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Temporal patterns of variability for prokaryotic and eukaryotic diversity in the urban air of Madrid (Spain)

Andrés Núñez[a], Guillermo Amo de Paz[b,c], Alberto Rastrojo[d], Zuzana Ferencova[c], A. Montserrat Gutiérrez-Bustillo[c], Antonio Alcamí[d], Diego A. Moreno[a,**,1], Raúl Guantes[b,e,*]

[a] Escuela Técnica Superior de Ingenieros Industriales, Universidad Politécnica de Madrid (ETSII-UPM), Madrid, Spain
[b] Department of Condensed Matter Physics and Material Science Institute 'Nicolás Cabrera', Science Faculty, Universidad Autónoma de Madrid, Madrid, Spain
[c] Departamento de Farmacología, Farmacognosia y Botánica, Universidad Complutense de Madrid (UCM), Madrid, Spain
[d] Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid (CSIC-UAM), Madrid, Spain
[e] Institute for Condensed Matter Physics (IFIMAC), Science Faculty, Universidad Autónoma de Madrid, Madrid, Spain

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ABSTRACT

Although many microorganisms are ubiquitously present in the air, airborne microbial communities have been much less characterized than those in soil or aquatic environments. Besides its ecological importance, detection and monitoring of the wide diversity of these aerosolized microorganisms (bacteria, viruses, fungi and pollen) is relevant for understanding allergy and disease outbreaks, especially in highly populated cities. In this study, we describe the simultaneous biodiversity of bacteria, fungi and plants present in the urban atmosphere of Madrid (Spain) along different seasonal periods, using DNA sequencing. Sampling in two different locations (downtown and peri-urban) we found that changes in the composition of each community are mainly driven by environmental factors, rather than by the features of the specific sampling microenvironments. While pollen particles are dominated by a few taxa characteristic of each season, bacteria and fungi show a high diversity but stable core communities along the year. The prokaryotic core is governed by soil and leaf surface bacteria, with predominance of *Actinobacteria* (*Frankiales* and *Micrococcales*) and *Alphaproteobacteria* (*Sphingomonadales*, *Rhodobacterales*, *Rhizobiales* and *Acetobacterales*). Fungal diversity is characterized by the steady presence of members of *Capnodiales* and *Pleosporales*. Pathogenic bacterial and fungal taxa were also detected across the year. We also correlated the airborne biodiversity with environmental variables. Air temperature has a strong influence on the community composition of bacteria, while pollen and fungi seasonal variations are mainly correlated with precipitation. Our results contribute to the characterization of airborne prokaryotic and eukaryotic communities in urban areas and show the suitability of this method for biosurveillance strategies.

1. Introduction

In addition to chemical compounds, the human population is constantly exposed to a vast diversity of microscopic biological entities present in the urban atmosphere (viruses, bacteria, archaea, fungi, pollen, etc.), which are emitted from diverse sources such as soil, surface waters, plant cover, etc. (Després et al., 2012). While most of them are innocuous, some pollen grains such as grasses, mugwort, ragweed and birch, and atmospheric moulds like *Cladosporium* spp., *Alternaria* spp. or *Aspergillus* spp. are proven allergens or pathogens, exacerbating asthma symptoms and increasing the number of allergy cases worldwide, currently over several million people (Bousquet et al., 2008; Crameri et al., 2014). Bacteria from soil and plant surfaces are usually predominant in the urban atmosphere (Bertolini et al., 2013; Bowers et al., 2011a; Fierer et al., 2008). However, pathogenic bacteria causing varied disorders such as *Legionella* spp. (etiological agent of Legionnaires' disease) are commonly transmitted via aerosols produced by cooling towers and water sources in metropolitan areas (van Heijnsbergen et al., 2015).

Besides its relevance for understanding allergy and disease outbreaks, the study of the airborne microbiota has ecological value. These biological particles are easily transported both throughout the land and

* Corresponding author. Department of Condensed Matter Physics and Material Science Institute 'Nicolás Cabrera', Science Faculty, Universidad Autónoma de Madrid, Madrid, Spain.
** Corresponding author.
E-mail addresses: diego.moreno@upm.es (D.A. Moreno), raul.guantes@uam.es (R. Guantes).
[1] Present address: Facultad de Farmacia, Universidad de Castilla-La Mancha (FF-UCLM), Avda. Dr. José María Sánchez Ibañez s/n, E-02071, Albacete, Spain.

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across the vertical section of the atmosphere. Thus, their presence is not limited to a small area close to the source of origin but they can also be found in the upper troposphere, transported long distances and dropped in new lands after deposition (Barberán et al., 2015; Cáliz et al., 2018; Damialis et al., 2017; DeLeon-Rodriguez et al., 2013; Smith et al., 2012). During their stay in the air, it has been proposed that they may act as ice and cloud condensation nuclei, having an influence on meteorology at a local level (Huffman et al., 2013; Morris et al., 2013). Moreover, since bacteria and fungi play a significant role in biogeochemical cycles, trans-continental transport may modify these cycles where they are deposited, which is especially relevant in a climate change scenario (Kanakidou et al., 2018; Zhu et al., 2017). As a consequence, the knowledge of the total diversity, abundance and dynamics of these entities in the air is gaining importance both for ecological and health interests.

The collection problems for such variety of particles and the high influence of the meteorological factors affecting their presence in the atmosphere make difficult to successfully study all this biodiversity (Fierer et al., 2008; Fronczek and Yoon, 2015; Jones and Harrison, 2004). Pollen distribution, concentration and seasonality in urban environments are well known in certain cities thanks to aeropalynological surveillances that provide daily monitoring and manage long time series data for predictive evolution of each type of pollen (Oteros et al., 2013; Ziello et al., 2012). Significantly less is known regarding peaks and progression throughout the year for airborne fungi and bacteria, partly because they are frequently surveyed by culture-based methods.

Current shotgun or amplicon DNA sequencing techniques provide a wide coverage of microorganism diversity at a much deeper taxonomic resolution than microscopy or culture methods. Recent reports applying this technology to samples collected in urban spaces have revealed a huge variety of biological entities in the air (Be et al., 2015; Bertolini et al., 2013; Cao et al., 2014; Gandolfi et al., 2015; Woo et al., 2013; Yooseph et al., 2013). Most works addressing this matter are mainly focused on one particular group of these biological entities and usually for a short period of time, although a few studies have addressed the joint analysis of bacterial and fungal communities in aerosol samples (Barberán et al., 2015; Bowers et al., 2012; Du et al., 2018; Fierer et al., 2008; Woo et al., 2013), and one study analyzed the relative abundance of airborne bacteria, plants, fungi, invertebrates and viruses (Be et al., 2015).

The goal of the present study is to analyze the joint biodiversity of bacterial, fungal and plant communities in a highly populated urban area, identifying sources of variability along different temporal periods. We recently evaluated the suitability of a particle collector, the Hirst spore trap, to simultaneously monitor and characterize the diversity of bacteria, fungi and plants by amplicon DNA sequencing (Núñez et al., 2017). Using this method, we sampled in two different locations by synchronously running two traps along four different seasonal periods (Table 1). One of the locations chosen is placed downtown (Urban, U) and represents a metropolitan area with an adjacent small park surrounded by highly trafficked streets. The other sampling location is in an area immediately adjacent to the city (Peri-urban, P), characterized by larger green zones (parks or natural areas), low buildings and light traffic. Both locations are representative cases of two different scenarios found in Madrid, in terms of built and green areas.

In addition to characterizing the main composition of the airborne communities and their patterns of long-term variation, we also analyze the influence of different meteorological and pollution factors, identifying the most relevant to explain the bioaerosols' variability. This work provides the first simultaneous characterization of prokaryotic and eukaryotic diversity in the urban atmosphere of Madrid, compiling meaningful information about these entities of ecological and human health interest.

2. Material and methods

2.1. Locations, sampling methodology and DNA extraction and quantitation

Two different locations were surveyed in Madrid (Spain) by running synchronously two volumetric spore traps (Burkard Manufacturing Co., England, UK), collecting 7-days samples for each season. The city of Madrid is located near the center of the Iberian Peninsula, 657 m AMSL and ≈320 km from the closest coastal point, with > 3 million of permanent inhabitants. We chose two different sampling sites: the urban location (U) at Escuela Superior de Ingenieros Industriales, Universidad Politécnica de Madrid (40.439881° N 3.689409° W, 21 m AGL, 705 m AMSL) represents a downtown metropolitan area, with 99.1% of the surface built in 1 Km around. The other sampling site is a peri-urban location (P) at Facultad de Farmacia, Universidad Complutense de Madrid (40.446025° N 3.725141° W, 18 m AGL, 637 m AMSL) in the surroundings of the city center, with 90.1% of the surface built in a radius of 1 Km. Both locations are separated by a distance of about 3 km. To evaluate how representative are these sampling sites in terms of built/green areas, we estimated the same percentage of urbanized area around 10 random sites, distributed uniformly on a total area of 45 km^2, considered the urban center of the city. The built percentages are in the range 93.9–99.9 (mean = 97.1; median = 97). The two sampling sites chosen are thus representative examples of the most different scenarios found in the city, in terms of proportion of built/green areas around.

