# SPATIO-TEMPORAL VARIABILITY OF AIRBORNE BACTERIAL COMMUNITIES AND THEIR CORRELATION WITH PARTICULATE MATTER CHEMICAL COMPOSITION ACROSS TWO URBAN AREAS

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**Abstract**

The study of spatio-temporal variability of airborne bacterial communities has recently gained importance, due to the evidence that airborne bacteria are involved in atmospheric processes and can affect human health. In this work, we described the structure of airborne microbial communities in two urban areas (Milan and Venice, Northern Italy) through the sequencing, by the Illumina platform, of libraries containing the V5-V6 hypervariable regions of the 16S rRNA gene, and estimated the abundance of airborne bacteria with qPCR. Airborne microbial communities were dominated by few taxa, particularly *Burkholderiales* and *Actinomycetales*, more abundant in colder seasons, and Chloroplasts, more abundant in warmer seasons. By partitioning the variation in bacterial community structure, we could assess that environmental and meteorological conditions, including variability between cities and seasons, were the major determinants of the observed variation in bacterial community structure, while chemical composition of atmospheric particulate matter (PM) had a minor contribution. Particularly, Ba, $SO_4^{2-}$ and $Mg^{2+}$ concentrations were significantly correlated with microbial community structure, but it was not possible to assess whether they simply co-varied with seasonal shifts of bacterial inputs to the atmosphere, or their variation favoured specific taxa. Both local sources of bacteria and atmospheric dispersal were involved in the assembling of airborne microbial communities, as suggested, to the one side, by the large abundance of bacteria typical of lagoon environments (*Rhodobacterales*) observed in spring air samples from Venice, and to the other by the significant effect of wind speed in shaping airborne bacterial communities at all sites.

**Keywords:** air pollution, bioaerosol, particulate matter, NGS, Milan, Venice

## INTRODUCTION

Microorganisms are ubiquitous on the Earth since they can be found in every environment, including the most extreme (O'Malley 2008). Although the atmosphere can be considered an extreme environment for microbes due to its harsh conditions, such as low nutrient concentration, low relative humidity and high UV intensity, bacteria and fungi have been detected in various atmospheric layers (Wainwright 2003; Griffin 2007).

In recent years, the study of airborne bacteria has gained interest due to the emerging evidences that microorganisms are involved in several atmospheric processes and can also impact human health. Due to this relevance for healthcare, researches on atmospheric bacteria in outdoor environments often focused on potentially harmful bacteria sourced from specific hotspots (e.g. hospitals, farms, waste treatment plants) (Gandolfi et al 2013).

Our comprehension of microbial communities and of their spatial and temporal variability in the atmosphere has significantly improved only recently, particularly by taking advantage of Next-Generation Sequencing-based (NGS) technology, which allows describing in details diversity and composition of microbial communities. NGS technology is now widely applied to the study of bacterial diversity in several environments, owing to its high throughput and to the rapidly reducing cost per sequence (Rinsoz et al 2008; Bowers et al 2009; Bowers et al 2011b; Bertolini et al 2013; Bowers et al 2013).

Microbiological surveys of airborne bacteria often focused on the atmosphere of urban areas, because air pollution is particularly intense in these areas (European Environment Agency 2014). Indeed, it has been recognized that people living in industrialized cities suffer from increased risk of inflammation and lung irritation because of the more intense exposition to PM (Schwartz et al 2002). Some studies also investigated the relationships between microbial community structure and meteorological conditions and found that the structure of airborne microbial communities was generally affected by air temperature and relative humidity (Maron et al 2006; Brodie et al 2007; Fierer et al 2008; Bertolini et al 2013). However, since bacteria live in tight association with PM, we may expect that PM composition can also alter the structure of microbial communities. This hypothesis is corroborated by the results of a few studies that reported an effect of chemical composition of PM on microbial abundance in atmospheric samples (Vaïtilingom et al 2012; Smith et al 2012), but to the best of our knowledge, no study of airborne bacteria conducted so far has investigated the effect of the chemical composition of PM in shaping the airborne bacterial community. For example,

Sánchez de la Campa and colleagues (Sánchez de la Campa et al 2013) fully characterized the chemical composition of Total Suspended Particulate (TSP) collected during a Saharan dust event in Spain and analysed the microbial community composition, but they did not investigated the relationships between chemical composition of TSP and the structure of associated microbial communities.

In this work we focused on the urban areas of Milan and Venice, two large and industrialized cities of the Po Valley (Northern Italy), which is one of the most polluted areas in Europe due to large concentration of industrial activities and to its peculiar climatic conditions. Indeed, this flatland is enclosed between the Alps to the North and (in most part) the Apennines to the South. These mountain ranges reduce atmospheric circulation and favour pollutant accumulation. Moreover, the highly recurring occurrence of thermal inversion conditions enhances atmospheric pollution (Mantecca et al 2012).

