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Bioaerosols in the Athens Metro: Metagenetic insights into the PM_{10} microbiome in a naturally ventilated subway station

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ABSTRACT

To date, few studies have examined the aerosol microbial content in Metro transportation systems. Here we characterised the aerosol microbial abundance, diversity and composition in the Athens underground railway system. PM_{10} filter samples were collected from the naturally ventilated Athens Metro Line 3 station "Nomismatokopio". Quantitative PCR of the 16S rRNA gene and high throughput amplicon sequencing of the 16S rRNA gene and internal transcribed spacer (ITS) region was performed on DNA extracted from PM_{10} samples. Results showed that, despite the bacterial abundance (mean = 2.82×10^5 16S rRNA genes/m^3 of air) being, on average, higher during day-time and weekdays, compared to night-time and weekends, respectively, the differences were not statistically significant. The average PM_{10} mass concentration on the platform was 107 μg/m^3. However, there was no significant correlation between 16S rRNA gene abundance and overall PM_{10} levels. The Athens Metro air microbiome was mostly dominated by bacterial and fungal taxa of environmental origin (e.g. *Paracoccus*, *Sphingomonas*, *Cladosporium*, *Mycosphaerella*, *Antrodia*) with a lower contribution of human commensal bacteria (e.g. *Corynebacterium*, *Staphylococcus*). This study highlights the importance of both outdoor air and commuters as sources in shaping aerosol microbial communities. To our knowledge, this is the first study to characterise the mycobiome diversity in the air of a Metro environment based on amplicon sequencing of the ITS region. In conclusion, this study presents the first microbial characterisation of PM_{10} in the Athens Metro, contributing to the growing body of microbiome exploration within urban transit networks. Moreover, this study shows the vulnerability of public transport to airborne disease transmission.

1. Introduction

Metro systems constitute a unique, confined and typically heavily occupied type of public indoor micro-environment that millions of people spend a considerable amount of time in on a daily basis. Assessment of indoor air quality and exposure of commuters to air pollution in Metro systems has drawn much attention due to its public health importance (Loxham and Nieuwenhuijsen, 2019; Xu and Hao, 2017; Klepczyńska Nyström et al., 2010). The majority of studies have focused on suspended particulate matter (PM), investigating its concentration and chemical constituents, on station platforms and inside trains (Moreno et al., 2018; Nieuwenhuijsen et al., 2007). However, only a few studies have examined the biological fraction of PM (i.e. bioaerosols) in subway networks. Despite the recognition of the impact of bioaerosol exposure on human health (Kim et al., 2018; Walser et al., 2015; Douwes et al., 2003) and the critical role of transit networks in airborne transmission of infectious micro-organisms (Goscé & Johansson, 2018; Nasir et al., 2016; Mohr et al., 2012), relatively little is known about the microbial airborne particles in Metro systems.

Most microbial investigations of bioaerosols use culture-based approaches (Table S1), with only a few studies to date applying molecular methods for characterising the aerosol microbiota (Dybwad et al., 2017, 2014, 2012; Zhou and Wang, 2013; Birenzvige et al., 2003). Recently, high throughput sequencing (HTS) has started expanding our knowledge of the aerosol microbial diversity in Metro systems. HTS studies carried out within the Metro networks of New York (Robertson et al., 2013), Hong Kong (Leung et al., 2014), Barcelona (Triadó-Margarit et al., 2017) and Oslo (Gohli et al., 2019) demonstrated that the subway air microbial assemblages are composed of a mixture of human commensals (e.g. skin-associated bacteria) and outdoor-air originating

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micro-organisms (e.g. soil-dwelling bacteria).

There is increasing interest on mapping the microbiome in underground railways as part of the urban infrastructure, as our knowledge of the microbial communities associated with the urban environment lags behind that of natural ecosystems. Recently, an international consortium was launched with the aim of profiling the microbiome of transit networks (The MetaSUB International Consortium, 2016). However, the particular surveys are based on surface-borne DNA obtained with swab samples (Klimenko et al., 2020; Walker et al., 2018) and air sampling is not included in their study design. Therefore, additional efforts are necessary in characterising the largely unexplored aerosol microbiome in underground transport settings.

The aim of this study was to characterise the microbial component of aerosols in the Athens underground railway system (Athens Metro, Greece). The Athens Metro consists of two underground lines (Lines 2 and 3 including 40 stations, at the time of sampling) and one almost entirely overground railway line (Line 1 with 24 stations) (Fig. S1A). The subway part of the network serves an estimated 938,000 passengers on a daily basis (www.ametro.gr). To our knowledge, investigations on the air quality of the Athens subway system have been limited to the physicochemical characterisation of PM on platforms and the interior of train cabins (e.g. Mendes et al., 2018; Assimakopoulos et al., 2013). So far, PM measurements at several stations have shown that the mass concentrations in the Athens subway environment are relatively elevated (Mammi-Galani et al., 2017) and considerably higher compared to those outdoors (Martins et al., 2016, Barmparesos et al., 2016).

Here, we provide the first biological characterisation of PM with an aerodynamic diameter $\leq$ 10 μm (PM_{10}) in the Athens Metro by presenting a description of the aerosol bacterial and fungal composition in a subway station platform based on HTS. To our knowledge, this is the first study to describe the mycobiome diversity in the air of a Metro environment based on amplicon sequencing of the internal transcribed spacer (ITS) region. Such studies are vital to expanding our knowledge of the bioaerosol microbiome in the public transport environment as well as providing an insight into human exposure to airborne particulate matter with potential for disease transmission within urban transit networks.

2. Material and methods

2.1. The study

This study was part of an extensive sampling campaign, performed during April-May 2014, in collaboration with the National Centre of Scientific Research (N.C.S.R) "Demokritos" (Institute of Nuclear & Radiological Sciences & Technology, Energy & Safety - Environmental Radioactivity Laboratory, Athens, Greece) for characterising the airborne particulate matter in the Athens Metro system (Mendes et al., 2018; Martins et al, 2016).

2.1.1. Sampling site

Sampling was conducted on the platform of the underground station "Nomismatokopio" (Fig. S1B), which is part of the Athens Metro Line 3 (Blue Line), situated in northeast Athens and commenced operation in September 2009. The particular station was chosen due to its proximity (1.8 km) to N.C.S.R "Demokritos" and it offered easy access. The 110 m long dual-side platform station is 20 m below ground, underneath a highly trafficked road, and is naturally ventilated (Mendes et al., 2018). The double-track tunnel design and ventilation conditions of this station are common characteristics of the majority of the network. Trains run from 05:30 until 00:30 every day, except Friday and Saturday when they run until 02:30, with a frequency between 4 and 10 min, depending on the day (weekday or weekend) and time of day (peak hours: 07:00–11:00 and 13:30–17:30).

2.1.2. Sample collection for bioaerosols analysis

Six PM_{10} samples were collected on 47 mm diameter polytetrafluoroethylene (PTFE) filters (0.45 μm pore size, PALL Life Sciences, USA) using a low-volume gravimetric sampler (ENCO PM, TCR TECORA, Italy). The sampler was equipped with a PM_{10} inlet, meeting the EN 12341 standard, and operated at 38 L/min. The instrument was placed in a central position on the platform, inside a metal cage with gridded walls, with the sampling inlet height being 1.8 m. To provide a representative description of the microbial component of aerosols to which individuals are exposed, sampling was carried out during both day-time (07:00–00:00) and night-time (00:00–05:30) hours on weekdays and weekends over a 2-week period. Sampling was performed continuously during the dates and times shown in Table 1. Prior to sampling, the sampler was sterilised with 70% (v/v) ethanol. After sampling, filters were placed in sterile petri-slides, sealed with parafilm and stored at −20 °C. Two unexposed filters (field blanks) were also analysed to account for contaminants introduced during sample collection and processing. PM_{10} mass concentration (μg/m^3) was measured using a DustTrak DRX Aerosol Monitor 8533 (TSI Inc., USA) with a 5-minute time resolution, duly calibrated using concurrent gravimetric measurements in the Metro station. Environmental conditions (temperature and relative humidity) recorded during sampling are shown (Table 1).