To evaluate the influence of environmental factors on airborne biodiversity, we collected a set of environmental variables from the two

Table 1
Meteorological and pollution data included in the analysis for both locations. Please note that in the Proof view the columns of Location P and Location U appear with different width and shifted. All columns of Location P and Location U should be alligned with the shadowed headings.

Assay	Sampling date	Air Temperature[b] [°C]	Relative humidity[b] [%]	Precipitation[c] [mm]	Wind speed[c] [m/s]	PM_{10}[c] [μg/m^3]	NO_2[c] [μg/m^3]
Location P – "Facultad de Farmacia, Universidad Complutense de Madrid", peri-urban site (90.1% built[a])							
Winter	2-9/Mar 2015	10.3	52	0.0	3.0	12	24
Spring	21-28/Apr 2015	14.3	53	29.2	3.7	15	14
Summer	20-27/Jul 2015	29.2	35	8.5	2.2	28	14
Fall	23-30/Nov 2015	9.1	60	0.2	1.7	18	34
Location U – "Escuela Técnica Superior de Ingenieros Industriales, Universidad Politécnica de Madrid", urban area (99.1% built[a])							
Winter	2-9/Mar 2015	11.3	56	0.0	2.0	14	42
Spring	21-28/Apr 2015	15.1	65	41.3	2.2	16	32
Summer	20-27/Jul 2015	29.8	41	3.6	1.9	22	32
Fall	23-30/Nov 2015	10.0	67	0.6	1.2	21	55

Data from the closest meteorological station (< 1 Km for both cases).
[a] Percentage calculated for the area covered in a radius of 1 Km around the sampling location.
[b] Average of two weeks (sampling week and the previous one).
[c] Total registered in two weeks (sampling week and the previous one).

meteorological and air pollution stations closest to the sampling sites (within 0.2–1.5 Km distance), and shown in Table 1.

Sampling, DNA extraction (MO BIO Laboratories, CA, USA) and quantification (Quant-iT™ PicoGreen® dsDNA Assay Kit; Invitrogen, MolecularProbes®) procedures were performed as described previously in Núñez et al. (2017). An additional negative control was set as a 7-days sample keeping the vacuum of the sampler off. No amplification by PCR was obtained for this specimen during the preparation of the amplicon libraries. Nonetheless, this sample was included in the sequencing batch to discard contaminations and sequencing issues.

2.2. High-throughput sequencing

High-throughput sequencing analyses were performed using the purified DNA from each sample. Universal primers attached to adaptors and multiplex identifier sequences were used to amplify V3–V4 regions from 16S rRNA for bacteria (Bakt_341 (F): 5′- CCTACGGGNGGCWGCAG -3'; Bakt_805 (R): 5′- GACTACHVGGGTATCTAATCC -3' (Herlemann et al., 2011) and 5.8S – ITS2 for fungi (ITS86 (F): 5′-GTGAATCATCGAATCTTTGAA-3' (Turenne et al., 1999) ITS-4 (R): 5′-TCCTCCGCTTATTGATATGC -3′(White et al., 1990), and plants (ITS-D (F): 5′- YGACTCTCGGCAACGGATA-3' (Cheng et al., 2016) ITS-4 (R): 5′-TCCTCCGCTTATTGATATGC -3′, in a targeted amplicon sequencing (TAS) approach. Purified-amplicon libraries were sequenced in Illumina® MiSeq platform (2 × 300 bp reads) at "Parque Científico de Madrid" (Madrid, Spain), with a minimum sequencing depth of 100,000 reads/amplicon (see Table 2 for details).

2.3. Sequence assembly and preprocessing

Data from NGS were first submitted to a general checking (total number of sequences, sequence quality per base and sequence length distribution) with FastQC software (version 0.11.3, Babraham Bioinformatics Group, Babraham Institute, UK [www.bioinformatics.babraham.ac.uk/projects/fastqc/]). Paired-ends sequences were assembled with PANDAseq (Masella et al., 2012) (version 2.8, https://github.com/neufeld/pandaseq/wiki/PANDAseq-Assembler), which filtered the sequences by Q-score quality (default: 0.6), trimmed the primers sequences and removed the sequences exceeding the length of the amplicon (bacteria: min: 400 bp, max: 500; plants: min: 320, max: 550). For fungal ITS2 library, as the sequencing protocol exceeded the length of the amplicon, we employed "read_fastq" from Biopieces (version 2.0, http://maasha.github.io/biopieces/) to remove the primer sequence at the end of the amplicon followed by "fastq-join" (Aronesty, 2013) (https://github.com/brwnj/fastq-join) to pair the reads. Global processing of the sequences was carried out in Qiime suite environment (Caporaso et al., 2010) (version 1.9.1, http://qiime.org). Potential chimeras were subtracted using default values from USEARCH v8.1 (https://drive5.com/usearch/), and OTUs clustering and taxonomic assignments were performed with the default algorithm of Qiime (pick_open_reference_otus.py), using UCLUST as method for picking OTUs (Edgar, 2010), which were clustered with 97% similarity cutoff. Taxonomic assignments (UCLUST, minimum consensus: 0.51, similarity > 0.9, max accepts: 3) were performed with Silva database for bacteria (Quast et al., 2013), release_132, and UNITE (Kõljalg et al., 2013), version 7.0, https://unite.ut.ee/) for fungi. A customized database was created for plants assignment as described previously in Núñez et al. (2017). The OTUs assigned to chloroplast and mitochondria in 16S analysis were filtered out. For the sake of the analyses, the OTUs without taxonomic assignment at least at the level of phylum were removed (2% of the OTUs in bacteria, 0.1% in plants and 0% in fungi). We checked a possible contamination in our samples by looking for 'outlier' OTUs of very large abundances present in only one of the samples, and manually identifying the corresponding species by blasting their sequences against the NCBI nucleotide database. While pollen and fungi samples were free of contamination, we found in one of the bacterial samples (P_Summer) two OTUs assigned to parasitic bacteria of arthropods (*Wolbachia* sp. and *Occidentia* sp.), which were removed.

2.4. Filtering and normalization

The probability of spurious OTUs with one or very low count values in amplicon sequencing increases with sequencing depth, sometimes due to PCR or sequencing noise (Quince et al., 2011; Weiss et al., 2017). As a pre-analysis step, we estimated a 'noise floor' in our experiments (a lower limit to count detection) using the following procedure: we first identified all OTUs with a mix of zero and non-zero values in all the samples, and for each of these OTUs we took the smallest non-zero count value. The median of all these values was considered as our technical detection limit, which were 2 counts for the three different entities (bacteria, fungi and plants). We filtered out the OTUs that were below this detection limit in all of the samples. These discarded OTUs constituted a noticeable fraction of all the identified OTUs: ~34% in bacteria (3693 out of 10,850 OTUs), ~22.6% in fungi (967 out of 4279 OTUs) and ~49% in plants (1573 out of 3138 OTUs), but their cumulative abundances were low (between 1% and 5% in bacterial samples, 0.1–0.5% in fungi and 0.1–0.4% in plants).

To account for biases due to differences in sequencing depth between samples, we employed cumulative sum scaling normalization (Paulson et al., 2013) as implemented in the "metagenomeSeq" package (version 1.18.0, http://cbcb.umd.edu/software/metagenomeSeq). This normalization procedure gave results qualitatively similar to rarefaction in our statistical analyses, with the advantage that potentially

Table 2
Sampling periods, locations and sequencing reads/OTUs.