In 2011-2012 we collected a total of 95 samples of $PM_{10}$ at three sampling sites, one urban site in Milan and two sites in the urban area of Venice. Concentrations of inorganic ions and elements in PM of each sample were assessed, as well as the structure of airborne bacteria communities, which was investigated through the sequencing of 16S rRNA gene libraries with the NGS Illumina platform. Furthermore, quantitative PCR was used to estimate the total number of airborne bacteria in each sample.

We aimed at fully describing the structure of microbial communities and their variability among seasons and sampling sites in order to gain insight into the processes assembling microbial communities. In addition, we aimed at assessing the impact of environmental and meteorological conditions and of PM inorganic ions and elements on microbial community structure. In particular, we aimed at: i) assessing the relative contribution of environmental and meteorological conditions on the one side and PM ions and elements on the other on the structure of airborne bacterial communities, and ii) identifying the environmental factors whose variation affected the structure of airborne microbial communities.

## MATERIALS AND METHODS

### Sample collection and DNA extraction

$PM_{10}$ was sampled for eight days per season at three different sites: 1) a urban site in the northern part of Milan (Northern Italy; 45°30’35.430’’ N, 9°12’38.5239’’ E); 2) one urban site

located near a high-traffic road in Mestre, in the urban area of Venice (Northern Italy) (Venice-Mestre hereafter; 45°29'12.426'' N, 12°13'20.327'' E); 3) one site in the suburban industrial area of Porto Marghera, in the North-western part of Venice (Venice-Porto Marghera hereafter; 45°26'18.623'' N, 12°12'13.388'' E). Unfortunately, one summer sample from Venice-Porto Marghera was unavailable for accidental reasons (Table S1). Each $PM_{10}$ samples was collected on quartz fibre filters (Whatman, Maidstone, England) by a high-volume sampler (ECHO HiVol, TCR TECORA, Milan, Italy) that worked for 24 h with the following conditions: flux speed = 200 L $min^{-1}$ in Milan and 500 L $min^{-1}$ in both Venice-Mestre and Venice-Porto Marghera. A quarter of each filter was cut into small pieces and loaded into the bead tube of the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) extraction kit, after the adding of 1M $CaCO_3$ in order to increase the pH, and shaking at 200 rpm for 60 min. The remaining steps of the DNA extraction were performed according to the manufacturer's instructions.

**Quantitative PCR (qPCR)**

The plasmid pCR2.1 (Life Technologies Italia, Monza, Italy) containing a fragment of the bacterial 16S rRNA gene was used for standard concentration curves. The target 466-bp fragment (331-797 according to *E. coli* position) was obtained by PCR amplification with the universal primer-set 331 Forward (5'- TCCTACGGGAGGCAGCAGT -3') and 797 Reverse (5'- GGACTACCAGGGTATCTAATCCTGTT-3') (Nadkarni et al 2002). The concentration of plasmidic DNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Serial dilutions of the plasmidic DNA were used as the standard for qPCR. Each qPCR reaction was carried out in a total volume of 10 μL using the FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 μM forward and reverse primer final concentration. The amplification was carried out with the Eco Real-Time PCR system (Illumina, CA, USA) under the following conditions: 95°C for 4 min, and 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, with acquisition of the fluorescence on the FAM canal at the end of each 72°C elongation step. The standards and the samples were included in triplicate in each run.

**16S rRNA fragment libraries by Illumina Hiseq 1000**

For Illumina HiSeq sequencing, the V5-V6 hypervariable regions of the 16S rRNA gene were amplified, pooled and purified as previously reported (Bertolini et al 2013). Multiplexed sequencing of all the pooled samples were performed on a single Illumina HiSeq 1000 lane, using a paired-end 2 × 100 base-pair protocol and the 4.0 sequencing chemistry. The cluster extraction and base-calling processing analyses were performed by using the Illumina CASAVA Analysis software, version 1.8. Illumina HiSeq 1000 sequencing was carried out at BMR Genomics, Padua, Italy.

**Sequence data**

Sequence data were deposited at the European Nucleotide Archive (ENA) with the study accession number PRJEB7001 (sample accession numbers from ERS528729 to ERS528823).

**Sequence analyses**

Each sequence was assigned to its original sample according to its barcode. Consistently with our previous work in the same area (Bertolini et al 2013), after sorting, the reverse read of each paired-end sequence was reverse-complemented and merged with the corresponding forward read, inserting 10 Ns in between (Claesson et al 2009). A quality cut-off was then applied in order to remove sequences i) that did not contain the barcode, ii) with an average base quality value (Q) lower than 30. The barcode was removed from sequences before further processing. The taxonomic attribution of filtered sequences was carried out using the stand-alone version of RDP Bayesian Classifier, using 50% of confidence as suggested for sequences shorter than 200 bp (Wang et al 2007; Claesson et al 2009). OTUs were defined on the basis of the classification at fourth taxonomic rank of the RDP classifier which, in most cases, corresponds to Order level.