2.2. Sample processing

2.2.1. DNA extraction

All procedures except those involving phenol/chloroform, which were conducted in a chemical fume hood, were performed in a Biosafety Level II cabinet (laminar-flow hood) using aerosol resistant pipette tips (ZAP Premier Filter Tips, Alpha Laboratories Ltd., UK) with surfaces cleaned with 0.5% (v/v) sodium hypochlorite solution. Pre-sterilised 5 ml screw cap tubes (RNase-/DNase-free, Axygen Inc., USA) filled with 0.75 g sterile 0.1 mm zirconium/silica lysing beads (BioSpec Products Inc., USA) were used. Filters were aseptically inserted into the 5 ml tubes using sterile forceps with the top (exposed) side facing inward. Cells were lysed by bead beating in 1 ml extraction buffer [2.5% (w/v) sodium dodecyl sulfate (SDS), 10 mM Tris-HCl pH 8, 25 mM Na_2EDTA pH 8, 100 mM NaCl and molecular biology grade water] using a Vortex-Genie 2 (Scientific Industries Inc., USA) with a MO BIO vortex adapter for 5 ml tubes (MO BIO Laboratories Inc., USA) at maximum speed (3200 rpm) for 12 min, followed by incubation in a 75 °C water bath for 30 min. The extracts were purified using phenol:chloroform method and DNA was recovered by isopropanol precipitation. To enhance DNA recovery, 2.5

Table 1
Sampling details.

Sample identity	Sampling date (Start time)	Sampling duration	Mean Temperature	Mean Relative humidity
Weekday-A	Tuesday 29/4 (07:00)	16 h 57 min	23.6 °C	38.0%
Week-night	Thursday 01/5 (00:00)	05 h 28 min	24.8 °C	36.6%
Saturday-day	Saturday 03/5 (07:00)	16 h 57 min	23.6 °C	45.5%
Weekday-B	Wednesday 07/5 (07:00)	16 h 57 min	24.2 °C	30.7%
Saturday-night	Saturday 10/5 (00:00)	05 h 28 min	23.6 °C	33.4%
Weekday-C	Tuesday 13/5 (07:00)	16 h 57 min	26.3 °C	37.2%

μl of co-precipitant glycogen (Ambion GlycoBlue, 15 mg/ml, Invitrogen, Thermo Fisher Scientific Inc., USA) was added. An extra precipitation step was included by addition of 2.5 vol absolute ethanol and 1/10 vol 3 M sodium acetate (pH 5.2). Finally, the DNA pellet was washed in ice-cold 70% (v/v) ethanol, air dried at room temperature and resuspended in 35 μl of sterile water (Fermentas, Thermo Fisher Scientific Inc., USA).

2.2.2. qPCR of the 16S rRNA gene

Bacterial abundance was determined by qPCR of 16S rRNA gene, using a SYBRgreen-based assay with the bacterial primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) (See Supplementary Information). PCR amplifications were performed in triplicate on a Bio-Rad CFX96 Real-Time System/C1000 Thermal Cycler (Bio-Rad Laboratories Inc., USA). The reaction mixture (10 μl) included 5 μl (1×) SensiFAST SYBR No-ROX Kit (Bioline, UK), 0.2 μl forward and reverse primers (10 μM), 3.6 μl DNA-free water (QIAGEN, Germany) and 1 μl DNA template under the following cycling conditions: 95 °C for 3 min, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. No-template controls were also included. Data were acquired by CFX Manager Software (Bio-Rad Laboratories Inc., USA) and results were reported in units of 16S rRNA gene copies per cubic meters of air.

2.2.3. Amplicon sequencing of the 16S rRNA gene and the internal transcribed spacer (ITS) region

HTS was performed using Illumina MiSeq platform (Illumina Inc., USA) with either the bacterial PCR primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) or the fungal primers ITS1F (Gardes and Bruns, 1993) and ITS2 (White et al., 1990). In addition to samples and filter blanks, one negative control (no template) was also included as a procedural control. For the fungal ITS sequencing, field blanks were not further processed, as they did not show any amplification of ITS1 region. Although no PCR amplification was observed for the negative control during the ITS library preparation, it was included in the ITS sequencing run for quality control. Library preparation was carried out using Illumina's Nextera XT Index kit in accordance with the Illumina dual-indexing protocol (Supplementary Information) and as previously described (Clark et al., 2020). Raw sequence data were submitted to the NCBI Sequence Read Archive (SRA) database under BioProject PRJNA597004.

2.2.4. Bioinformatics analysis of sequence libraries

The quality of the raw sequencing data was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences were trimmed to a minimum nucleotide Phred quality score of 20 and minimum sequence length threshold of 180 bp using the fastq_quality_trimmer from the FastX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Quality-trimmed sequences were processed using QIIME version 1.9.1 (Caporaso et al., 2010). Chimeric sequences were identified and removed using UCHIME algorithm (Edgar et al., 2011) implemented in VSEARCH (Rognes et al., 2016) and clustering of sequences into operational taxonomic units (OTUs) was performed using open-reference OTU picking protocol (97% similarity). Taxonomy assignment to the bacterial and fungal OTUs was performed using the RDP (Cole et al., 2014) and the UNITE (Nilsson et al., 2018) databases, respectively.

The resultant bacterial and fungal OTU tables were filtered to remove singleton OTUs, unassigned reads, non-bacterial and non-fungal sequences, respectively, and possible contaminant sequences/OTUs resulting from the blank/control samples. Details of the OTUs found in the negative controls are presented (Supplementary Tables S2-S5, Fig. S2). The numbers of sequences per sample were normalised based on the number of sequences obtained from the smallest library to account for differences in sequencing depth between samples. Supplementary filtering was carried out to remove low-abundance OTUs with 5 or fewer counts in order to minimise the error associated with spurious OTUs and artefactual reads (Brown et al., 2015; Kunin et al., 2010). For bacteria, a total of 47,695 16S rRNA gene sequences were recovered. These sequences were distributed across 1421 bacterial OTUs at 97% identity. For fungi, a total of 694,426 ITS sequencing reads, comprising 3146 fungal OTUs at 97% identity, were retained for downstream analysis.

Fungal OTUs were also assigned to ecological categories, known as guilds, based on the FUNGuild database (Nguyen et al., 2016) using the online Guilds application (http://www.stbates.org/guilds/app.php). Out of the 3146 fungal OTUs, a total of 1945 OTUs were assigned to ecological guilds. However, only 1292 were ranked as "highly probable" or "probable" (i.e. likelihood that the OTU belongs to a specific guild) and were taken into account for the purposes of the analysis. All OTUs parsed into the same ecological guilds were grouped together.

The potential sources of the identified aerosol microbial taxa were explored by matching the representative sequences of the most dominant OTUs to environment types these micro-organisms have been previously found in, based on isolation source metadata described by Environment Ontology (EnvO) terms (Buttigieg et al., 2013), using the bioinformatic pipeline *SEQenv* (Sinclair et al., 2016). *SEQenv* was run on both the 16S rRNA gene and ITS sequences using a 99% minimum identity threshold.

2.2.5. Statistical analysis

Real-time PM_{10} mass concentrations were plotted using *openair* package (Carslaw and Ropkins, 2012) within R computing environment (R Core Team, 2014). Mean values per each sampling duration were presented alongside with the time-integrated estimates of 16S rRNA gene abundance, as determined by qPCR. The normality of data distribution was checked by the Shapiro-Wilk test and non-parametric tests were used when the assumptions for the parametric equivalents were not met. Group means of PM_{10} mass as well as qPCR quantification data were compared using a *t*-test. Correlations between bacterial abundance, PM_{10} mass concentration and environmental variables (temperature, relative humidity) were examined using the Pearson product-moment correlation coefficient. Spearman's rank correlation analysis was also performed to assess the relationship between the relative genus abundance of the dominant bacterial/fungal taxa and the PM_{10} mass concentration levels. Statistical significance for all tests was set at $P < 0.05$.

Within-sample (i.e. alpha) diversity was evaluated using OTU richness and Shannon's index. The alpha diversity measures were estimated and visualised using the microbiome analysis package *phyloseq* (McMurdie and Holmes, 2013) within R. Correlations between bacterial richness and qPCR-based abundance were assessed by Spearman's rank coefficient analysis. The number of core OTUs, defined as the OTUs that are present in all samples, was determined using compute_core_microbiome.py within QIIME.

To assess differences in the microbial composition among samples (i.e. beta diversity), the abundance-based Bray-Curtis dissimilarity and the incidence-based binary Jaccard distance metrics were computed using *phyloseq*. The resulting distance matrices were visualised with Principal Coordinates Analyses (PCoA) 2D-plots generated by *ggplot2* package (Wickham, 2009) within *phyloseq*. Statistical differences in bacterial composition among groups of samples was determined using permutation-based multivariate analysis of variance (PERMANOVA) (Anderson, 2001) with 9999 permutations, performed with compare_categories.py within QIIME. Statistical significance was set at $P < 0.05$.