Sampling date	02–09 March 2015		21–28 April 2015		20–27 July 2015		23–30 November 2015	
Sample ID[a]	U_Winter	P_Winter	U_Spring	P_Spring	U_Summer	P_Summer	U_Fall	P_Fall
DNA concentration (pg/m^3)	1050.6	1485.2	462.4	1008.2	30.6	130.8	49.0	24.4
No. reads 16S (Bacteria)	164,505	187,059	203,147	246,262	177,074	200,235	133,695	132,682
• No. OTUs(% RA[b])	3111(84% RA)	3782(84% RA)	3169(92% RA)	4145(89% RA)	4435(12% RA)	3969(13% RA)	3426(31% RA)	4654(32% RA)
• Final No. OTUs analyzed	1955	2433	1699	2272	2956	2689	2625	3358
No. reads ITS (Fungi)	177,371	180,039	234,183	265,640	188,725	176,228	166,905	198,801
• No. OTUs	2524	2844	4123	4218	1379	1449	2542	2922
• Final No. OTUs analyzed	836	1032	1785	1826	503	560	1283	1447
No. reads ITSD (Plants)	282,449	221,852	235,007	243,993	292,587	200,333	196,112	212,650
• No. OTUs	1381	1321	738	729	949	490	622	588
• Final No. OTUs analyzed	742	743	362	361	342	257	329	352

[a] Sample ID: U_: Urban location, "Escuela Técnica Superior de Ingenieros Industriales, Universidad Politécnica de Madrid"; P_: Peri-urban location, "Facultad de Farmacia, Universidad Complutense de Madrid".

[b] RA: proportion of the total abundance compiled by the sequences assigned to mitochondria and chloroplasts.

useful OTUs are not lost (McMurdie and Holmes, 2013).

To characterize OTUs that are present only in one season ('unique') or appearing along the four seasonal periods ('common') we imposed a restrictive criterion of 'presence' that takes into account experimental variability. We analyzed three previous duplicate experiments using two Hirst-spore traps simultaneously monitoring during a 7-day period in a given location (Núñez et al., 2017). First, we identified all the OTUs present only in one of the duplicate traps. Then, we took the 95% quartile of the abundance distribution of these OTUs as a threshold to reliably assess the presence of an OTU in two duplicate and independent sampling devices. In other words, abundances above this threshold value (which was around 0.032% in the three entities) can be detected with 95% confidence during the sampling period, while OTUs with smaller counts may pass undetected (since they may be observed in only one of the duplicates). We consider a species as 'unique' of a season if its relative abundance is higher than this threshold in both locations during the sampling period of the season, and lower in the rest of the seasons. Species 'common' to all seasons are those showing abundances higher than the threshold in both locations during all seasonal periods.

2.5. Richness and evenness estimates

Richness in Fig. 1 is calculated as the total number of different OTUs observed in each sample after filtering by the detection limit and normalizing as described above (2.4. Filtering and normalization). For both richness and alpha-diversity (evenness) estimates, we subtracted the detection limit from all the counts, so that counts below the detection limit where considered as zero (absent), and one count above the detection limit as singletons. To check for the possible underestimation of richness when many rare (low abundance) taxa are present, we tested another two non-parametric estimators of richness, Chao1 and abundance-based coverage estimator (ACE), that correct this effect (Hughes et al., 2001), which retrieved slightly higher values of richness but identical trends to those shown in Fig. 1.

We used Pielou's evenness index as a measure of the similarity in species relative abundance in a community. Pielou's evenness, which is calculated as the Shannon information scaled by the maximum information, is a proper measure of 'relative evenness' (Jost, 2010) and ranges between 0 and 1, with larger values representing more even distributions in abundance among species.

2.6. Correlation with environmental variables

We collected daily environmental variables (maximum, minimum and average temperature, average wind speed, precipitation and relative humidity) as well as pollution data (concentration of particulate matter $PM_{2.5}$ and PM_{10}, NO_2) from two meteorological stations close to the sampling locations, recorded during the sampling week and two previous weeks. Since linear dependencies between some of the variables were high (for instance between PM_{10} and $PM_{2.5}$) we worked only with the set of variables shown in Table 1. For these, we calculated one, two and three week averages to test associations between environmental variables and sample composition changes between locations and seasons. These associations were found by constraining our PCoA ordinations to the environmental variables taken as explanatory variables (distance-based Redundancy Analysis (Borcard et al., 2011), using Bray-Curtis distance). The variance explained by the variables involved was corrected as in Peres-Neto et al. (2006) (adjusted R^2).

To find the relevant environmental variables associated to the three different entities, we systematically performed model selection of one, two and three week averages of our variables against the composition data for each entity. Model selection was assessed by permutation tests (1000 permutations) and maximizing the adjusted R^2 for the explanatory variables included (Borcard et al., 2011). We tested model selection both against the whole set of environmental variables (Table 1) and against meteorological and pollution factors separately, obtaining consistent results. Two and three week averages of environmental variables gave qualitatively similar results, with slightly better significance for the two-week averages (calculated by permutation tests). One-week averages sometimes failed to identify relevant variables or identified only one of them. We thus chose the two-week averages as the most consistent set of data to test associations. Our analyses yielded two main environmental variables for each entity, which accounted for around 50% of the variance and passed tests of statistical significance in all the cases (Table S4). We checked that adding another variable only increased very little the amount of variance explained and did not pass significance tests. We therefore kept the two-variable models as the most explanatory and reliable, and performed constrained ordinations only against these variables.

2.7. Statistical analyses and software

All statistical analyses were performed in R software environment (version V3.4.2, https://www.r-project.org/). We used the "phyloseq" package version 1.20.0 (McMurdie and Holmes, 2013) for data processing, multivariate unconstrained statistical analysis (PCoA), richness and diversity estimates and bar plots. The "vegan" package, version 2.4–472, was used for the following analyses: permutational multivariate analysis of variance (PERMANOVA, "adonis" function) for studying the influence of location and seasonality associated to PCoAs using the Bray-Curtis distance matrices and 999 permutations; distance-based redundancy analysis (db-RDA) to study the association between environmental variables and microbial community composition ("capscale" function), using Bray-Curtis distance; permutation tests to validate constrained ordination models ("anova.cca" function); model selection to explore the relevance and choose the most significant environmental variables ("ordistep" and "ordiR2step" functions), and the function "forward.sel" in library "packfor" in R.

The environmental origin of the OTUs assigned to bacteria and fungi was assigned using Seqenv pipeline (Sinclair et al., 2016), analyzing the top 10 matches and choosing the most frequent annotation.

2.8. Fungal and pollen identification and quantification by microscopy

One half of the Melinex® tape from each sample was used for morphological determination and quantification by microscopy following the procedures of the Spanish Aerobiological Network (Oteros et al., 2013) and described previously (Núñez et al., 2017). Total pollen or fungal spore counts per week were expressed as the sum of daily mean counts per cubic meter of air (Tables S1–S3 in the Supplementary Material).

2.9. Sequence accession numbers

Raw Sequence Data obtained in this study are available in the National Center for Biotechnology Information, Sequence Read Archive under the Accesion No. SRP126725 (Bioproject PRJNA422354).

3. Results and discussion

3.1. Richness and diversity estimates for two urban locations across seasons

Previous works have shown that microscopic airborne biodiversity is affected by changes both from nearby sources and environmental factors (Bowers et al., 2011b; Fierer et al., 2008; Jones and Harrison, 2004). To reduce short-term variability, we collect and analyze the accumulated biomass in our particle traps running continuously during seven-day periods (see Section 2.1. Locations, sampling methodology and DNA extraction and quantitation). These samples are then representative of the biodiversity found in a particular site along a week. Experiments with duplicate traps ((Núñez et al., 2017), and section 2.4.

Filtering and normalization) showed a concordance of ~90% in relative abundance of common OTUs, confirming the reproducibility of the method employed.

As a first characterization of the total diversity found in the two different locations, we calculated the richness in each sampling site along the four seasonal periods (Fig. 1a and b). Both, the urban and peri-urban locations show very similar values and trends of richness along seasons for the three communities. Bacteria show a marked rise in the number of OTUs in Summer in agreement with former studies (Be et al., 2015; Bertolini et al., 2013; Bowers et al., 2012, 2011a, 2011b) and likely due to the increase in temperature (Genitsaris et al., 2017), while some other studies reported higher bacterial diversity in different seasons, suggesting a large influence of the meteorological characteristics of the region (Du et al., 2018; Lee et al., 2017). Richness in fungi peaks in Spring and Fall. Unexpectedly, richness in plants is the largest in the Winter sample, which is explained by the fact that our ITS marker is able to differentiate a wide intraspecific genetic diversity. This is the case of the genus *Cupressus* spp. (order Pinales), with almost 500 OTUs assigned to this genus in the Winter sample, spanning around 60% of the total abundance in this season.

We next analyzed the differences in diversity between locations and seasonal periods, using Pielou's evenness index (Section 2.5. Richness and evenness estimates), Fig. 1c–d. Evenness and richness patterns are similar in fungi and bacteria, with higher values in this last group. This suggests that the new taxa appearing in different seasons (mainly in Summer and Fall in bacteria, Spring and Fall in fungi, Fig. 1a–b) have similar relative abundances to those present in all seasons. In contrast, evenness for pollen changes in Summer and Fall compared to richness values, and notably exhibits a high peak in Summer in the peri-urban location (P; Fig. 1d). This peak is the consequence of a wider diversity of plants flowering in this period, mostly belonging to the groups *Poaceae* (60 OTUs summing up 35% abundance in P) and *Fabaceae* (45 OTUs summing up 25% abundance). This is in contrast with other seasons in which there is a dominant pollen type in the atmosphere.