**Environmental factors and chemical data**

*Meteorological data and PM concentration*

Meteorological data (daily average temperature, daily rainfall, wind speed, global solar radiation, relative humidity) and $PM_{10}$ concentrations relative to the sampling periods were retrieved from the Regional Agencies of Environmental Protection (respectively ARPA-

Lombardia for Milan, data available at: www.arpalombardia.it, and ARPA-Veneto for Venice, data available at: www.arpa.veneto.it).

*Analysis of inorganic ions end elements*

Ions were extracted from a quarter of each filter by ultrasonic method in MilliQ® water for 60 min and then analysed by ion chromatography (IEC) to determine water soluble inorganic ions ($Na^+$, $NH_4^+$, $Mg^{2+}$, $Ca^{2+}$, $K^+$, $F^-$, $Cl^-$, $NO_3^-$, $NO_2^-$, $SO_4^{2-}$) as previously described (Squizzato et al 2012).

To determine the elemental concentration, another quarter of each filter was digested by microwave digestion system (Microwave Digestor Rotor Ethos 1600, Milestone) (Jarvis et al 2003) and then analysed by ICP-MS (Inductively Coupled Plasma Mass Spectrometry) and ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry). The acid mix used to mineralize the samples was composed by $HNO_3$, $H_2O_2$ and HF (Karthikeyan et al 2006). The elements analysed were Al, S, Fe, Ca, Mg, K, Ti, Cr, Mn, Zn, Cu, Ba, As, Ag, Cd, Ni, Pb, Sb, V, Co.

**Statistical analyses**

*Univariate analyses*

OTUs found in one sample only (singletons) were removed because they may inflate variance explained by models (Legendre and Legendre 1998). The fifteen OTUs (including *Chloroplasts* and excluding unclassified sequences), whose mean relative abundance was larger than 1%, were considered abundant (abundant OTUs hereafter). The number of ribosomal operons was log-transformed to achieve normality (Shapiro-Wilk test of normality after log-transformation: $W = 0.987$, $P = 0.727$). Variation in the log-number of ribosomal OTUs was analysed by Analysis of Variance corrected for inhomogeneity of variance, while variation in the number of OTUs was analysed in a Generalized Linear Model assuming a Poisson error distribution.

*Multivariate analyses*

The hierarchical cluster analysis of the relative abundance of OTUs was performed with the complete linkage method on the chi-square distance between samples. Canonical Correspondence Analysis (CCA) was used to relate the bacterial community structure to PM

composition and environmental variables (Legendre and Legendre 1998; Córdova-Kreylos et al 2006). Collinear variables were removed before the analyses, and CCA models were simplified by backward removing non-significant predictors. Post-hoc pairwise comparisons were performed whenever a categorical predictor was significant. P-values of post-hoc tests were adjusted using the FDR procedure (Benjamini and Yekutieli 2001). The variation partitioning (VarPart) in Canonical Analysis was used to quantify the variation explained separately and jointly by different subsets of variables, while controlling for the effect of the other subsets (Borchard et al 2011). Multivariate Regression Trees (MRT) were used to identify thresholds in the values of environmental variables and PM composition that determined changes in the structure of bacterial communities (Borchard et al 2011). The Indicator Taxa analysis was used to identify taxa that are typical of different groups of samples (Dufrene and Legendre 2007).

All the analyses were performed in R 3.0.2 (Team 2008) with the only exception of the Variation Partitioning analysis, which was performed with the VarCan software (Peres-Neto et al 2005). The Supplementary Materials provide full details on statistical analyses and the R packages used.

## RESULTS

### Quantification of airborne bacteria

A total of 27 samples out of 95 resulted non quantifiable since the amount of DNA extracted from them was too low (see Table S1 for details). The number of ribosomal operons per sample, which was used here as a measure of bacterial abundance (Bertolini et al 2013), ranged between $2.376 \times 10^2$ and $4.876 \times 10^5$ ribosomal operons per $m^3$ of air, with a mean value of $3.026 \times 10^4 \pm 0.867 \times 10^4$ SE. Log-transformed number of ribosomal operons varied significantly according to season ($F_{3,56} = 3.619$, $P = 0.018$) and sampling site ($F_{2,56} = 3.449$, $P = 0.039$), but not according to their interaction ($F_{6,56} = 1.872$, $P = 0.102$). Post-hoc tests indicated that summer samples contained a significantly lower number of ribosomal operons than spring ones ($t_{56} = 2.734$, $P = 0.041$; Fig. 1a) and that Milan samples contained significantly larger numbers of ribosomal operons than both those of Venice-Mestre and Venice-Porto Marghera ($t_{56} \leq 3.117$, $P \leq 0.008$; Fig. 1b).