3. Results

3.1. PM_{10} concentration and bacterial 16S rRNA gene abundance

The average PM_{10} mass concentration on the platform, based on the mean values per sampling period, was 107 $\mu g/m^3$ (Fig. 1). The highest

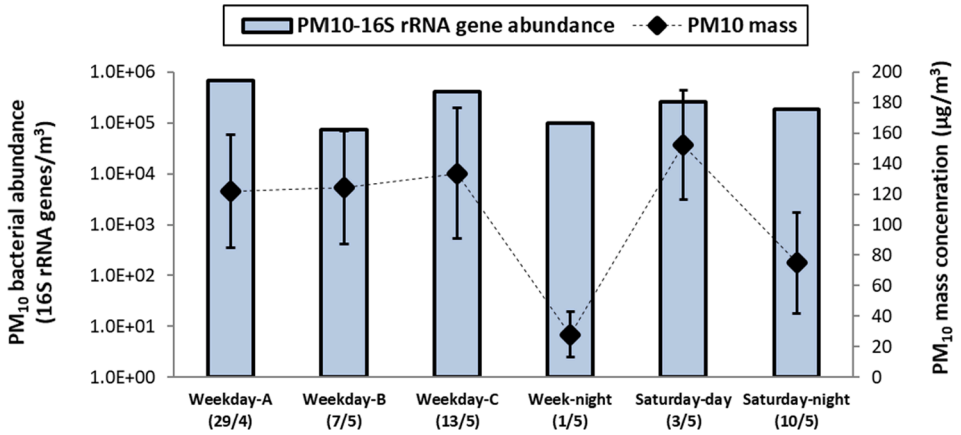

Fig. 1. PM_{10} bacterial abundance at the Athens Metro station "Nomismatokopio", as determined by qPCR. Primary axis y is on log scale and concentrations are presented in 16S rRNA gene copies/m^3 of air per each sampling period. Estimated means of the PM_{10} mass concentrations (µg/m^3) are also presented (secondary y-axis). Error bars represent standard deviation.

concentration (152 µg/m^3) occurred during Saturday (day), whereas the lowest value (28 µg/m^3) was found during Week-night. Time series and overall diurnal variation plots are presented (Fig. S3-S4). Overall, the concentration of PM_{10} during the Metro day-time operating hours (estimated mean from "Weekday A", "B", "C" and "Saturday-day" samples = 133 µg/m^3) were significantly higher (independent samples *t*-test, $t(4) = 4.27$, $P = 0.01$) compared to those measured during the night (estimated mean from "Week-night" and "Saturday-night" samples = 53 µg/m^3). The weekday PM_{10} mass was, on average, relatively lower than the one estimated for the weekend (with and without taking into account night-time values). The mean concentration during Week-night was 2.68 times lower than the one measured during Saturday night.

Bacterial 16S rRNA gene abundances varied between 7.26×10^4 (Weekday-B) and 6.71×10^5 (Weekday-A) 16S rRNA genes/m^3 of air, with an estimated mean abundance of 2.82×10^5 16S rRNA genes/m^3 of air (Fig. 1). Similar to PM_{10}, the day-time bacterial abundance (mean = 3.52×10^5 16S rRNA genes/m^3 of air) was, on average, higher compared to night-time (mean = 1.42×10^5 16S rRNA genes/m^3 of air). However, there was no significant difference (independent samples *t*-test, $P > 0.05$) between day-time and night-time values. In contrast to PM_{10} mass, bacterial gene abundances on the weekdays were on average higher than the one on the weekend (with and without taking into account night-time samples), although the difference was not significant. It is worth noting that the night-time abundance during the weekend (Saturday-night) was greater than the one observed for Week-night, as also noted for the PM_{10} concentration. No significant correlation was found between 16S rRNA gene abundance and overall PM_{10} mass concentration (Pearson's coefficient $r = 0.42$, $P = 0.41 > 0.05$) or any of the environmental parameters measured (Pearson's coefficient test, $P > 0.05$).

3.2. Aerosol bacterial and fungal composition

Overall, the top four phyla in the bacterial 16S rRNA libraries were: Proteobacteria (44.6%), Actinobacteria (33.5%), Firmicutes (12.5%) and Bacteroidetes (5.7%), comprising > 96% of all reads (Fig. 2). Among the 18 bacterial classes detected at mean relative abundance > 0.1% across all samples, the most abundant ones were *Actinobacteria* (33.5%), *Alphaproteobacteria* (27.9%), *Gammaproteobacteria* (10.1%) and *Bacilli* (9.2%). At the genus level, there were 122 groups found (>0.1%) across samples, accounting for almost 91% of the total reads. *Paracoccus* (within the *Alphaproteobacteria*) was the most abundant genus in the air of the Athens station, with relative abundances ranging from 10.5% (Weekday-B) to 6.4% (Week-night) and a mean value of 8.0%, followed by *Sphingomonas* (Saturday-night 7.7% – Weekday-B 5.0%) (Fig. 2). Other highly represented members of the aforementioned classes included *Kocuria* (5.6%, *Actinobacteria*), *Acinetobacter* (3.9%, *Gammaprotebacteria*) and *Staphylococcus* (2.8%, *Bacilli*). Among other genera, less abundant taxa detected at proportions <1% (not shown in Fig. 2) included *Dietzia, Streptococcus, Enterobacter*, *Enterococcus*, *Anaerococcus*, *Blautia*, *Burkholderia* and members of *Ruminococcaceae.*

It is worth noting that whilst some bacteria were more abundant during day-time compared to night-time, such as *Corynebacterium* (day-time mean 3.79% > night-time mean 1.38%), *Acinetobacter* (day-time mean 4.73% > 2.32%), *Staphylococcus* (day-time mean 3.51% > night-time mean 1.35%) and *Micrococcus* (day-time mean 1.62% > night-time mean 0.28%), the average proportions of the majority of the top genera appeared to be relatively increased during night-time (e.g. *Sphingomonas* day-time mean 5.53% < night-time mean 7.66%, *Rubellimicrobium* day-time mean 4.32% < night-time mean 6.11%, *Arthrobacter* day-time mean 3.91% < night-time mean 6.44%). When looking at the differences between weekday and weekend samples, the relative abundance of the taxa that were enriched during the day-time Metro operating hours, also appeared relatively elevated during the weekdays compared to the weekend (e.g. *Corynebacterium* weekday mean 3.85% > Saturday 3.62%, *Staphylococcus* weekday mean 3.64% > Saturday 3.12%, *Micrococcus* weekday mean 2.05% > Saturday 0.31%). However, when looking at the difference between the weekday and weekend night-time samples, the proportions of the aforementioned genera were increased during Saturday night compared to the Week-night (e.g. *Corynebacterium* Week-night 0.90% < Saturday-night 1.86%, *Staphylococcus* Week-night 0.84% < Saturday-night 1.85%, *Acinetobacter* Week-night 1.71% < Saturday-night 2.93%, *Pseudomonas* Week-night 1.06% < Saturday-night 1.68%).

Within the fungal ITS libraries, the majority of taxa belonged to Ascomycota (62.9%) followed by Basidiomycota (36.4%). Overall, there were 18 fungal classes detected (>0.1%) across all samples, with *Dothideomycetes* (42.5%), *Agaricomycetes* (28.9%), *Leotiomycetes* (9.1%) and *Eurotiomycetes* (4.3%) predominating (Fig. 2). A total of 89 genera (comprising 93% of the total reads) were identified at > 0.1% average proportions. The two most dominant fungal genera detected were ascomycetes *Cladosporium* and *Mycosphaerella* (*Dothideomycetes*) with relative abundances ranging from 30.0% (Weekday-C) to 11.1% (Week-night) and 18.6% (Saturday-night) to 1.9% (Week-night), respectively (mean values 23.9% and 10.7%, respectively), followed by *Antrodia* (*Agaricomycetes*, mean = 7.0%), which was highly enriched during (week) night-time hours (21.5%) (Fig. 2). In lower proportions, *Sistotrema* (2.4%), *Bjerkandera* (2.3%), *Penicillium* (2.3%), *Alternaria* (1.4%), and *Aspergillus* (1.3%) among others, were also found. Various other fungal genera commonly found in indoor environments were also detected albeit at low proportions, such as *Botrytis*, *Aureobasidium* and