Overall, the similar trends in richness and diversity between both sampling sites hint to a larger influence of the seasonal period, as reflected in former studies (Bertolini et al., 2013; Bowers et al., 2012; Franzetti et al., 2011; Innocente et al., 2017), rather than the specific location, in determining community composition of airborne fungi and bacteria. However, the diversity of pollen types in a particular period may be strongly influenced by the presence of different local sources (gardens or parks).

3.2. Seasonal factors shape species communities

To visualize the relative influence of sampling period versus sampling site in the composition of airborne communities, we ordinated all the samples along the two main components obtained by principal coordinate analysis (PCoA), using Bray-Curtis distance (Fig. 2a–c). For the three different organisms, our samples tend to cluster by sampling period (Season), while sampling site (Location) seems to have a minor effect. This qualitative result is also observed using other ecological distances or ordination methods (Fig. S1). However, the community structure across seasons shows different patterns for each biological entity. For bacteria, we notice that composition during Spring is more related to that observed in Winter. This result may be due to the fact that both sampling periods are closer in time, with similar values of air temperature and relative humidity, Table 1. On the other hand, the structure of the fungal communities in Summer and Winter are more similar between them compared to the rest of the year. This can be explained by the fact that Summer and Winter fungal communities are both characterized by a lower diversity (Fig. 1), with a high abundance of *Capnodiales* (mainly *Cladosporium* spp., Table S3) which are typically found during dry weather (Oliveira et al., 2009).

Since the biological diversity detected is considerable, we asked whether a subset of the most abundant taxa is able to explain the dominant gradients in the space of the two main components. If this is the case, we can delimit the number of OTUs needed to define a biological community in a particular period. We found that the ~100 most abundant OTUs of each biological group are enough to obtain a graphical distribution similar to that employing all the diversity, Fig. 2d–f. These taxa are gathered selecting the most abundant OTUs in each sample, and although they constitute a small fraction of the total number of OTUs detected (< 7% for bacteria, < 15% for fungi and <

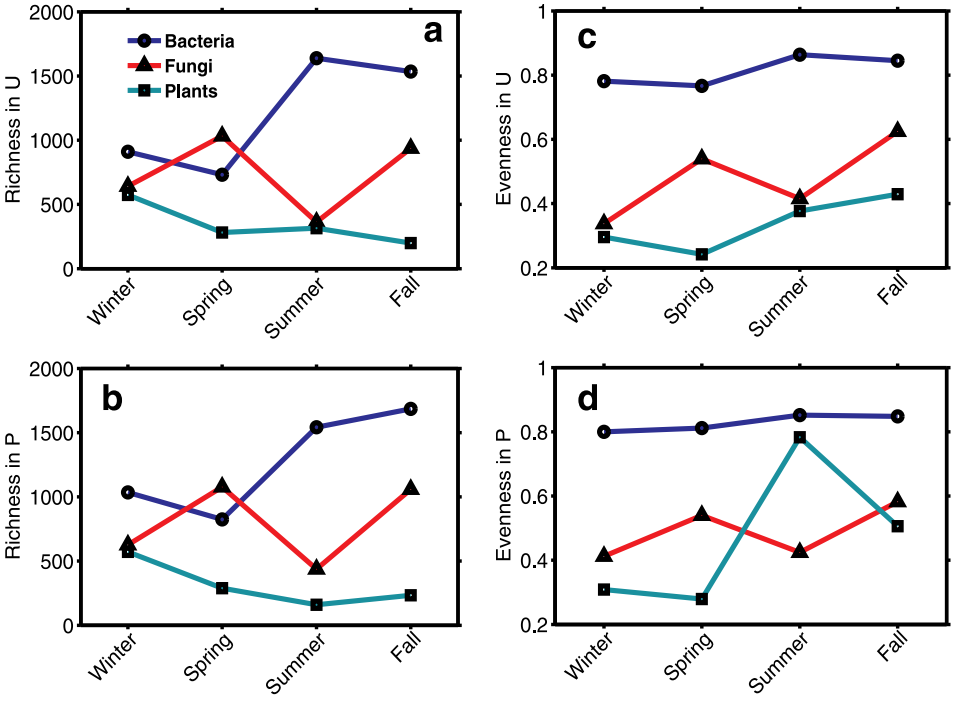

Fig. 1. Comparative of richness and evenness across seasons. *(Figure in Color)*.
Richness (number of OTUs observed) and evenness (Pielou's evenness index) of bacteria (circles), fungi (triangles) and pollen (squares) in the four seasonal sampling periods. Color lines are plotted to guide the eye. **a, c**, Urban (U) sampling site; **b, d,** Peri-urban (P) sampling location. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

29% for plants), their relative abundances represent a considerable fraction of the sample (52–68% for bacteria, 83–95% for fungi and 97–99% for plants). These results suggest that, despite the large biodiversity, the most abundant representatives and their relative abundances are sufficient to characterize the beta diversity across communities, a statement consistent with other works (Be et al., 2015; Weiss et al., 2017). There are however marked differences between the three groups analyzed. The taxa involved in the characterization of the fungal and plant communities are less diverse, in terms of number of different phylotypes (defined as any unique taxonomic rank), than bacterial samples. Although a potential bias may exist due to the primers and region selected, this result suggests that the communities of the former are dominated by few very abundant groups (in agreement with the microscopy analyses, Tables S2 and S3, and previous studies in the area (Díez-Herrero et al., 2006; Gutiérrez et al., 2006)), while the latter are characterized by many taxa with similar but smaller relative abundances (and thus higher evenness, see Fig. 1). Moreover, the PCoA biplots in Fig. 2d–f show that many of the dominant taxa in bacteria and fungi are present in all the seasons (gathered around the origin in the two component plane), whereas in plants these are more specific of a given season.

Taken together, these analyses suggest that long-term environmental changes, rather than the local microenvironment, drive week-long variability in airborne microbial communities. This is in agreement with other studies showing a large influence of season (Bertolini et al., 2013; Gandolfi et al., 2015; Genitsaris et al., 2017; Lee et al., 2017; Maron et al., 2006; Woo et al., 2013). Although urbanized areas are more homogeneous in microbial composition than rural areas (Barberán et al., 2015), local sources and fine scale characteristics of the sampling site, such as plant cover, play an important role in explaining short-term variability in nearby urban locations (Fan et al., 2019; Mhuireach et al., 2016, 2019). Therefore, we cannot rule out the influence of these local factors in our samples, which may be obscured by the effects of week-long and seasonal sampling.