## Spatial and seasonal variability in microbial community structure

The number of paired reads obtained in each sample ranged from 18,089 to 2,089,122. In each sample, 22.2% to 42.3% of the sequences (mean value: 29.1%) could not be classified and were discarded from subsequent analyses. To avoid differences in coverage among the samples, 10,000 randomly-selected paired reads were chosen from each sample. This number of sequences still allows a detailed description of the community structures and a proper evaluation of alpha- and beta-diversity (Caporaso et al 2012). The Operational Taxonomic Units (OTUs) were defined according to the RDP classification at the fourth taxonomic rank. Overall, 108 OTUs were identified in the PM of the three sampling sites, 102 of which were identified in more than one sample (non-singletons), while the number of OTUs identified in each sample ranged between 45 and 78. The number of OTUs per sample did not differ significantly according to season ($\chi^2_3$ = 3.793, P = 0.285), sampling site ($\chi^2_2$ = 1.776, P = 0.411), or their interaction ($\chi^2_6$ = 3.251, P = 0.777).

The taxonomic classification of bacteria based on the fourth taxonomic rank showed a low spatial variability in all seasons (Table 1 and Fig. S1). The three analysed sites were dominated by few taxa: particularly *Burkholderiales*, *Actinomycetales* and *Chloroplasts* constituted about 50% of all communities. Dominance by these taxa was particularly evident in summer and autumn, when the communities at the three sites were more similar to each other. Indeed, the abundance of *Enterobacteriales* (5.8%) in the suburban industrial site of Venice-Porto Marghera in summer represents the only relevant variation in the otherwise similar bacterial community structures in these seasons. Community structure differed more between sites in winter and spring. Nevertheless, *Burkholderiales* were abundant at all sites in winter, while *Rhodobacterales* were particularly abundant (between 25% and 30%) in both Venice sites in spring. In the urban sites of Milan and Venice-Mestre, temporal variability of airborne bacterial communities followed a well-known trend: the sum of *Actinomycetales* and *Burkholderiales* decreased from winter to summer. At the same time *Chloroplasts* increased with increasing ambient temperature (Brodie et al 2007; Franzetti et al 2011; Bertolini et al 2013). Thus, cold seasons were dominated by microorganisms generally retrieved in soil while in warm seasons taxa typically associated with plants mainly prevailed.

Cluster analysis showed five main clusters when applied both to all OTUs and to the most abundant ones (Fig. 2 and Fig. S2). Sample classification was generally consistent between the analyses, with 89 over 95 (i.e. 93.7%) of samples classified in the same cluster in both

analyses. Particularly, two clusters included exactly the same samples. One of these clusters, entirely composed by spring samples from both sites of Venice, was characterized by a high abundance of *Rhodobacterales*, while the other one, composed by spring and some summer samples from both Venice and Milan, was characterized by a high abundance of *Chloroplasts*.

**Microbial community structure, environmental conditions, and chemical composition of PM**

'Environmental conditions' here refers to meteorological conditions, plus a four-level factor defining the season, a three-level factor defining the sampling site, and the two-way interactions among them. Variables Ca, Cr, Co, Cu, Fe, K, Ni, Ti, Zn, were removed from the analyses because they were strongly correlated with other chemical variables ($r^2 \geq 0.655$, see methods). Square correlation among the remaining variables was $\leq 0.576$.

Canonical Correspondence Analysis (CCA) performed on all OTUs or only on the most abundant ones gave consistent results. Indeed, both analyses indicated that bacterial community structure varied according to the concentration of the same chemical constituents of PM, namely Ba, $SO_4^{2-}$ and $Mg^{2+}$, and according to the same environmental variables, namely city, season, their interaction, wind speed, and relative humidity (Table 2). The first two CCA axes obtained from a linear combination of these variables indeed explained as much as 72.8% of total variability in bacterial communities. Post-hoc pairwise comparisons on both all and the most abundant OTUs (Fig. S3) indicated that spring samples significantly differed from those of all other seasons ($F_{1,38} \geq 7.068$, $P_{FDR} \leq 0.001$), while comparison among the other seasons were non-significant ($F_{1,38} \leq 2.154$, $P_{FDR} \geq 0.334$). Bacterial communities at Venice-Porto Marghera were generally different from those at the other two sites, irrespective of season ($F_{1,52} \geq 3.789$, $P_{FDR} \leq 0.005$). The significant interaction effect was investigated by pairwise comparisons among seasons within city run on both all and the most abundant OTUs. These comparisons indicated that there was a significant difference among spring and autumn samples of Venice-Mestre only ($F_{1,12} \geq 13.928$, $P_{FDR} \leq 0.024$), while all the other comparisons were non-significant ($F_{1,12} \geq 5.566$, $P_{FDR} \geq 0.234$).

