. Most of the detected fungal sequences belong to the phylum *Ascomycota*. Some pathogenic bacterial and fungal taxa were also found. This study provides the first information about microbial diversity in the outdoor air above Sofia and contributes to better understanding of urban airborne microbiota.

Key words: airborne microorganisms, metagenomics, urban air

Introduction. According to the World Health Organization, air pollution is the most significant environmental risk factor for the health of the European population. Regardless of technological and methodological advances in

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recent years, our knowledge of microbial diversity in outdoor air is currently extremely scarce. Due to the lack of standardization, different studies show considerable variation, both in concentration and species composition, depending on the sampling methods used. The varying concentration of microorganisms in air, combined with the negative effects of some abiotic factors, are limiting culture-dependent approaches in the qualitative and quantitative analysis of microbial diversity in air. These shortcomings have largely been overcome by the application of metagenomic analysis and have become an increasingly preferred method for studying species diversity in the air. Studies concerning the air above highly urbanized areas are particularly relevant and important since air pollution generally affects people in cities much more than those in rural areas. Changes in microbial concentration and diversity are affected by urbanization specifics of the settlement, geographical location, and climate. At the same time, each of the conducted studies were carried out in cities drastically different in terms of their geographical, climatic, and anthropogenic characteristics. A number of studies have shown that microbial communities in urban air are mainly composed of *Firmicutes* (*Thermoactinomyces*, *Bacillus*, *Staphylococcus*, *Streptococcus*, *Abiotrophia*), *Alpha-*, *Beta-* and *Gamma-proteobacteria* (*Acinetobacter*, *Stenotrophomonas*, *Pseudomonas*, *Sphingomonas*, *Massilia*, *Delftia*, *Brevundimonas*), *Bacteroides* (*Empedobacter*, *Pontibacter*, *Adhaeribacter*, *Hymenobacter*), *Actinobacteria* (*Thermobifida*, *Streptomyces*, *Kitasatospora*, *Propionibacterium*, *Friedmanniella*) and *Cyanobacteria* (*Crinalium*) [1–6]. It should be noted that among the identified OTUs (Operational taxonomic units) in urban air, in addition to typical soil dwellers, some pathogenic microorganisms and allergens were also found. So far, no studies have been conducted to investigate microbial diversity in outdoor air over Sofia city (Bulgaria), the geographic location of which determines a longer retention of polluted air.

Materials and methods. ***Location and sampling methodology.*** The samples were collected from the outdoor air over the highly urbanized central part of Sofia, Bulgaria. The sampling location was the rooftop (20 m above the ground) of the Faculty of Biology, Sofia University (42°41′01.9″ N, 23°19′58.3″ E). Samples were collected during spring (May 2020) and winter (January 2021). For sampling aspiration device – Higitest (Maimex106) was used with four glass fibre filters (Whatman™, ∅ 80 mm/ pore size 1.6 µm). The holders of the device were disinfected with 70% ethanol before each sampling. The flow rate of the air stream was 100 m^3/h. The total volume of the air passed through the filters was ~ 1000 m^3. Immediately after sampling, the filters were removed from the device and stored at $-20\,^{\circ}C$ until further analysis.

DNA isolation. Prior to the DNA isolation, a modified pretreatment protocol was used to detach the cells from the filters and to yield more DNA [7]. Quick-DNA™ Fecal/Soil Microbe MiniPrep kit (Zymo Research Corp., USA) was used for the isolation of metagenomic DNA. A Quawell UV Spectrophotometer

Q3000 was used to determine the concentration and purity (A260/280) of the isolated DNA.

Next-generation sequencing. Next-generation sequencing (NGS) of the extracted DNA was performed via an Illumina HiSeq platform by Novogene Co., Ltd., UK. For bacterial identification the V3–V4 region of the 16S rRNA gene was amplified using primer pair 341F/806R. For fungal identification primers ITS3/ITS4 targeting the ITS2 region of the 18S rRNA gene were used. The effective tags were analyzed by software Uparse v7.0.1001 [8]. OTUs were obtained by clustering with 97% identity on the Effective Tags of all samples, and then identified [9,10]. An interactive tool Krona was used to visualize and explore the result of the taxonomic annotation analysis.