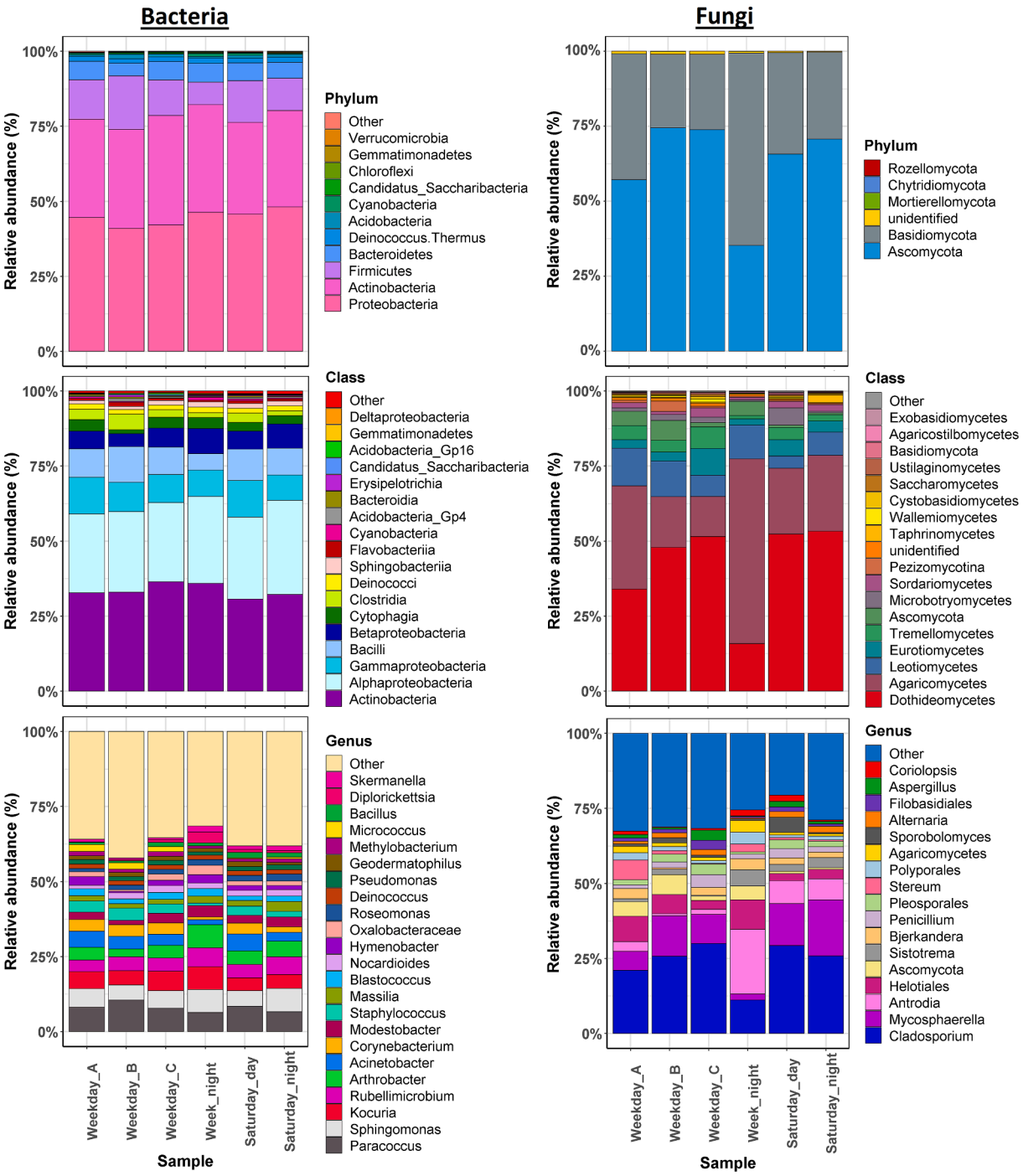

Fig. 2. Relative abundance of bacterial (left) and fungal (right) OTUs at the phylum, class and genus level per each PM_{10} sample collected from the Athens Metro station "Nomismatokopio". For phyla/ classes "Other" denotes the taxa observed at <0.1% mean relative abundance, whereas for genera "Other" denotes the taxa detected at <1% mean relative abundance across samples. The rest of the phyla, classes and genera are ordered according to overall average abundance across samples, from least abundant (top) to most abundant (bottom).

Acremonium (<1%) as well as *Malassezia*, *Candida*, *Cryptococcus*, *Rhodotorula* and *Trichosporon* (<0.5%).

As revealed by the FUNGuild classification (Table S6), the majority of the airborne fungi detected in the Metro station were, on average, plant pathogens (32.9% of total assigned sequences), including taxa such as *Mycosphaerella* and *Blumeria*, wood saprotrophs (32.1%), e.g. *Antrodia*, *Bjerkandera*, *Trametes* and other *Polyporaceae*, or undefined saprotrophs (21.1%), including genera such as *Stereum*, *Steccherinum*, *Tetrachaetum*, *Wallemia* and *Filobasidium*.

The substantially reduced proportions of the predominant genera *Cladosporium* and *Mycosphaerella* during the weekday night-time hours (11.1% and 1.9%, respectively) compared to the regular Metro operating hours (day-time mean values, including Saturday, 26.5% and 10.8%, respectively) largely mirrored the big decrease of Ascomycota and *Dothideomycetes* observed in the particular air sample (Week-night). On the other hand, the (week) night-time enrichment of Basidiomycota and *Agaricomycetes* was highly represented by some of the dominant fungi such as *Antrodia* (night-time 21.5% > day-time mean 3.3%), *Sistotrema* (night-time 5.4% > day-time mean 1.4%) and *Steccherinum* (night-time 2.8% > day-time mean 0.6%). However, the same trend was

not observed during Saturday night, which mostly followed the day-time patterns.

3.3. Aerosol bacterial and fungal diversity

Bacterial richness was strongly associated with bacterial abundance (Spearman's rank correlation coefficient $\rho = 0.94$, $P = 0.017$). Approximately 15.9% of the total observed bacterial OTUs were present across all Metro aerosol samples (i.e. the core microbiome). In addition, 41.5% was common among Weekdays A, C and Saturday (day-time), whereas 21.2% of total identified OTUs was shared among all day-time samples including Weekday-B. A percentage of 44.5% of total observed OTUs was present in both night-time samples. Alpha diversity estimated using Shannon's Diversity Index ranged between 5.60 (Week-night) and 5.90 (Saturday-day) for the airborne bacteria (Fig. S5).

Fungal richness demonstrated higher weekday values compared to the weekend ones, for both day-time and night-time hours (Fig. S5). Across all days, 7.5% of total observed OTUs were found to be present in all samples (core mycobiome). 13.9% of the OTUs were shared among day-time samples, whereas approximately 15.2% of total detected OTUs were common between the night-time samples. The Shannon's Diversity Index estimated that the greatest level of fungal diversity was obtained for Weekday-A (5.03), while the lowest level was observed during Saturday night (4.07) (Fig. S5).

Principal coordinate analysis (PCoA) was performed in order to assess clustering (and potential separation) of bioaerosol samples collected during different days, in terms of bacterial and fungal beta diversity (Fig. 3). For bacteria, PCoA analysis, based on both the Bray-Curtis metric and the Jaccard index, showed that samples collected during day-time (Weekday A, C and Saturday) were closer together, with the exception of the sample collected during Weekday-B, which appeared to be the most dissimilar compared to the rest of the samples. The two night-time samples (Week-night and Saturday-night) were less similar than the ones obtained during day-time (Weekday A, C and Saturday), with the Saturday-night sample being more similar to the day-time samples. When examining the similarity of the samples in terms of fungal OTUs by PCoA analysis, there were no distinct groupings formed. The variation between weekdays (A, B, C and Week-night) and weekend (Saturday-day and night) or between day-time and night-time, in terms of both PM_{10} bacterial and fungal composition, was assessed but no statistical significance was found (PERMANOVA, $P > 0.05$).

3.4. Associated habitats of most abundant bacterial and fungal OTUs

The 15 most abundant bacterial and fungal OTUs, averaged across all samples, and their associated environmental habitats (annotated Environmental Ontology - EnvO terms) indicating potential source environments, according to *SEQenv* analysis (Tables S7-S8), was investigated. Based on the results for bacteria, the most dominant OTUs had diverse source habitats (Fig. 4), with the majority identified as soil-