VarPart analysis indicated that bacterial communities seemed mostly related to environmental conditions, which alone explained 25.3% and 29.0% of the variation in bacterial community structure in the analysis run on all OTUs and in that run on the abundant OTUs, respectively. Conversely, PM composition explained only 1.6% and 1.9% of variance, respectively, while

the common contribution of the environmental conditions and PM composition in shaping bacterial community structure was 22.9% and 25.5%, respectively (Fig. S4).

Multivariate Regression Trees (MRT) were used to identify thresholds in the values of environmental and PM variables that were related to changes in the structure of bacterial communities. MRT analysis indicated that bacterial communities can be divided in 7 groups according to PM composition and environmental variables selected in the previous analyses (Fig. 3). Finally, the Indicator Taxa approach (Dufrene and Legendre 2007) was used to identify OTUs typical of different seasons and groups indicated by the MRT analysis (Borchard et al 2011) (Table 3).

## DISCUSSION

### Quantification of airborne bacteria

The concentrations of airborne bacteria in the near-surface atmosphere of the urban areas of Milan, Venice-Mestre and Venice-Porto Marghera (Northern Italy) were much larger than those reported in the only other study carried out in the urban area of Seoul (South Korea) that used qPCR to assess the number of ribosomal operons in the lower atmospheric layers (Lee et al 2010), as well as in previous culture-based reports from other urban areas (Fang et al 2007). Culture-dependent methods underestimate bacterial abundance in the atmosphere (Peccia and Hernandez 2006), so that higher values from culture-independent methods are not surprising. Conversely, bacterial concentrations even higher that those found in our samples were obtained in other studies that applied culture-independent techniques other than qPCR, such as epifluorescence microscopy (Maron et al 2005; Bowers et al 2009; Bowers et al 2011a; Bowers et al 2011b). (Bowers et al 2009; Bowers et al 2011a; Bowers et al 2012), (Maron et al 2005). Our results are therefore in the lower range of variation (or even lower) of previous estimates of airborne bacteria based on culture-independent techniques.

In a previous study carried out in Milan, we assessed bacterial abundance in the TSP with the same analytical technique (Bertolini et al 2013) and found a slightly larger value than that we found in the $PM_{10}$ in the present study. In particular, in winter and in spring the number of ribosomal operons per gram of PM was in the same order of magnitude in both studies, while summer and autumn samples collected during this study showed a lower bacterial abundance than in the previous one. These differences may be partly due to the fact that $PM_{10}$ is a sub-fraction of TSP and can, therefore, contain a lower abundance of bacteria. However, we

cannot exclude the existence of annual fluctuations due to slightly different environmental conditions from year to year.

In addition, the lower abundance of ribosomal operons in summer than in spring samples (Fig. 1a) is quite surprising, as it contrasts with previous observations, at least for Milan site (Bertolini et al 2013), and we have no clear explanation for it. Finally, the causes of the larger number of ribosomal operons observed in Milan than in both Venice-Mestre and Venice-Porto Marghera (Fig. 1b) should be further investigated.

**Seasonality in microbial community structure**

The Variation Partitioning analysis indicated that bacterial communities seemed mostly related to environmental conditions, which, alone or in combination with $PM_{10}$ composition, explained about 50% of the variation in bacterial community structure (Fig. S4). Variation in environmental conditions, in turn, is mostly related to seasonality, as also indicated by CCA, where season is the factor that explained the largest fraction of variance (Table 2). Therefore, airborne microbial community structure seems to vary seasonally at all sampling sites. More in detail, CCA analysis identified wind speed and relative humidity as variables affecting airborne microbial community structure (Table 2). However, a precise identification of the environmental factors that actually affect the structure of airborne bacterial communities is challenging, since their variations are often tightly related to seasonality. In this way, the effects of each environmental factor are difficult to disentangle from those of season considered as a whole. To date, only few studies were able to identify one single meteorological factor that affected the structure of microbial communities in the atmosphere. (Maron et al 2006) (Bowers et al 2012)

Spring communities were consistently indicated as different from those of the other seasons by cluster analysis, MRT and CCA (respectively Fig. 2 and 3, Table 2). Moreover, spring samples hosted different airborne microbial communities in Milan and Venice. This is mainly due to the high abundance of *Rhodobacterales* in spring microbial communities from both Venice-Mestre and Venice-Porto Marghera. In fact, one of the five clusters highlighted by the cluster analysis (Fig. 2 and S2) as well as Group 2 identified by the MRT (Fig. 3) were entirely composed by spring samples from both Venice-Mestre and Venice-Porto Marghera and were characterized by a high abundance of *Rhodobacterales*. To the best of our knowledge, this is the first work in which high abundance of *Rhodobacterales* is reported in

air samples (Franzetti et al 2011; Bertolini et al 2013). However, the presence of this taxon is not surprising in Venice, because this city is surrounded by a lagoon. Indeed, bacteria belonging to this order are purple non-sulphur bacteria and include species that can be retrieved in both freshwater and marine environments (Fu et al 2013). In particular, abundance of *Rhodobacterales* in spring samples may be due to the seasonal temperature increase and, possibly, to concomitant algal blooms, which are the conditions that can promote the growth of this particular bacterial taxon (Gilbert et al 2012).