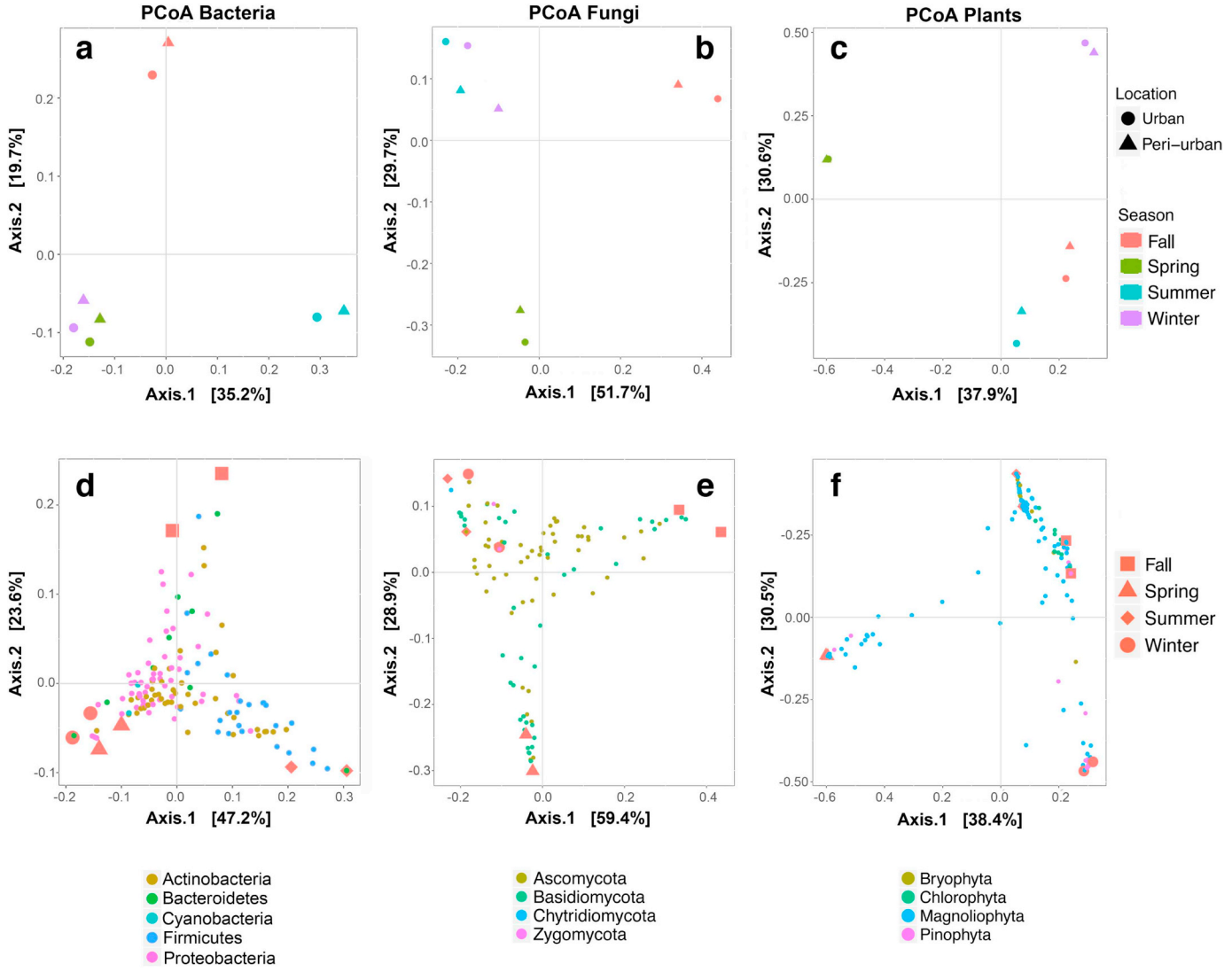

Fig. 2. Differences in community structure are mainly determined by season. *(Figure in Color)*
Top panels: Principal Coordinate Analysis (PCoA) of bacteria, fungi and plants using Bray-Curtis distance, **a-c**. All OTUs filtered and normalized as explained in section 2.4. *Filtering and normalization* are included in the ordination. The eight samples used are shaped according to sampling site and coloured by seasonal period, PERMANOVA tests to differentiate grouping by Season/Location yielded the following explained variances and p-values, calculated with permutation tests (1000 permutations): Bacteria (**a**): $R^2 = 0.12$, n.s for Location; $R^2 = 0.64$, ** for Season. Fungi (**b**): $R^2 = 0.06$, n.s. for Location; $R^2 = 0.85$, ** for Season. Plants (**c**): $R^2 = 0.05$, n.s. for Location; $R^2 = 0.86$, ** for Season. (***$p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$, n.s. Not significant). **d-e**: PCoA biplots showing simultaneously ordination of samples and taxa on the two main coordinates with the most abundant OTUs in each sample (top 50 OTUs per sample for bacteria, and top 30 for fungi and plants). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Temporal variation of the most abundant taxa

We next analyzed the main taxonomic groups present in the urban air of Madrid. Since sampling site plays a smaller role in community composition in our samples, we combined the measurements in both locations to describe the overall diversity captured in the four sampling periods across the year. Thus, we ignore the smaller variability due to location particularities to focus on long-term changes (seasonal). For each organism, we selected the orders spanning at least the 90% of the relative abundance in each season. In Fig. 3 we show the dominant orders in each group and their distributed abundances in the different sampling periods (see also Fig. S2 for abundances across samples). For bacteria and fungi, the main orders are present during the whole year, with variations in relative abundance, while for pollen some abundant orders are specific of each period. We also investigated the potential sources of airborne bacteria and fungi using Seqenv (Sinclair et al., 2016, section 2.7. Statistical analyses and software). Statistical analyses and software). Our analysis showed that, for bacteria, around 70% of the relative abundance for each seasonal period is due to soil-related sources, 3–8% to plants, 1–2% to water, and 1–4% to animal. Fungal communities are originated mainly from soil sources (> 70%), as most of them are saprophytes.

The prokaryotic community is dominated by members of *Actinobacteria* and *Proteobacteria* commonly found in soil (Delgado-

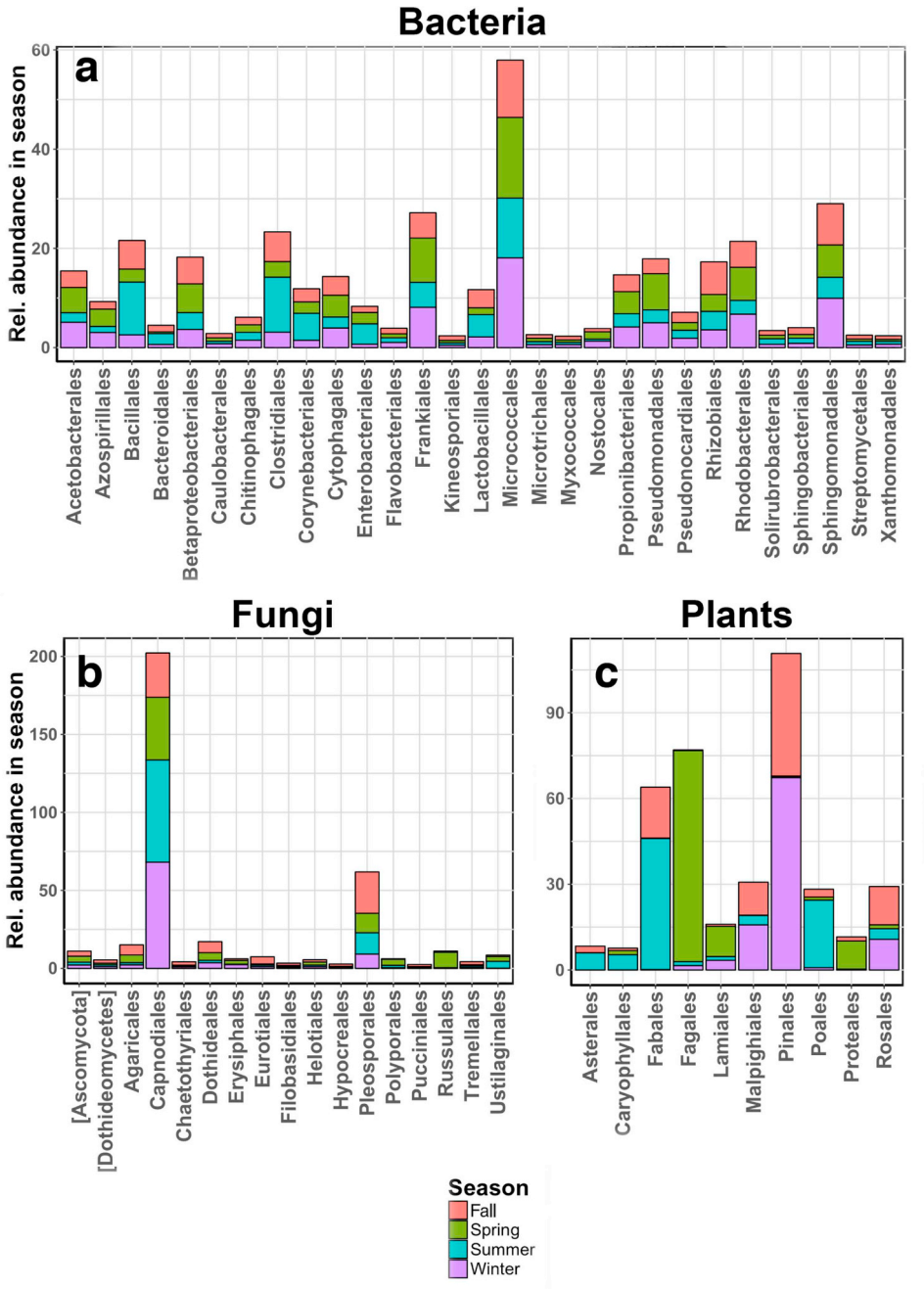

Fig. 3. Main taxonomic orders across seasons. *(Figure in Color)*
Stacked bar plots of the predominant taxonomic orders across seasons for the three different organisms: **a**, Bacteria, **b**, Fungi, **c**, Plants. The number of orders in each season was chosen to span at least the 90% of relative abundance per sampling period. Colors indicate seasons and the height of each color bar is the relative abundance in that season of the corresponding taxonomic order. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Baquerizo et al., 2018; Janssen, 2006), also registered by others authors in the air of different metropolitan areas (Bowers et al., 2011a; Fierer et al., 2008). In fact, many of these taxa are characteristically found in the soils of urban parks (Xu et al., 2014). *Micrococcales* (*Actinobacteria*) is the most abundant order and shows a high and consistent presence between seasons. *Alphaproteobacteria* are also abundant throughout the year (> 14%) with members of the orders *Sphingomonadales*, *Rhodobacterales*, *Rhizobiales* and *Acetobacterales* (Fig. 3a), usually found in leaves surfaces and soils (Bowers et al., 2011a; Kembel et al., 2014). The presence of orders including taxa which may potentially come from animal faeces, *Bacillales* and *Clostridiales* (Bowers et al., 2011b), turns especially abundant in Summer (> 21%).