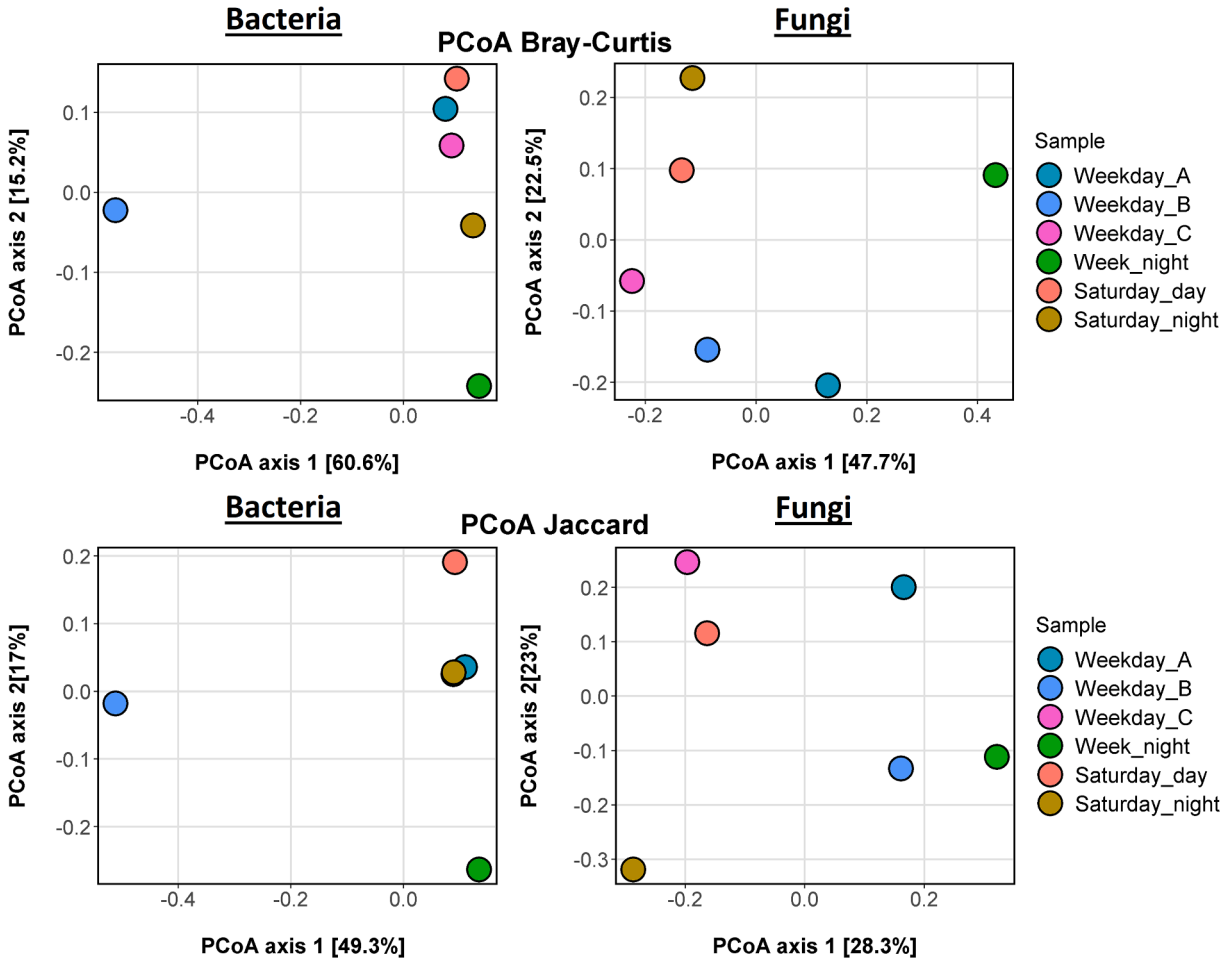

Fig. 3. Principal coordinate analysis (PCoA) plots of bacterial (left) and fungal (right) beta diversity based on Bray-Curtis dissimilarity (top) and Jaccard distance (bottom) matrices. Each point in the plot represents the microbial composition in the PM_{10} samples collected from the Athens Metro station "Nomismatokopio". Points in greater proximity correspond to more compositionally similar samples.

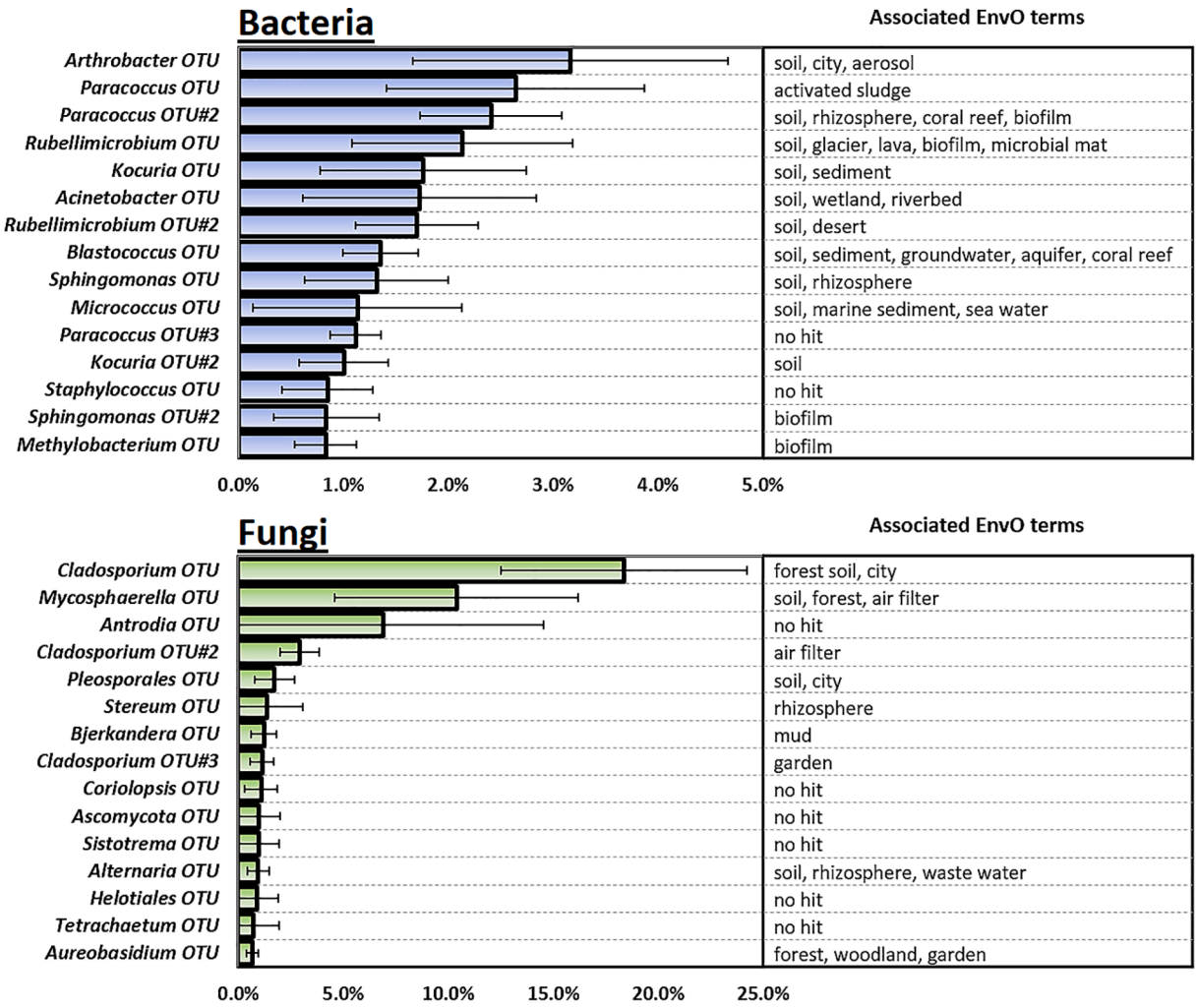

Fig. 4. Mean relative abundance of the top 15 most enriched bacterial (top) and fungal (bottom) OTUs found across all air samples from the Athens Metro station "Nomismatokopio". The most resolved taxonomic ranking for each OTU was included. Error bars represent standard deviation ($n = 6$). Bacterial taxa shown represent 24% of total sequences (on average), whereas fungal taxa shown represent 50.3% of total sequences (on average). List on the right shows the corresponding matched Environmental Ontology (EnvO) terms of other environment types these micro-organisms have been found in, based on *SEQenv* analysis.

associated. Other matched terms included environments such as "rhizosphere", "city", "desert", "groundwater", "sediment", "sea water" and "aerosol". The most dominant fungal OTU sequences were linked to terrestrial and plant-associated source habitats-terms (Fig. 4) such as "forest", "forest soil", "rhizosphere" and "garden". Other highly represented fungal taxa were associated with urban settings, i.e. "city".

3.5. Correlation between microbial taxa abundance and PM_{10} concentration

Potential relationships between the most abundant bacterial and fungal genera and the average values of the concurrently recorded PM_{10} mass levels were examined (Fig. 5). Results, based on Spearman's rank coefficient analysis, revealed positive correlations between PM_{10} concentration and the bacterial genera *Paracoccus, Acinetobacter*, *Corynebacterium*, *Staphylococcus, Geodermatophilus, Methylobacterium* and *Micrococcus*. However, only *Paracoccus, Acinetobacter* and *Geodermatophilus* demonstrated strong positive associations ($\rho > 0.5$) with the particle levels. In terms of fungal genera, *Cladosporium*, *Penicillium*, *Aspergillus*, *Sporobolomyces*, *Pleosporales* (unidentified genus) and *Filobasidiales* (unidentified genus) were found to be positively and strongly associated with PM_{10} mass, whereas negative correlations were found between the majority of the rest of the taxa (mostly members of *Agaricomycetes*) and particulate concentration.