Results and discussion. ***Number of OTUs and taxonomic annotation.*** All sequences were clustered into OTUs at the 97% sequence similarity value. The detected bacterial OTUs in spring were 1010, whereas in winter they were 238, likely due to the influence of the unfavourable meteorological factors. Two hundred and ninety-three fungal OTUs were detected in the spring sample. The low fungal concentration in January was the reason for the insufficient amount of DNA for metagenomics in winter. The results showed high microbial diversity of bioaerosols, which supports the idea that air can be a habitat for a huge variety of microorganisms belonging to different taxonomic groups.

Taxonomic bacterial and archaeal diversity in spring. The Next-generation sequencing of the spring sample shows not only representatives of the domain *Bacteria* (99.93%), but also detection of archaeal sequences – 0.07% (Fig. 1). In the domain *Bacteria*, 29% of all sequences belonged to unclassified representatives of the class *Oxyphotobacteria*. The most abundant phylum was *Actinobacteria* (27%).

Bacterial sequences attributed to the genera *Kocuria*, *Bifidobacterium*, *Friedmanniella*, *Gardnerella*, *Nocardioides*, *Microbacterium*, *Arthrobacter* had the largest share. The discovery of an anaerobic species from genus *Bifidobacterium* in the air is surprising, but sequences of the same genus have also been detected in another study of bioaerosols [11].

Twenty percent of all sequences were assigned to the phylum *Proteobacteria*. Within this phylum, 70% of representatives belong to *Alpha-*, 29% to *Gamma-* and 1% to *Deltaproteobacteria*. The most representatives within the *Alphaproteobacteria* were assigned to the genera *Paracoccus*, *Rubellimicrobium*, *Sphingomonas*, *Methylobacterium*, *Wolbachia*, *Roseomonas*, *Skermanella*; the most sequences assigned to *Gammaproteobacteria* belonged to genera *Gilliamella*, *Pantoea, Pseudomonas*, *Massilia, Ralstonia*. The species *Sphingomonas endophytica*, *Sphingomonas faeni*, *Pseudomonas aeruginosa*, *Pseudomonas psychrophila* and *Pseudomonas stutzeri* were detected. The share of unclassified representatives of *Deltaproteobacteria* was large (over 60%), most of the identified representatives were assigned to the genus *Oligoflexus*. Representatives of the phylum *Firmicutes*