Fungal communities are dominated by the presence of the phylum *Ascomycota* along the four sampling periods, consistent with similar studies (Bowers et al., 2012; Fierer et al., 2008; Oh et al., 2014). This phylum is mainly composed by *Capnodiales,* which exceed the 60% of the total abundance in Winter and Summer samples (Fig. 3b). *Pleosporales* have also a significant presence throughout the year, with a peak in Fall sample of around 25% of abundance. These groups have very frequent conidia (asexual spores) and live mainly on soil as saprophytes and vegetal parasites.

The pollen community shows a pattern of high seasonality (Fig. 3c), also supported by the morphological identification (Tables S1–S2) and phenological studies that have stated the pollen calendar of the city (Gutiérrez et al., 2006). For instance, the Winter sample is dominated by *Pinales* (67% of relative abundance), while *Fagales* govern the community composition in Spring.

To assess whether variations in relative abundance across seasons in bacteria and fungi are due to changes in abundance of few taxa, or to the appearance of new species under particular environmental conditions, we increased the taxonomic resolution to the Genus level, and gathered the most abundant genera in each time period, Fig. 4. We observe that a core of prevalent genera dominates bacterial community composition, with changes in abundance along the year (Fig. 4a). Among the group of *Proteobacteria*, it is remarkable the shift of *Pseudomonas* spp., with high abundance during the sampling periods with low temperatures (Winter and Spring samples). This fact could favour the growth of some species of this genus, since they have been previously described as promoters of ice nucleation in the atmosphere (Attard et al., 2012). It is also noticeable the increase in abundance in Spring and Summer samples (> 3% abundance in Summer) of *Pantoea agglomerans*, a well-known phytopathogen (formerly named *Enterobacter agglomerans* = *Erwinia herbicola*) (Brady et al., 2008), likely because of the proliferation of plant cover.

Fungal communities (Fig. 4b) follow a similar pattern of dominance by few abundant taxa present along the year, especially *Davidiella/Cladosporium* spp., making most of the *Capnodiales* abundance in Fig. 3b, while the genera *Alternaria* and *Epicoccum* dominate the abundance of *Pleosporales*. We observe, however, more clusters of taxa that are specific of a given season, mainly belonging to *Basidiomycota* (Fig. 4b).

The pattern for pollen (Fig. 4c) shows that the seasonality noticed in Fig. 3c is originated by specific highly abundant genera: *Cupressus* in Winter (order *Pinales*) and *Quercus* in Spring (order *Fagales*), both anemophylous (distributed by wind), while the most abundant Genus in Summer, *Styphnolobium* (order *Fabales*), is entomophylous (spread by insects).

To further investigate seasonal specificity, we quantified the number and relative abundance of OTUs that were observed in only one season (unique) and of those observed along the four seasonal sampling periods (common). To characterize OTUs as unique or common, we used a restrictive criterion of presence/absence for taxa taking into account the experimental variability of our sampling method, as explained in section 2.4. Filtering and normalization. We found that most of bacterial and fungal taxa are common to the four seasonal periods and span more than 50% of the relative abundance of each season, Figs. S3a–b. On the other hand, those unique of a given season are scarcer, especially in bacteria, and represent less than 10% in relative abundance. Summer and Spring are the samples with a larger outbreak of unique taxa in bacteria and fungi, respectively, consistent with the pattern of richness shown in Fig. 1. For plants we found that unique taxa appear frequently, and in Spring and Summer samples their number and abundance exceeds those of the species present throughout the whole year, Fig. S3c.

The most abundant genera corresponding to the unique taxa for each type of biological entity are shown in Fig. S4. The increase in number and abundance of unique bacterial genera in the Summer samples is due to the appearance of the soil-related genera *Kurthia*, *Bhargavaea* and *Lechevalieria*. Gut bacteria (*Nosocomiicoccus*, *Anaerococcus*, *Agathobacter*, and members of the families *Ruminococcaceae* and *Christensenellaceae*) are also detected only in Summer, contributing to the general trend observed for *Bacillales* and *Clostridiales*, and probably associated to the changes in the city dynamics (more people walking their pets, increase of trash in the streets, etc.). The appearance of *Cellvibrio* spp., a bacterium with a reported capability to degrade plant material (Attia et al., 2018), coincides with the decay of vegetation during the Fall period.

With respect to fungi, it is significant the presence and abundance of the genera *Schizophyllum* (wood-rotting fungi) and *Ustilago* (parasite of grasses) in the Spring samples, when the plant cover increases in Madrid.

In the case of plants, the appearance of unique taxa follows the flowering dates of the main pollen type in the city (Gutiérrez et al., 2006), although high-throughput sequencing provides a better resolution for cryptic groups such as *Poaceae*. Moreover, since pollen may act as a carrier for bacterial microbiome (Ambika Manirajan et al., 2016; Oteros et al., 2019), it is quite interesting that the increase of unique species of plants during the Summer period is concomitant with the same phenomenon in bacteria.

3.4. Potential pathogenic bacteria and fungi are present in urban air at all seasons

In addition to allergenic pollen and harmless microorganisms, the air may transport human pathogens, especially those causing respiratory diseases (Abd Aziz et al., 2018; Be et al., 2015; Cao et al., 2014; Fan et al., 2019; Kowalski and Bahnfleth, 1998). Although with much lower abundance than soil and leaf surface bacteria, a total of 39 bacterial genera (596 OTUs) with pathogenic representatives were found in our samples and compiled in Table 3.

Our results show that most of these genera were present in all the seasons. *Roseomonas* and *Corynebacterium*, alongside with the well-known pathogenic genera *Staphylococcus* and *Streptococcus* were consistently found among the most abundant, in agreement with other studies (Zhang et al., 2019). The cumulative abundance of the pathogens peaked in the Summer samples (14.47%), and the greatest diversity was found during the Fall period (38 genera). This is consistent with the general trends in richness of our bacterial samples, showing the largest diversity in Summer/Fall (Fig. 1a–b). Recently, Fan et al. (2019) showed a strong association between the proportion of pathogenic bacteria and particulate matter (PM). We notice that the concentration of PM_{10} particles is significantly higher during our Summer and Fall sampling periods (Table 1). *Enterococcus*, *Bacteroides*, *Prevotella*, *Fusobacterium* and several members of the family *Enterobacteriaceae* (*Enterobacter*, *Escherichia-Shigella*, *Klebsiella*), all of a likely animal/faeces origin, increased their relative abundance in the Summer samples, in accordance with the observed increase of this potential source during this period (Section 3.3).

Detected fungal pathogens, mainly from soil origin, spanned 27 genera (428 OTUs), with similar maximum cumulative abundances in Summer and Winter samples (~77%), but a greater diversity in the latter. The allergenic fungi *Cladosporium/Davidiella* spp. and *Alternaria*