4. Discussion

4.1. Bacterial abundance

Traditional culture-based methods greatly underestimate the microbial densities in air (Rappé and Giovannoni, 2003). Given the limited number of culture-independent bioaerosol surveys in underground transport systems and the lack of qPCR-based estimates of microbial concentrations, there is little information about the overall subway bioaerosols levels. In our study, the mean bacterial concentration found in the Athens Metro station Nomismatokopio (2.82×10^5 16S rRNA genes/m^3 of air equivalent to 4.03×10^4 *E. coli* genome equivalents/m^3 of air) were similar to the average bacterial load reported for the Barcelona subway system (4.46×10^4 equivalent *E. coli* genomes/m^3 of air), based on qPCR for samples collected inside trains, on platforms and lobbies (Triadó-Margarit et al., 2017). Robertson et al. (2013) estimated an average value of 2.2×10^4 cells/m^3 for the total airborne microbial load of several New York subway platforms, based on fluorescence microscopy. However, it has to be taken into account that quantitative metrics are incomparable when sample collection and processing parameters vary, including quantification assay design and efficiency, and caution must be taken when comparing results obtained from different studies. Moreover, the bacterial abundance estimated herein represents the inhalable fraction PM_{10} and not the total suspended particulate

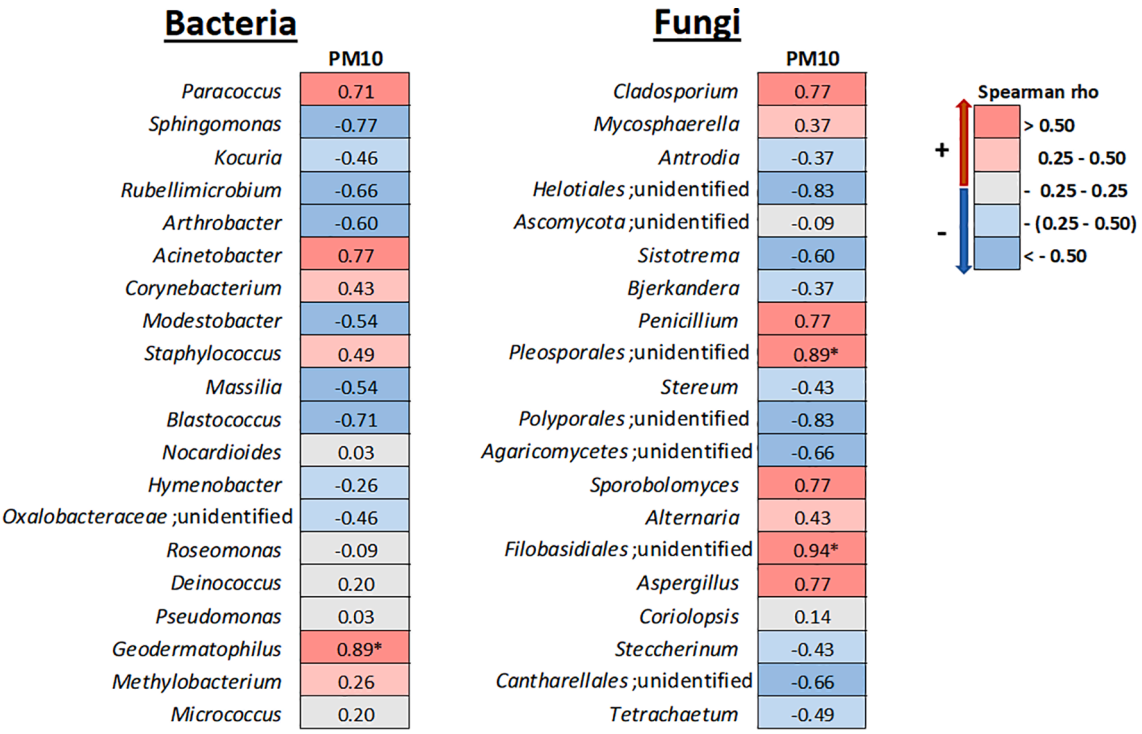

Fig. 5. Relationship between relative genus abundance and PM_{10} mass concentration, calculated using Spearman's rank correlation coefficient rho (ρ). The top 20 most abundant bacterial (left) and fungal (right) genera are presented in the figure. Red represents a positive correlation, whereas blue represents a negative correlation. Asterisks denote statistical significance ($P < 0.05$).

matter that has been typically analysed in most bioaerosol molecular studies (Triadó-Margarit et al., 2017; Robertson et al., 2013).

Despite the fact that microbial aerosols constitute part of airborne particulate matter, PM concentrations are rarely included in bioaerosol studies. Nevertheless, due to the different origin of biological and non-biological components of the airborne particulates, the bioaerosol levels are not always in agreement with the PM concentrations. In our study, although 16S rRNA gene copy numbers were on average higher on the weekdays compared to the weekends, the highest PM_{10} concentration was measured on a Saturday (day-time). The weekday/weekend trends in bacterial levels observed in the Athens station are, notwithstanding, in accordance with the overall higher $PM_{2.5}$ concentration levels reported for the weekdays, compared to the weekends (the difference was not substantial), by Martins et al. (2016) who performed measurements at the same station during the same period. Besides this being related to the higher frequency of trains and increased numbers of commuters on the weekdays, this agreement could indicate that the bacterial aerosols in the station could be mostly associated with the $PM_{2.5}$ size fraction. Furthermore, bacterial levels were increased during the regular Metro operating hours compared to the night-time service interruption hours, which is in accordance with both the PM_{10} measured concentrations and the day-time/night-time $PM_{2.5}$ trends reported for the Nomismatokopio station (Martins et al., 2016). Our findings are also in line with culture-based studies in other subway systems that included night-time bioaerosol monitoring (Dybwad et al., 2014, 2012; Birenzvige et al., 2003). It should be noted that the considerable high PM_{10} levels (>100 μg/m^3) recorded during operational hours, which are within the range of concentrations previously measured in other Metro stations (Xu and Hao, 2017), have been reported to be directly linked to the train traffic rate (Raut et al., 2009). In contrast, significantly reduced particle levels, reflecting background ambient concentrations, are commonly observed when the Metro is closed for the public.

4.2. Microbial diversity

Overall, microbial community diversity did not demonstrate any statistically significant differences in terms of day-time/night-time as well as weekday/weekend variation. In our study, the aerosol bacterial composition exhibited a higher level of overlapping OTUs compared to the airborne mycobiome which was more diverse across sampling days. The detection of higher inter-day variability in bioaerosol fungal composition suggests that passengers commuting on a daily basis are likely to be exposed to a wider range of airborne fungi, compared to bacteria, indicating that different factors contribute to shaping the PM_{10} bacterial and fungal assemblages in the subway environment.

4.2.1. Bacterial composition

In terms of bacterial composition, Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes were prevalent as has been reported using HTS in the New York (Robertson et al., 2013), Hong Kong (Leung et al., 2014), Barcelona (Triadó-Margarit et al., 2017) and Oslo subways (Gohli et al., 2019). The predominance of these phyla has also been consistently observed in various non-transportation indoor settings (e.g. Gao et al., 2018; Prussin et al., 2016; Miletto and Lindow, 2015) and in the atmosphere (Behzad et al., 2015). Minor contributions from other phyla detected in the air of the Athens Metro station have also been reported previously, such as Deinococcus-Thermus (Triadó-Margarit et al., 2017; Leung et al., 2014), Acidobacteria (Triadó-Margarit et al., 2017) and Cyanobacteria (Robertson et al., 2013).