**Potential sources of airborne bacteria**

MRT highlighted that, in spring, samples from Milan differed from those at both Venice sampling sites, while in the other seasons variability in microbial communities of the atmosphere was lower between Milan and Venice-Porto Marghera, which are more than 200 km apart, than between Venice-Mestre and Venice-Porto Marghera, which are few kilometres apart.

To explain this pattern, we can hypothesize that sources of airborne bacteria may give a different contribution to airborne communities across seasons. Indeed, previous works suggested that soil, leaves, sea water and animal faeces are potential sources of airborne bacteria and demonstrated a temporal variability in their relative contribution (Bowers et al 2011b; Bertolini et al 2013; Bowers et al 2013). In addition, Bowers and co-workers (Bowers et al 2011a) suggested that the composition of the airborne bacterial communities could be more affected by variation in local bacterial sources than by changes in local meteorological conditions (e.g. wind direction). Hence, local sources of bacteria may possibly play a more important role in shaping airborne microbial communities than previously hypothesized. Our results partly confirmed this strong influence of local sources in shaping airborne bacterial communities, since *Rhodobacterales* were typical of spring samples from Venice-Mestre and Venice-Porto Marghera. However, we could not exclude the possible contribution of meteorological conditions in shaping airborne bacterial communities, as CCA revealed a significant effect of wind speed and relative humidity. Furthermore, the significant contribution by wind speed may indicate that dispersal and transport of bacteria play an important role in assembly processes of airborne microbial communities.

*Sphingobacteriales* and *Actinomycetales* were identified as indicator taxa of autumn samples. Bacteria belonging to these orders are typically found in soil, thus suggesting that soil is the

main source of airborne bacteria in this season. Similar results were also found in our previous study on Milan urban area (Bertolini et al 2013). Furthermore, *Chloroplasts* and *Rhizobiales* were typical of summer samples, thus suggesting that plants are an important bacterial source in this season. Conversely, the taxa that were indicated as associated with winter samples (*Flavobacteriales, Pseudomonadales, Burkholderiales,* and *Xanthomonadales*) mainly include ubiquitous microorganisms. Thus, for this season it is more difficult to clearly identify the potential sources of airborne bacteria.

Our results also suggested that the composition of the microbial community in the atmosphere was influenced not only by changes in the relative contribution of the different sources in different seasons (Bowers et al 2011a; Bowers et al 2012; Gandolfi et al 2013; Bowers et al 2013), but also by the seasonal dynamics of the microbial communities in the sources themselves. In fact, the massive presence of *Rhodobacterales* in Venice spring communities (Table 3 and Fig. 3) may indicate not only a shift in the relative contribution of lagoon water to atmospheric bacteria, but might also reflect changes in microbial populations of the lagoon water across seasons. Future researches should therefore support the study of temporal variability of airborne microbial communities with the parallel study of temporal variation in the bacterial communities at potential sources of airborne bacteria, especially those located close to sampling sites.

**Relation between PM ions and elements and microbial community structure**

Despite the recent advancement in the knowledge of the structure of airborne microbial communities, there is still a lack of information about community functions (Polymenakou 2012). A first, though indirect, insight into this topic might come from the assessment of relationships between the presence, in the atmosphere, of peculiar groups of bacteria and of particular chemical elements and ions that can be related *a posteriori* with bacterial metabolism. Variables accounting for PM composition included concentrations of a wide variety of ions and elements (see methods), several of which could potentially have a role in bacterial metabolism. However, both CCA and MRT indicated that only Ba, $SO_4^{2-}$ and $Mg^{2+}$ concentrations were significantly related to microbial community structure. Since $SO_4^{2-}$ is an essential compound for bacterial growth, its concentration might differentially affect the survival and growth of the microbial populations, thus influencing their relative abundance (Scherer and Sahm 1981), while Ba might be differentially tolerated by different bacterial

taxa. However the results from the Indicator Taxa approach, although revealing the existence of a co-variation between Ba and $SO_4^{2-}$ concentrations and some microbial taxa (Table 3), did not allow identifying a definite causal relation between some PM chemical characteristics and microbial community structure.