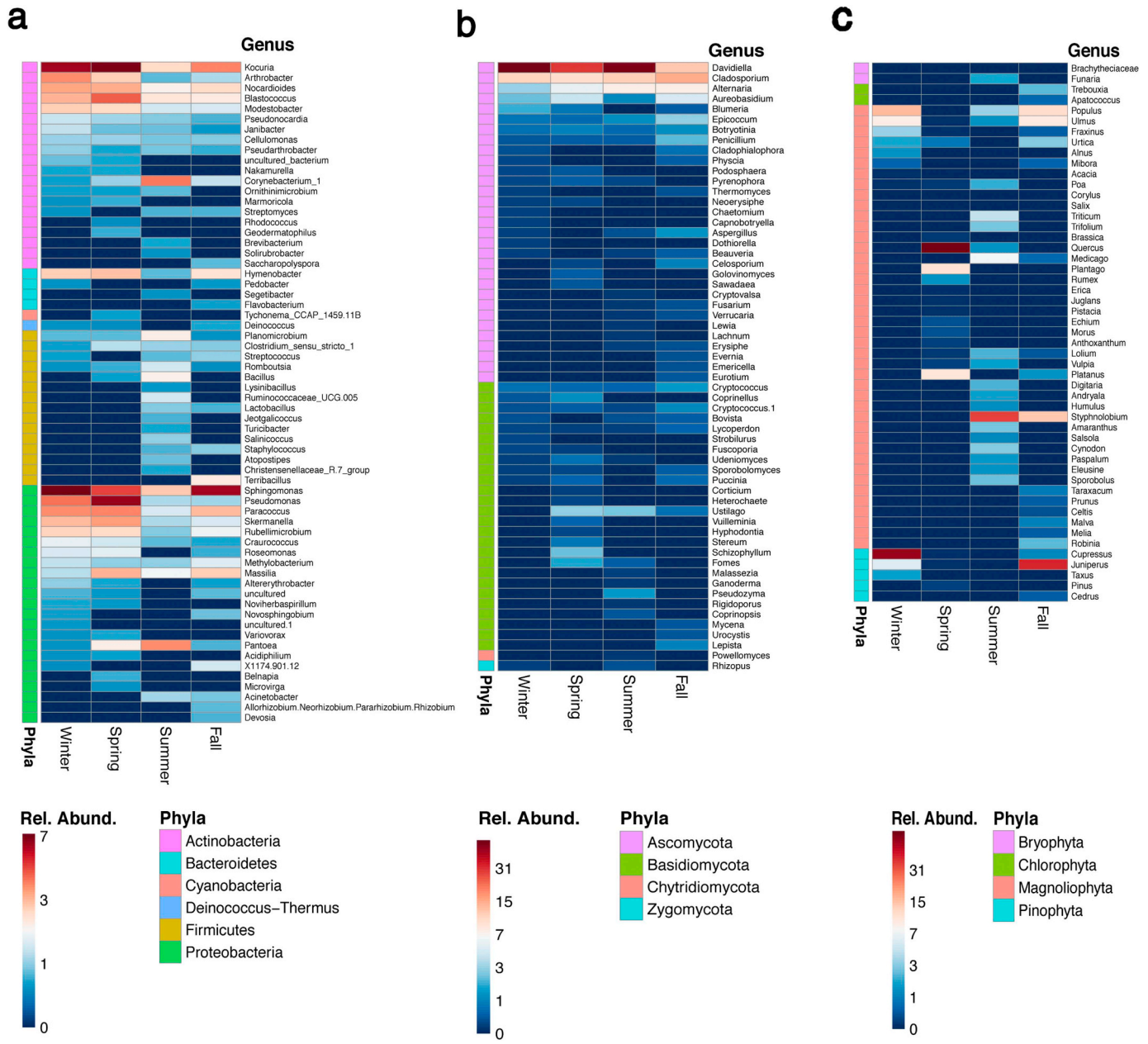

Fig. 4. Seasonal variations of the most abundant genera. *(Figure in Color)*
Heatmaps of the most abundant genera in each sampling period for the three different organisms: Bacteria (left), Fungi (middle), Plants (right). The collected taxa are the top 40 per season in bacteria, top 30 in fungi and top 20 in pollen. These top genera span the 50%–70% abundance in bacteria (out of 1145 total genera), 60–84% in fungi (576 total genera) and 80–99.6% in pollen (165 total plant genera). Color scale indicates relative abundance (%) of the corresponding genera (rows). Taxa are ordered by decreasing abundance in Winter within each Phylum type. Phyla are shown with different colors as a left bar in each heatmap (see legends). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

spp., which may also cause human diseases, contributed greatly to these harmful taxa. The genera *Aureobasidium*, *Epicoccum* and *Cryptococcus* were also abundant and present at all seasons.

3.5. Environmental factors correlated with airborne biodiversity

In order to assess the relative influence of different environmental variables on seasonal changes in community composition, we performed a constrained analysis of principal coordinates (CAP, see section 2.6. Correlation with environmental variables) with environmental factors (air temperature, relative humidity, rainfall and wind speed) and atmospheric pollution data (PM_{10}, NO_2), Table 1. Permutation tests obtained significant values ($p < 0.05$) for all constrained ordinations, Fig. 5, showing a clustering of samples very similar to those obtained in the previous PCoAs (Fig. 2). Other statistical tests and variance in the species data explained by the different environmental factors are shown in Table S4.

For bacteria, we see that average temperature plays an important role in the dispersion of samples along the main axis, Fig. 5a, with PM_{10} concentration also contributing to this dispersion. These two variables show strong associations with the variations in species composition found in Summer and Fall, and partially explain the separation of these samples from those of Winter/Spring. The influence of temperature on airborne bacterial communities has been previously reported by other authors (Bowers et al., 2012; Fang et al., 2008; Genitsaris et al., 2017; Lee et al., 2017). In our case, high temperatures (Summer) coincide

Table 3
Potential pathogenic genera found in air samples and their relative abundance distribution (%).

Organism	Genus[a]	Winter	Spring	Summer	Fall	Source
Bacteria						
	Acinetobacter (45)	0.37	0.46	1.12	0.90	Soil/Water
	Actinomyces (8)	0.00	0.00	0.03	0.01	Soil
	Aerococcus (4)	0.07	0.07	0.27	0.15	Soil
	Aeromonas (3)	0.02	0.02	0.01	0.05	Water
	Arcobacter (4)	0.05	0.05	0.02	0.07	Water
	Bacillus (55)	0.28	0.63	2.00	0.36	Soil
	Bacteroides (52)	0.11	0.07	0.30	0.27	Animal
	Campylobacter (6)	0.01	0.01	0.03	0.02	Animal
	Clostridium_sensu_stricto (39)	0.65	1.23	1.14	1.00	Soil
	Corynebacterium (72)	0.64	1.08	4.18	1.32	Animal/Soil/Water
	Enterobacter (6)	0.02	0.04	0.02	0.06	Animal/Soil/Water
	Enterococcus (7)	0.04	0.01	0.21	0.09	Animal/Soil/Water
	Erysipelothrix (9)	0.01	0.00	0.02	0.05	Animal
	Escherichia-Shigella (3)	0.01	0.02	0.14	0.06	Animal/Soil/Water
	Fusobacterium (5)	0.09	0.03	0.13	0.15	Animal
	Geodermatophilus (11)	0.41	0.71	0.55	0.41	Soil
	Gordonia (8)	0.02	0.02	0.01	0.09	Soil
	Haemophilus (3)	0.02	0.01	0.04	0.02	Animal
	Helicobacter (3)	0.00	0.00	0.04	0.00	Animal
	Klebsiella (1)	0.03	0.01	0.14	0.07	Animal/Soil/Water
	Lactococcus (3)	0.01	0.00	0.00	0.08	Soil/Water
	Legionella (7)	0.00	0.00	0.04	0.02	Soil/Water
	Micrococcus (1)	0.02	0.02	0.06	0.04	Soil
	Micromonospora (5)	0.11	0.06	0.14	0.09	Soil
	Mycobacterium (17)	0.16	0.21	0.33	0.36	Animal/Soil/Water
	Mycoplasma (1)	0.00	0.00	0.00	0.02	Animal/Water
	Neisseria (5)	0.00	0.00	0.00	0.02	Animal/Soil
	Nocardia (5)	0.00	0.03	0.04	0.01	Soil
	Porphyromonas (4)	0.00	0.00	0.04	0.00	Animal
	Prevotella (25)	0.09	0.06	0.24	0.16	Animal
	Pseudomonas (81)	3.99	6.43	1.15	1.14	Phyllosphere/Soil/Water
	Roseomonas (27)	1.47	1.61	0.26	0.73	Soil
	Serratia (6)	0.02	0.04	0.02	0.08	Soil
	Staphylococcus (21)	0.40	0.19	0.77	0.93	Animal
	Stenotrophomonas (7)	0.02	0.05	0.10	0.15	Soil
	Streptococcus (25)	0.62	0.41	0.81	1.03	Animal
	Thermoactinomyces (4)	0.02	0.02	0.06	0.14	Soil
	Thermomonospora (5)	0.01	0.01	0.01	0.04	Soil
	Vibrio (3)	0.00	0.00	0.00	0.01	Water
	Total (596)	9.79	13.60	14.47	10.20	
Fungi						
	Acremonium (12)	0.03	0.02	0.00	0.14	Soil
	Alternaria (57)	3.07	5.67	7.59	7.70	Soil
	Aspergillus (35)	0.17	0.13	0.32	1.28	Soil
	Aureobasidium (18)	2.33	4.07	1.10	4.81	Soil/Water
	Botrytis/Botryotinia (8)	0.74	1.01	0.61	1.18	Soil
	Candida (10)	0.01	0.00	0.03	0.06	Animal/Soil/Water
	Chaetomium (18)	0.18	0.07	0.11	0.16	Soil/Water
	Cladosporium/David. (39)	67.42	39.44	64.85	26.94	Soil
	Cryptococcus (44)	1.02	1.10	0.77	2.55	Animal/Soil
	Emericella (3)	0.02	0.01	0.05	0.30	Soil
	Epicoccum (24)	0.82	0.63	1.23	2.81	Soil
	Eurotium (2)	0.13	0.09	0.08	0.40	Water
	Exophiala (13)	0.07	0.05	0.02	0.16	Soil
	Fusarium (16)	0.16	0.05	0.16	0.32	Soil
	Geomyces (4)	0.05	0.00	0.00	0.03	Soil
	Helminthosporium (1)	0.00	0.00	0.00	0.02	Soil
	Mucor (6)	0.05	0.04	0.10	0.03	Soil
	Oidiodendron (6)	0.02	0.00	0.02	0.02	Soil
	Paecilomyces (5)	0.03	0.01	0.00	0.02	Soil/Water
	Penicillium (61)	0.42	0.46	0.46	2.06	Soil
	Phoma (18)	0.11	0.13	0.04	0.28	Soil
	Rhizopus (4)	0.18	0.04	0.30	0.13	Soil
	Scopulariopsis (5)	0.16	0.03	0.09	0.21	Soil
	Stachybotrys (2)	0.03	0.01	0.00	0.00	Soil
	Talaromyces (11)	0.05	0.03	0.00	0.09	Soil
	Trichoderma (4)	0.01	0.01	0.02	0.03	Soil
	Ulocladium (2)	0.00	0.01	0.00	0.00	Soil
	Total (428)	77.28	53.1	77.95	51.73	