At the genus level, the Athens Metro station was dominated by a mixture of environmental taxa including *Paracoccus*, *Sphingomonas*, *Rubellimicrobium*, *Arthrobacter*, *Modestobacter*, *Massilia*, *Blastococcus*, *Nocardioides*, *Hymenobacter*, *Deinococcus*, *Pseudomonas*, *Geodermatophillus*, *Methylobacterium*, *Bacillus* and *Skermanella*. This study also recovered *Corynebacterium*, *Staphylococcus*, *Acinetobacter*, *Kocuria* and *Micrococcus*, collectively comprising, on average, 16.5% of the total

reads and containing many species associated with humans, mainly skin commensals (Grice et al., 2009, 2008; Berlau et al., 1999). However, some members of these genera have also been isolated from various environmental sources. For instance, *Acinetobacter*, apart from being a human skin commensal, is also ubiquitous in nature (Doughari et al., 2011). The New York subway platforms were also found to be mainly comprised of airborne bacterial genera associated with both terrestrial/aquatic environments (e.g. *Arthrobacter*, *Acinetobacter*) and human skin commensals (*Staphylococcus*, *Kocuria*, *Micrococcus*) (Robertson et al., 2013). Likewise, the most common bacterial genera found during commuting within the subway network in Hong Kong were human skin-associated (*Micrococcus*, *Enhydrobacter*, *Propionibacterium*, *Staphylococcus, Corynebacterium*), whereas environmental bacteria (*Sphingobium*, *Blastomonas*, *Xanthomonas*) were also among the most commonly detected airborne micro-organisms (Leung et al., 2014). Major genera identified in the air of subway stations in Oslo (e.g. *Micrococcus*, *Staphylococcus*, *Sphingomonas*, *Hymenobacter*) were also detected in the present study (Gohli et al., 2019). In contrast, Triadó-Margarit et al. (2017) found that the proportions of human-related bacteria (*Staphylococcus*, *Neisseria*, *Streptococcus*, *Corynebacterium*, *Enhydrobacter*) were below 1% and that aerosols in the Barcelona subway (various settings) were more highly enriched with environmental taxa (e.g. *Methylobacterium*, *Chitinophagaceae*, *Bradyrhizobium* and *Paracoccus*). Several of the bacteria detected in the Athens Metro (e.g. *Corynebacterium*, *Kocuria*, *Staphylococcus*, *Bacillus*, *Pseudomonas*) have also been found by culture-based studies in other Metro systems worldwide (Table S1), implying that a large fraction of the observed subway bioaerosol diversity may represent living micro-organisms and possibly metabolically active rather than dead cells or microbial fragments suspended in the air.

4.2.2. Fungal composition

To our knowledge, this is the first study to apply high throughput amplicon sequencing to characterise the fungal diversity of bioaerosols in a Metro system and therefore provides novel information on a previously largely unexplored mycobiome. The aerosol fungal diversity in the Athens station was mainly represented by Ascomycota and Basidiomycota, as commonly observed in studies of airborne fungi (Pashley et al., 2012). Likewise, the fungal composition in the New York network (determined by Sanger sequencing) was also dominated by the aforementioned phyla (Robertson et al., 2013). The predominance of *Dothideomycetes* and *Agaricomycetes* classes is also in line with other cultivation-independent indoor (Shin et al., 2015; Hoisington et al., 2014; Adams et al., 2013) and outdoor (Fröhlich-Nowoisky et al., 2012) bioaerosol surveys. However, in New York's subway aerosols *Agaricomycetes*, instead of *Dothideomycetes*, were the most prevalent class, followed by *Eurotiomycetes* (*Trichocomaceae*).

At the genus level, the most abundant taxon was *Cladosporium*, which is a ubiquitous phylloplane fungus with various ecological functions (e.g. endophytes, wood saprotrophs, plant pathogens), commonly found to dominate the air mycobiome indoors and outdoors (Bensch et al., 2012; Fröhlich-Nowoisky et al., 2012; Kaarakainen et al., 2008). *Cladosporium* has also been reported to be the most prevalent fungus outdoors in Athens (Richardson et al., 2019; Pyrri & Kapsanaki-Gotsi, 2015). The second most abundant airborne fungal genus found in the Athens Metro, *Mycosphaerella*, mainly includes plant leaf infecting species (Crous, 1998). Both *Cladosporium* and *Mycosphaerella* belong to the broader taxonomic group *Capnodiales*, which was among the identified fungal orders in the New York subway platforms (Robertson et al., 2013). *Penicillium*, *Aspergillus and Alternaria* spp., which were also among the prevalent airborne fungi at the Athens station, are known for their wide distribution in the indoor environment (Nevalainen et al., 2015). The rest of the Athens subway air comprised mainly of saprobic fungi (e.g. *Agaricomycetes*), suggesting a potential outdoor origin. Several identified basidiomycetous genera, members of *Agaricomycetes* (e.g. *Antrodia*, *Sistotrema*, *Bjerkandera*, *Stereum*, *Coriolopsis* and *Steccherinum)*, are known wood decomposers (Hibbett et al., 2014). Robertson et al. (2013) also reported the detection of fungal wood decayers in the New York subway aerosol mycobiome. Apart from the commonly detected (by culture-based studies) indoor fungi *Cladosporium*, *Penicillium*, *Aspergillus* and *Alternaria* spp. and a limited number of other ascomycetes such as *Botrytis* and *Acremonium*, which were found at low proportions in the Athens Metro, the presence of the majority of the aforementioned airborne fungal taxa in a subway environment has not been reported previously.

4.3. Bioaerosol sources

The cumulative relative abundance of bacterial genera (>1%) affiliated with species with presumptive human origin presented here (~16.5%) lies within the range of non-transportation studies in occupied settings, where human-associated bacterial taxa were found to comprise, on average, 12% (Gaüzère et al., 2014), 17% (Hospodsky et al., 2012; Qian et al., 2012) and 23.7% (Shin et al., 2015) of the total number of indoor air bacterial sequences. Minor enrichments (<1%) of taxa associated with the human oral or gut microbiome (e.g. *Streptococcus*, *Lactobacillus, Enterobacter*, *Enterococcus, Anaerococcus, Blautia, Burkholderia* and *Ruminococcaceae*) were also found (Rajilić-Stojanović and de Vos, 2014; Ma et al., 2012; Dewhirst et al., 2010; Rajilić-Stojanović et al., 2007; Hardie and Whiley, 2006). It is well-established that human occupancy is one of the predominant sources of airborne microbial particles in the non-industrial indoor environment (Meadow et al., 2015; 2014; Gaüzère et al., 2014; Hospodsky et al., 2012) and therefore, human microbiota originating from commuters contribute significantly to the aerosol microbiome in public transit systems. Humans can act as primary sources through direct emission of micro-organisms via talking, breathing, coughing, sneezing and skin shedding (Spendlove and Fannin, 1983). Moreover, the association of the human commensal bacteria with the large numbers of commuters was further supported by the increased proportions of *Corynebacterium*, *Staphylococcus*, *Acinetobacter* and *Micrococcus* observed during the Metro operating hours (day-time) compared to the night-time hours. Interestingly, the relative abundance of these taxa positively correlated with PM_{10} levels, indicating the influence of the human presence on both biological and non-biological particles. It is worth noting that *Kocuria* did not exhibit elevated proportions during day-time and was negatively associated with the PM_{10} concentration. However, *Kocuria* spp. apart from being part of the normal human skin and oral flora, have also been isolated from a wide variety of environmental sources (e.g. Kim et al., 2004; Kovács et al., 1999). Indeed, our analysis associated the two most prevalent *Kocuria*-affiliated OTUs with non-human related sources. *Corynebacterium*, *Staphylococcus* and *Micrococcus* also increased during the weekdays compared to the weekend (i.e. Saturday), which could be related to the more frequent service and the higher numbers of rail passengers.

Humans can also act as secondary sources due to the movement-induced resuspension of particles deposited on surfaces, floor, clothes and footwear (Licina and Nazaroff, 2018; Qian et al., 2014; Goebes et al., 2011). Resuspension of settled material, generated by air movement on the platform caused by the trains passing through the tunnel as well as the commuters, has been identified as one of the major sources of airborne particles in the subway environment (in addition to abrasion between rails, train wheels and brakes) (Moreno et al., 2014). Various human-affiliated bacteria as well as taxa not associated with commuters, identified in the present and previous Metro air microbiome studies, have also been found to dominate subway surfaces (Hernández et al., 2019; Hsu et al., 2016; Afshinnekoo et al., 2015). Biological particles suspended in the subway air may originate from the surface microbiome and may also contribute to it by depositing biological material on various surfaces and therefore, the detection of various common taxa is expected.

Since outdoor air samples were not obtained, tracking of outdoor

sources was mainly limited to identification of taxa indicative of outdoor sources such as soil, plants, water and sediments. Considering that the majority of the most enriched bacterial taxa represent non-human associated taxa, constituents from various ecological habitats, and given that all the dominant fungal taxa are non-occupant related saprotrophs or plant-pathogens, strongly suggests that outdoor-derived microbes have a significant influence on the Athens Metro bioaerosol composition. In addition, the increased proportions of several predominant taxa, such as *Agaricomycetes* (e.g. *Antrodia*, *Sistotrema, Steccherinum*), *Sphingomonas*, *Rubellimicrobium* and *Arthrobacter*, during the (week) night-time service interruption hours further supports the argument that a large fraction of the Metro station PM_{10} microbiota is not associated with commuters. Moreover, the higher night-time abundance of *Agaricomycetes* in the station is in line with the higher spore discharge rates generally observed for saprotrophic basidiomycetes during the night (Abrego et al., 2018). It should be noted that in most cases the night-time enrichment of the aforementioned taxa was not observed during Saturday night. This could be related to the prolonged operation of the Metro system on Friday, as trains run until 02:30 and therefore, any compositional change during Saturday night (i.e. early hours) is expected to be less pronounced. Furthermore, the higher PM_{10} levels observed during Saturday night, which is particularly illustrated in the real-time profiles where the PM_{10} levels remain elevated until 03:00, compared to the regular week-night, also indicates potential differences in the particle sources between the two nights (e.g. late trains/commuters during Saturday night) that could have affected the microbial composition profiles. To the best of our knowledge, no prior HTS bioaerosol studies in subway systems have included night-time samples.