Variation Partitioning analysis disclosed that the PM composition *per se* explained only less than 2% of total variation in airborne bacterial communities (Fig. S4). Therefore, the independent contribution of chemical composition of PM in shaping microbial communities appears small if compared to that of environmental conditions. Moreover, 23-26% of variance of airborne bacterial community structures is explained by the shared contribution of PM composition and environmental conditions. Thus, our results did not allow disentangling the effect of PM ions and elements on microbial community structure from that of environmental (including meteorological) conditions which exhibit a clear seasonality. Furthermore, the observed seasonal shifts in the concentrations of Ba, $SO_4^{2-}$ and $Mg^{2+}$ may be ascribed also to the possible seasonal changes in the sources that release PM in the atmosphere and that can have different chemical composition. In this case, PM composition and bacterial community structure would simply co-vary due to variations in their common sources, without implying that chemical compounds determine selective pressures able to shape bacterial communities in the atmosphere. Further research is therefore needed to elucidate the seasonal variation both of PM sources and of the potential sources of airborne bacteria. In addition, detailed investigation, possibly in experimentally controlled conditions, are needed to elucidate the independent effect of chemical composition of PM, if any, on the structure of airborne bacterial communities.


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## LEGEND TO FIGURES

**Fig. 1**. Number of ribosomal operons per cubic meter of air in A) different seasons and B) different sampling sites. The solid lines represent the median value, the top and the bottom of the boxes represent the first and the third quartile while whiskers include 95% of data. Segments with asterisks denote seasons or sampling sites that differ significantly at Tuckey post-hoc test ($|z| \geq 2.414$, $P \leq 0.042$).

**Fig. 2**. Hierarchical cluster analysis of all OTUs. Numbers represent sample ID. Colour bars at the bottom evidence the city (brown = Milan, violet = Venice-Mestre, purple = Venice-Porto Marghera) or the season (green = spring, red = summer, orange = autumn, blue = winter) in which a given sample was collected. Symbols denote the clusters that are consistently identified in analyses both with all OTUs and with only abundant OTUs.

**Fig. 3.** Multivariate Regression Tree (MRT). Histograms shows bacterial community structures. Only abundant OTUs are represented.

## TABLES

**Table 1**. Airborne bacterial community structure at each season and sampling site. For brevity we report the proportional abundance of the most abundant taxa that accounted for at least 5% of the classified sequences in at least one sample. Taxa were defined on the basis of the RDP classification at the fourth taxonomic rank.

| | Milan (%) | | | | Venice-Mestre (%) | | | | Venice-Porto Marghera (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SU** | **AU** | **WI** | **SP** | **SU** | **AU** | **WI** | **SP** | **SU** | **AU** | **WI** | **SP** |
| *Burkholderiales* | 19.6 | 14.0 | 43.7 | 13.3 | 12.6 | 20.4 | 18.3 | 15.5 | 35.9 | 47.7 | 37.3 | 9.2 |
| *Rhodobacterales* | 2.4 | 2.7 | 1.4 | 2.7 | 1.8 | 3.5 | 3.1 | 24.9 | 1.1 | 1.3 | 1.6 | 28.7 |
| *Chloroplasts* | 12.6 | 6.0 | 2.4 | 28.1 | 24.8 | 16.6 | 7.7 | 10.4 | 3.7 | 5.4 | 3.5 | 20.0 |
| *Actinomycetales* | 20.8 | 26.8 | 14.6 | 15.7 | 21.0 | 19.3 | 21.1 | 14.6 | 10.4 | 13.6 | 16.2 | 11.1 |
| *Flavobacteriales* | 2.5 | 2.3 | 1.5 | 2.1 | 2.4 | 1.7 | 9.8 | 2.0 | 1.3 | 1.8 | 4.2 | 1.4 |
| *Rhizobiales* | 5.6 | 6.0 | 4.6 | 6.3 | 4.0 | 5.4 | 4.4 | 7.1 | 2.9 | 3.0 | 3.5 | 8.5 |
| *Pseudomonadales* | 3.7 | 3.3 | 6.5 | 2.1 | 2.3 | 2.6 | 3.5 | 2.1 | 1.4 | 2.1 | 2.0 | 1.4 |
| *Sphingobacteriales* | 6.3 | 6.5 | 3.3 | 3.6 | 5.6 | 4.7 | 6.0 | 3.3 | 3.3 | 3.7 | 4.7 | 3.2 |
| *Enterobacteriales* | 1.6 | 1.8 | 0.5 | 0.8 | 1.3 | 1.3 | 2.8 | 0.9 | 5.8 | 3.3 | 1.2 | 0.6 |
| *Clostridiales* | 3.5 | 4.8 | 4.2 | 7.1 | 3.8 | 3.6 | 3.3 | 3.2 | 1.4 | 2.0 | 5.2 | 2.8 |
| *Bacillales* | 3.4 | 4.6 | 2.3 | 3.1 | 4.4 | 4.5 | 5.2 | 3.3 | 4.9 | 2.6 | 3.5 | 2.5 |