[a] List compiled from Abd Aziz et al. (2018), Fan et al. (2019), and Kowalski and Bahnfleth (1998). The no. of OTUs assigned to the genera are indicated between parentheses.

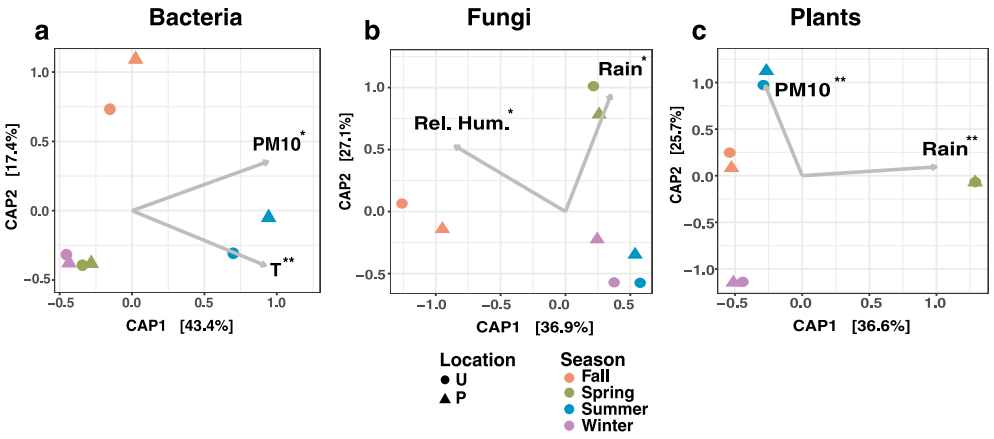

Fig. 5. Principal coordinate analysis constrained by environmental factors. *(Figure in Color)*
Distance-based Redundancy Analysis (db-RDA) for ordinations of the samples with Bray-Curtis distances, constrained by different environmental factors (Table 1). For each different organism we selected a parsimonious model with two environmental variables explaining ~50% of variation in community composition (adjusted R^2, see section 2.6. *Correlation with environmental variables* and *2.7. Statistical analyses and software*). Species data are restricted to the most abundant OTUs as in Fig. 2d–f. The ordinations shown correspond to correlation biplots: angles between samples and arrows reflect their correlations.Asterisks represent significance of the environmental factors under permutation tests, using a mimimum of 1000 permutations: *** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$. Other statistical information of the db-RDA analyses is collected in Table S4. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with high bacterial richness, Fig. 1, suggesting that the values reached favour the multiplication of these organisms. PM_{10} particles are also positively correlated with richness and diversity of bacterial samples, with the highest concentration of PM_{10} particles in Summer and lowest in Winter/Spring (see also Fig. 1). The relationship between particulate matter and microbial communities have been previously studied by different methods, from culture-dependent (Lee et al., 1973) to NGS (Bowers et al., 2012; Du et al., 2018; Franzetti et al., 2011; Gandolfi et al., 2015; Lee et al., 2017), finding also significant correlations. In fact, because of their size, these microorganisms could be attached to larger inorganic particles and be transported jointly (Ambika Manirajan et al., 2016; Oteros et al., 2019).

Precipitation and relative humidity are correlated with fungal communities Fig. 5b, Precipitation is usually a key factor related to fungal growth and dispersion. The major component in this community (*Cladosporium/Davidiella*, Fig. 4 and Table S3) has been reported to be negatively correlated with rainfall (Katial et al., 1997; Oliveira et al., 2009). This is in agreement with the decrease in relative abundance of this fungal spore observed in Spring and Fall, when the precipitation and relative humidity are higher.

In the case of pollen and other plant material, Fig. 5c, rain and PM_{10} concentration are the factors associated to most of the observed variation. In particular, the amount of precipitation clearly separates the Spring samples from those of the other seasons in ordination space. We notice that the statistical associations obtained with CAP do not imply causality, and in this case they seem to merely reflect the fact that rainfall is especially high in Spring, a season where the flowering of plants favors the appearance of many pollen species not present during the rest of the year. Moreover, the strong association between composition of the pollen samples and PM_{10} particles has no *a priori* ecological explanation since the average size of pollen grains is over that range. Therefore, these associations must be taken with caution, as also discussed in Sousa et al. (2008). For instance, it has been reported that wind speed and direction in a particular area is a main factor influencing pollen concentration (Recio et al., 2010; Rojo et al., 2015). In the present study, the influence of this factor may be masked by the stronger variation of other environmental factors.

4. Conclusions

The results of the present study suggest that long-term variability of airborne diversity in urban areas is largely influenced by environmental changes, in agreement with other works (Bertolini et al., 2013; Bowers et al., 2013, 2012; Franzetti et al., 2011; Innocente et al., 2017), and to a less extent by the features of the local microenvironment. However, each community (bacteria, fungi or pollen) shows characteristic patterns of temporal variation in composition and abundance. Thus, microbial communities (bacteria and fungi) have a steady core of taxa present in high abundance along the year. On the contrary, the main contributors to the airborne plant community change remarkably and show a clear seasonal pattern, dominated by the anemophilous species present in the metropolitan area and used as ornamental (genera *Cupressus*, *Ulmus*, *Populus*) and the plants of the natural areas in the surroundings (especially *Quercus* in Spring). DNA sequencing approaches not only yielded a better taxonomic identification than microscopy, but also allowed us to detect other representatives of the kingdom *Viridiplantae* as *Chlorophyta* and *Bryophyta* (usually overlooked in aerobiological studies), which may support and launch further studies in the field.

The core of the airborne bacteria is quite diverse but constituted by representatives typically associated to soil and plant surface, mainly *Actinobacteria* (*Kocuria*, *Nocardioides*, *Arthrobacter*, *Blastococcus*, *Modestobacter*), and *Proteobacteria* (*Sphingomonas*, *Pseudomonas*, *Paracoccus*). The fungal community is mainly composed of *Capnodiales* (mostly *Cladosporium/Davidiella*), *Pleosporales* (*Alternaria*) and *Dothideales* (*Aureobasidium*). Potentially harmful taxa were detected at all seasons, coming from soil, water and animal fecal matter, suggesting a steady presence of these microorganisms from environmental sources, which is relevant to the knowledge of the human exposome. Nonetheless, further studies with a higher resolution are necessary to discern the pathogenic species from those that are not, and also to address whether they are active. Finally, we observed that each biological kingdom (bacteria, fungi or plant) is correlated with different environmental factors: high temperatures promote an increase in bacterial diversity, while precipitation and relative humidity seem to have a strong influence on fungal and plant communities.

The present study constitutes a first step towards a more complete survey of the biological diversity of a highly populated city of a Mediterranean country. Further investigation would increase the number of sampling locations and involve longer time periods with higher temporal resolution, which would allow tracing a map of the main urban airborne species and the influence of different sources of microorganisms. This knowledge will be useful both from an ecological perspective, for instance by identifying ecological markers of long term climatic changes or chemical contamination of the air, as well as for designing preventive strategies in public health to minimize the exposure to airborne pathogens.

Author contribution

A. Núñez and G. Amo de Paz contributed equally to this work.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atmosenv.2019.116972.

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