Outdoor air appears to be an important contributor to the airborne microbial diversity in the Metro station. Ventilation strategy has been shown to play a major role in shaping the microbial composition in the built environment (Kembel et al., 2012; Kodama and McGee, 1986). Meadow et al. (2014) demonstrated that, in well-ventilated occupied settings, the introduction of unfiltered air to the indoor environment has a significant impact on the indoor bioaerosol microbial diversity and tends to increase the composition similarity between indoor and outdoor air microbiota. Moreover, findings from prior sequencing-based bioaerosol studies in underground rail transport systems strongly indicate that outdoor air is a major driver of the subway aerosol microbial composition (Leung et al., 2014; Robertson et al., 2013). Although both the outdoor air and the occupants have been identified as the main sources of micro-organisms found in indoor settings (Leung and Lee, 2016), the input of occupant-related direct emissions is greater for bacteria (skin-associated fungal yeasts *Malassezia*, *Candida*, *Cryptococcus*, *Rhodotorula* and *Trichosporon* were detected at fractions $< 0.5\%$), whereas fungal taxa found indoors mainly derive from the adjacent outdoor environment carried inside via outdoor air (Barberán et al., 2015; Adams et al., 2014) and therefore reflect the outdoor fungal diversity (Adams et al., 2013; Fradkin et al., 1987). This suggests that the distinct human-associated input of bacteria contributes to the shaping of less diverse airborne bacterial communities in the Metro station, compared to the more variable aerosol mycobiome observed across days. Further, the detectable airborne microbiome in naturally-ventilated indoor settings has been reported to be more diverse compared to mechanically-ventilated spaces (Kembel et al., 2012).

In addition, the positive association found between the indoor and ambient particle ($PM_{2.5}$) levels at the Athens station during the same period and the detection of higher levels of chemical components of crustal origin indoors (Martins et al., 2016), provide further evidence that the outdoor-derived particles accumulating in the station have a substantial influence on the overall subway air quality. The detection of crustal matter, which is indicative of soil material and road dust typically present outdoors, is in line with the identification of soil-related micro-organisms (e.g. *Paracoccus*, *Sphingomonas*, *Rubellimicrobium*, *Arthrobacter* etc.) in the air of the station, as soil particles may act as carriers of microbial material.

4.4. Health-related taxa

The densely populated underground transportation systems facilitate the rapid dissemination of infectious micro-organisms through the air and are ideal environments for the deliberate release of biohazardous agents. Among the highly represented airborne bacterial taxa identified in the Athens Metro station, there were various genera affiliated with opportunistic pathogens, including *Staphylococcus* (Ziebuhr et al., 2006; Sheagren, 1984), *Corynebacterium* (Bernard, 2012), *Kocuria* (Purty et al., 2013), *Acinetobacter* (Wong et al., 2017), *Roseomonas* (Romano-Bertrand et al., 2016; Lewis et al., 1997), *Pseudomonas* (Muder et al., 1987; Bodey et al., 1983), *Diplorickettsia* (Subramanian et al., 2012) and *Dietzia* (Koerner et al., 2009).

In terms of fungi, the abundantly detected *Cladosporium*, *Penicillium*, *Aspergillus* and *Alternaria* are well-known for their allergenic properties (Simon-Nobbe et al., 2008). *Aspergillus* is further important as it includes several species that cause severe infections in immunocompetent and immunocompromised individuals (Denning, 1998). *Bjerkandera* and *Sporobolomyces* spp. have also been associated with human health conditions (Ogawa et al., 2009; Plazas et al., 1994; Cockcroft et al., 1983). Detection of unusual high abundance of pathogenic airborne micro-organisms is still considered a good indicator of potential public health issues, provided that there is baseline microbiome information. Therefore, monitoring the bioaerosol background levels and characterising the microbial composition in Metro transportation systems is of major importance for defining potential hazards and assessing related health risks.

4.5. Limitations

This study provided a first in-depth description of the aerosol microbiome within the Athens Metro network, based on a sampling campaign conducted at the station “Nomismatokopio”. The station shares the same architectural design and ventilation type (i.e. air exchange occurring through draught relief shafts and the passenger pathways) with most of the other stations in the network (Mendes et al., 2018). Data presented here are representative of the microbial particles in the platform environment and not the commuting trains, as samples were not collected inside the carriages. As the occupant density is typically higher in the confined space of the train cabins, differences are expected in the contribution of passengers as sources of bioaerosols between the two types of microenvironments. The station microbial particles are, though, expected to have a substantial impact on the train indoor air microbiome, via the air exchange between the tunnels and the carriages, as some types of Metro carriages are naturally ventilated and windows are possible to open in all trains, including those with air-conditioning system (Mammi-Galani et al., 2017). Similarities between different types of Metro environments have been reported previously for the Barcelona subway system (Triadó-Margarit et al., 2017), as the authors did not observe any significant compositional differences between platforms and the interior of trains with windows not possible to open, as well as for different air-conditioning settings (on or off) in the trains.

In relation to sample collection, the filter-based static sampling strategy used, encompassing the whole-day Metro operating hours (~17 h) as well as non-operating hours during the night (~6h), allowed for the collection of sufficient biomass for downstream molecular analysis as well as providing a representative overview of the aerosol microbial assemblages encountered in the station and not just a “snapshot” view. Although looking into intra-day variations was not in the scope of this work, fluctuations in the microbial abundance throughout the day are expected, especially for taxa found to correlate with the PM_{10} concentrations. Nevertheless, a bigger sample size than the one that could be obtained in the current study would allow for a more robust evaluation of the temporal dynamics of the airborne microbial load. In addition, as

potential source samples were not collected from the station, a thorough source-tracking analysis was not possible and the potential origin of the observed airborne taxa was explored based on their isolation source habitats and the general knowledge of the microbial ecology.

In terms of assays used for molecular analysis, it has to be noted that the qPCR-based estimate of 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 limitations as a result of differences in cell DNA content and DNA extraction biases (Feinstein et al., 2009). qPCR of ITS region was decided not to be performed due to the known variability of the internal transcribed spacer region even within species or over the lifetime of the fungi (Lavrinienko et al., 2020). Finally, although results on microbial composition are discussed in the context of potential exposure to microbes that can have a negative impact on human health, limitations of amplicon-based sequencing approaches in providing sufficient resolution at the sub-genus level as well as the inability to provide an insight into the viability of the captured micro-organisms should be taken into account.

4.6. Future directions

Additional efforts are necessary to further examine the bioaerosol load in more stations and during different seasons, with concurrent outdoor air sampling as well as surface sampling, to provide a more comprehensive assessment of airborne micro-organisms and the contribution of their sources. Furthermore, sampling should also be conducted inside Metro train carriages, using portable devices, to achieve an overall evaluation of commuters' exposure to microbial aerosols. Although this study showed the vulnerability of public transport to airborne disease transmission in terms of potential bacterial and fungal human-health pathogens, future work is also needed to characterise the aerosol virome, which is particularly timely given the current global COVID-19 pandemic.

5. Conclusions

The current study presents the first microbial characterisation of PM_{10} in the Athens Metro underground railway system, providing the foundation for further investigation of subway air quality beyond the typical physicochemical aerosol determination. Our findings showed that the aerosol microbial diversity in the Athens Metro station environment is similar to the one encountered typically in naturally ventilated occupied indoor settings. Overall, we demonstrated that the Metro air microbiome is dominated by bacterial and fungal taxa of environmental origin (soil, vegetation, water etc.) with a lower contribution of human commensal bacteria, underscoring the importance of both outdoor air and commuters as sources in shaping the bioaerosol microbial composition. Moreover, this work contributed to the growing body of the microbiome exploration in urban settings and public transport systems by expanding our knowledge of the aerosol mycobiome in the subway environment. Finally, given that transport hubs are widely regarded as infection hotspots for spreading disease, our findings provide valuable information for the role of public transport in infectious disease transmission.

Author statement

I confirm that all of the reported work is original and all authors have seen and approved the final version. All local, national and international regulations, conventions and normal ethical practices have been respected. This paper has not been published or accepted for publication. It is not under consideration at another journal. Consent is given for publication in *Environment International* if accepted.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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