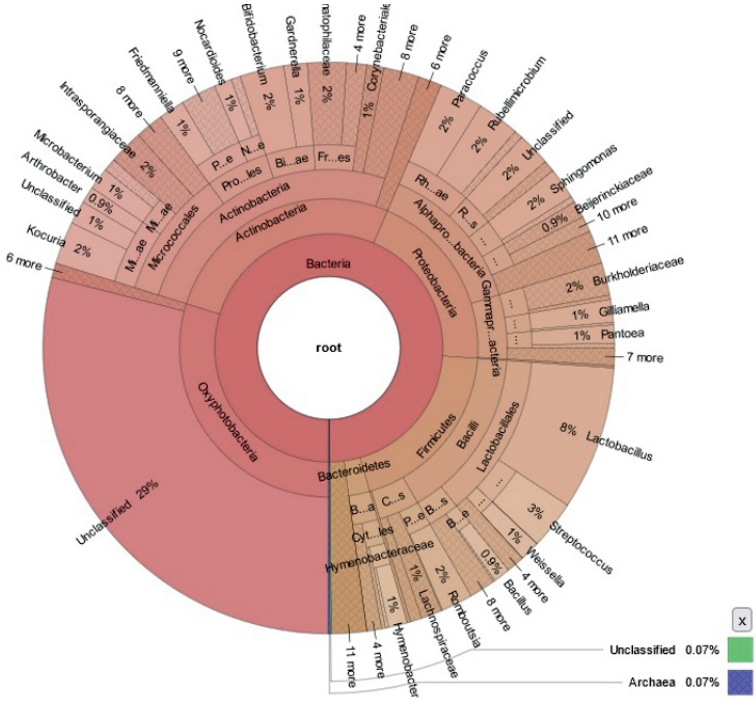


Fig. 1. Taxonomic distribution of airborne bacteria in spring

also had a large share, as 19% of the annotated sequences within the domain *Bacteria* were attributed to it. Within this phylum, 80% of the sequences were assigned to the *Bacilli* class. Most sequences belonged to the genus *Lactobacillus* (8%). Sequences of the species *Lactobacillus apis*, *Lactobacillus kunkeei*, *Lactobacillus murinus*, *Ligilactobacillus salivarius*, *Lactobacillus ingluviei* were identified. Three percent of all sequences belonged to representatives of the genus *Streptococcus*. *Streptococcus agalactiae* and *Streptococcus anginosus* subsp. were detected in the sample. The other widely represented genera were: *Romboutsia*, *Weissella*, *Bacillus*, *Aerococcus*, *Exiguobacterium*, *Blautia*, *Dorea*.

Archaeal sequences of phylum *Euryarchaeota* and the family *Nitrososphaeraceae* were detected in our samples. The sequences were attributed to the genera *Methanobrevibacter*, *Candidatus Nitrososphaera*, *Candidatus Nitrocosmicus*, *Methanosaeta*, *Halobaculum*, *Halarchaeum*. The presence of archaea in the atmosphere remains poorly understood. One of the few published studies of successful detection of archaeal DNA sequences in air samples is of three clades assigned to the *Crenarchaeota* [12]. *Thaumarchaeota* and *Euryarchaeota* presence in air samples was reported in Germany [13].

Taxonomic bacterial diversity in winter. In the winter sample only representatives of the domain Bacteria were found in contrast to the spring (Fig. 2). An impression is made by the dominant presence of the phylum *Proteobacteria* –

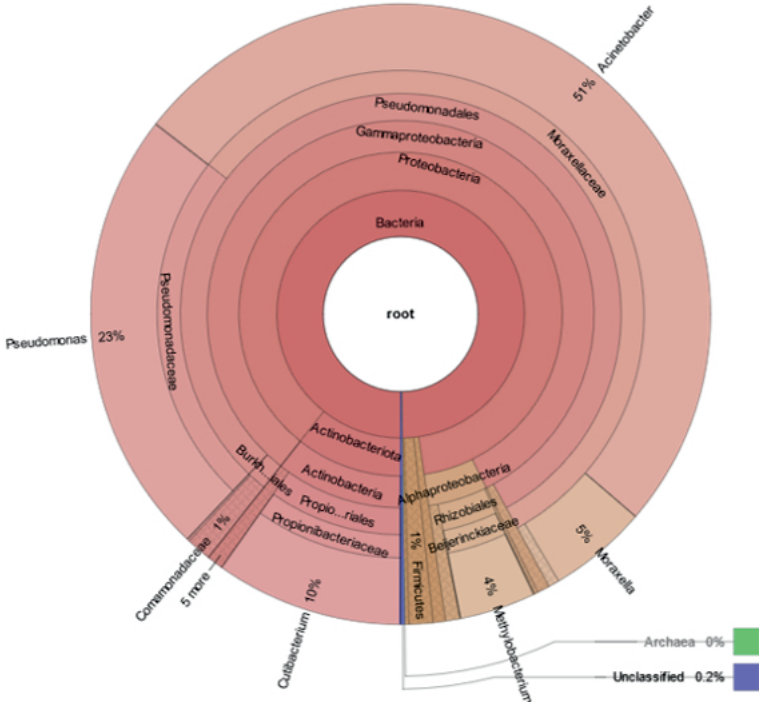


Fig. 2. Taxonomic distribution of airborne bacteria in winter

87% of all sequences during winter were assigned to it, whereas in spring the share was only 20%. Most sequences belong to the genera: *Acinetobacter*, *Pseudomonas*, *Moraxella*, *Methylobacterium-Methylorubrum*, *Comamonas*, *Sphingomonas*. The second most abundant phylum was *Actinobacteria*, with 11% of the sequences assigned to it. Most of the identified representatives belonged to the genera: *Cutibacterium*, *Corynebacterium*, *Georgenia*, *Cellulomonas*. Species *Acinetobacter indicus*, *Acinetobacter junii*, *Acinetobacter towneri*, *Cutibacterium acne*, and *Cutibacterium granulosum* were also detected. Only 1% of all bacterial sequences during winter belonged to the phylum *Firmicutes*, which is a significant difference from the spring sample. Within this phylum, the largest percentage of sequences was assigned to the genus *Staphylococcus*. The remaining more widespread genera were *Lactobacillus* and *Enterococcus*.

Regarding seasonality, our studies recorded a dominant share of *Actinobacteria* (27%), *Proteobacteria* (20%) and *Firmicutes* (19%) phyla in the spring season, while in winter 87% of the representatives belonged to *Proteobacteria* and 11% to *Actinobacteria*. There is a significant difference in the prevailing genera during the seasons. The most prevailing genus that was detected in both seasons was *Sphingomonas*. The dominant bacterial genera in outdoor air during spring were *Lactobacillus*, *Streptococcus*, *Kocuria*, *Bifidobacterium*, *Paracoccus*, *Rubellimicrobium*, *Sphingomonas*, *Romboutsia*, *Friedmanniella*, and *Pantoae*. The most prevalent

bacterial genera detected in winter were *Acinetobacter*, *Pseudomonas*, *Cutibacterium*, *Moraxella*, *Methylobacterium-Methylorubrum*, *Comamonas*, *Corynebacterium*, *Staphylococcus*, *Georgenia*, and *Sphingomonas*. Various metagenomic studies of the air over urbanized areas show that the bacterial communities mainly consist of representatives of the phyla *Firmicutes*, *Proteobacteria*, *Bacteroides*, *Actinobacteria*, and *Cyanobacteria* [1, 4, 5, 14, 15], which was also confirmed by the results obtained in our study. Seasonality in bacterial diversity in air samples from the city of Beijing was also found, where the abundance of *Cyanobacteria*, *Firmicutes* and *Actinobacteria* was highest in spring, and in winter: *Proteobacteria*, *Actinobacteria* and *Firmicutes* predominate [16]. In Seoul, South Korea results showed a dominant presence of genera *Actinobacteria*, *Proteobacteria* and *Firmicutes* in spring [17]. A metagenomic study conducted in winter in Italy established a dominant presence of representatives of the genus *Sphingomonas* (*Proteobacteria*) [18]. Similar results were obtained in Hong Kong during the cold months, where sequences belonging to the phylum *Proteobacteria* were predominantly identified [17].

We also detect some opportunistic pathogens and pathogenic species like *Streptococcus anginosus*, *Streptococcus agalactiae*, *Kocuria rosea*, *Pseudomonas aeruginosa*, *Pantoea agglomerans*, *Ralstonia picketti*, *Dietzia maris*, *Cutibacterium acnes*, *Mycobacterium* sp., *Acinetobacter lwoffii*, *Veillonella montpellierensis*, *Rhodococcus corynebacterioides*, *Staphylococcus epidermidis*, *Citrobacter freundii*, *Delftia tsuruhatensis*, *Enteroccocus faecalis*. Other pathogens detected in the samples were associated with the genera: *Roseomonas*, *Bacillus* and families *Rickettsiaceae* and *Burkholderiaceae*. Some plant pathogenic genera (*Ralstonia*, *Rathayibacter*) and species (*Curtobacterium flaccumfaciens*, *Rhodococcus fascians*) were also detected. In Madrid, Spain, opportunistic pathogens like *Enterobacter*, *Streptococcus*, *Acinetobacter*, *Corynebacterium*, *Cutibacterium*, *Klebsiella* were also established [2]. Sequences of opportunistic pathogens and phytopathogens were also detected in Mexico [6]. At the species level, the presence of some opportunistic pathogens *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *Streptococcus salivarius*, *Propionibacterium acnes* and *Pseudomonas aeruginosa* were also found [17].

Taxonomic fungal diversity in spring. The metagenomic analysis of the ITS2 region for the identification of fungal representatives showed that 98% of all sequences were assigned to phylum *Ascomycota* (Fig. 3).

The most abundant fungal genus was *Cladosporium*, to which 64% of all fungal sequences in the sample were assigned to. Fifteen percent of all sequences were assigned to the genera *Alternaria*, 2% to *Pseudopithomyces*, 2% to *Aureobasidium*, and 11% were unclassified. Of the sequences assigned to *Basidiomycota* (1.94%), the genera *Rhodotorula*, *Cryptococcus*, *Ustilago* were most abundant. The *Chytridiomycota* division was least represented, with 0.04% of all fungal sequences assigned to the genus *Olpidiaster*. OTUs belonging to the *Chytridiomycota* were

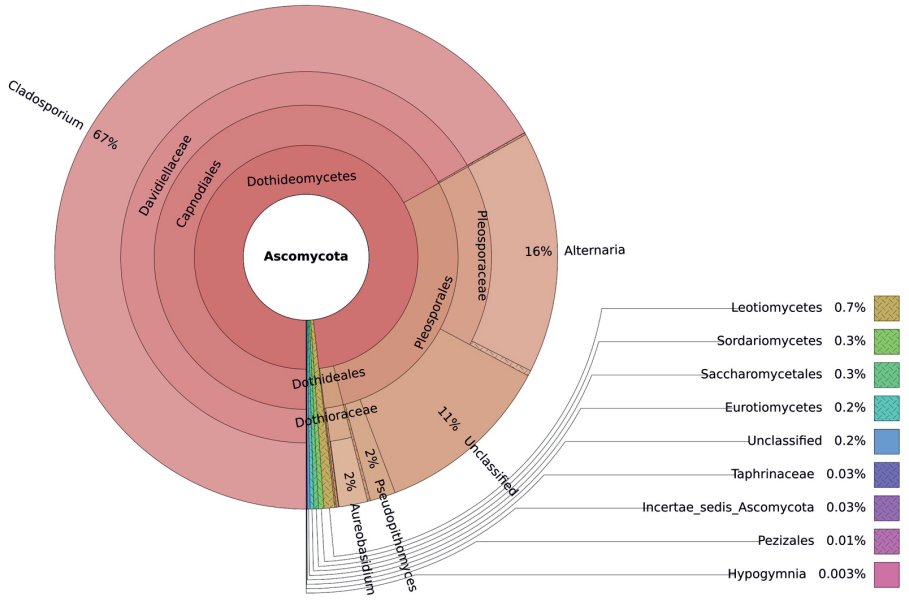


Fig. 3. Taxonomic distribution of airborne fungi in spring

found in only one of the samples in a similar study in Seoul, South Korea [16]. The dominant presence of fungi from the phylum Ascomycota and genus *Cladosporium* was also reported in other metagenomic studies [6, 14, 16, 17, 19].

Sequences of the species *Aureobasidium pullulans*, *Periconia byssoides*, Phoma macrostoma, *Sawadaea bicornis*, *Rutstroemia firma*, *Candida humilis*, *Saccharomyces cerevisiae*, *Fusarium solani*, and *Khuskia oryzae* were detected. Among the fungal representatives identified in spring, a number of seasonal allergenic and pathogenic fungi were identified – *Cladosporium cladosporioides*, *Fusarium solani* and representatives of the genus *Alternaria*. Several plant pathogens were also found: *Cl. cladosporioides*, *Pyrenophora tritici-repentis*, *Khuskia oryzae*, *Olpidium brassicae*, *Fusarium solani*, *Neocatenulostroma germanicum*, *Monilinia fructigena*, *Phaeosphaeria junicola*, *Diaporthe helianthi*, *Gaeumannomyces graminis*, *Drechslera andersenii*, *Sydowia polyspora*, as well as representatives of species of the genera *Alternaria*, *Botrytis*, and *Stemphylium*. Airborne fungal presence is a significant risk factor for human health, as many species are significant pathogens and allergens.

Conclusions. Despite the apparently hostile environment, we found a high taxonomic diversity with pronounced seasonality differences in the bioaerosols in urban outdoor air over Sofia. Microbial diversity was higher during spring, likely due to more favourable abiotic factors of the environment. Many of the detected representatives are typical soil and plant surface dwellers. Potentially pathogenic taxa were also detected, as many of these microorganisms are typical soil, water, and animal fecal inhabitants, implying the constant presence of these

microorganisms coming from different sources. Further investigation would be necessary to distinguish pathogenic potential of species found in urban air. If so, airborne microorganisms can be considered a potential air pollutant if they can cause allergic diseases and infections. Studies of urban air are of great importance due to urbanization rates and the need for developing public health strategies for minimizing exposure to air pollution and airborne pathogens.

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