**Table 2.** Canonical Correspondence Analyses of bacterial community structure based on all OTUs or only abundant OTUs in relation to variables accounting for PM composition and environmental conditions. The main effects of city and season were calculated by including in the model appropriate Helmert contrasts. N. Per. is the number of permutations used to assess significance.

| | | All OTUs | | | | | Abundant OTUs | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Effect** | **df** | **$\chi^2$** | **F** | **N. Per.** | **P** | **df** | **$\chi^2$** | **F** | **N. Per.** | **P** |
| **PM composition** | **Ba** | 1 | 0.016 | 4.917 | 199 | 0.005 | 1 | 0.015 | 5.855 | 199 | 0.005 |
| | **$SO_4^{2-}$** | 1 | 0.010 | 3.101 | 199 | 0.005 | 1 | 0.009 | 3.521 | 199 | 0.010 |
| | **$Mg^{2+}$** | 1 | 0.010 | 3.139 | 699 | 0.029 | 1 | 0.009 | 3.336 | 799 | 0.035 |
| **Environmental conditions** | **Season** | 3 | 0.107 | 11.503 | 199 | 0.005 | 3 | 0.107 | 14.692 | 199 | 0.005 |
| | **City** | 2 | 0.025 | 4.085 | 199 | 0.005 | 2 | 0.025 | 5.083 | 199 | 0.010 |
| | **Wind speed** | 1 | 0.008 | 2.508 | 499 | 0.026 | 1 | 0.008 | 3.006 | 999 | 0.031 |
| | **Relative humidity** | 1 | 0.012 | 3.847 | 199 | 0.005 | 1 | 0.011 | 4.417 | 199 | 0.005 |
| | **Season × City** | 6 | 0.123 | 6.404 | 199 | 0.005 | 6 | 0.120 | 7.836 | 199 | 0.005 |
| | | *Overall test* | | | | | *Overall test* | | | | |
| | ***Model*** | *16* | *0.391* | *7.617* | *199* | *0.005* | *16* | *0.391* | *9.563* | *199* | *0.005* |
| | ***Residual*** | *78* | *0.250* | | | | *78* | *0.199* | | | |

**Table 3.** Results from Indicator Taxa analyses identifying OTUs typical of different seasons and groups identified by the MRT analyses.

| Group | Environmental condition | Taxon | IndVal statistic | P |
|---|---|---|---|---|
| | **SEASON** | | | |
| 1 | Summer | *Rhodobacterales* | 0.861 | 0.005 |
| | | *Chloroplasts* | 0.689 | 0.005 |
| | | *Rhizobiales* | 0.601 | 0.005 |
| 2 | Autumn | *Sphingobacteriales* | 0.538 | 0.045 |
| | | *Actinomycetales* | 0.534 | 0.005 |
| 3 | Winter | *Flavobacteriales* | 0.635 | 0.005 |
| | | *Pseudomonadales* | 0.609 | 0.005 |
| | | *Burkholderiales* | 0.592 | 0.005 |
| | | *Xanthomonadales* | 0.570 | 0.035 |
| 4 | Spring | NO OTU | | |
| | **MRT Group** | | | |
| 1 | Spring in Milan | *Chloroplasts* | 0.641 | 0.005 |
| 2 | Spring in Venice-Mestre and Venice-Porto Marghera | *Rhodobacterales* | 0.838 | 0.005 |
| 3 | Other seasons in Venice-Porto Marghera with relative humidity < 89% | NO OTU | | |
| 4 | Other seasons in Venice-Porto Marghera with relative humidity ≥ 89% | *Burkholderiales* | 0.549 | 0.010 |
| 5 | Other seasons in Milan or Venice-Mestre with Ba concentration < 1021 ng/$m^3$ | *Sphingomonadales* | 0.477 | 0.030 |
| | | *Sphingobacteriales* | 0.442 | 0.050 |
| 6 | Other seasons in Milan or Venice-Mestre with Ba concentration ≥ 1021 ng $m^{-3}$ and $SO_4^{2-}$ concentration < 3556 ng $m^{-3}$ | *Actinomycetales* | 0.450 | 0.005 |
| 7 | Other seasons in Milan or Venice-Mestre with Ba concentration ≥ 1021 ng $m^{-3}$ and $SO_4^{2-}$ concentration ≥ 3556 ng $m^{-3}$ | *Pseudomonadales* | 0.562 | 0.010 |
| | | *Xanthomonadales* | 0.554 | 0.005 |

Figure 1

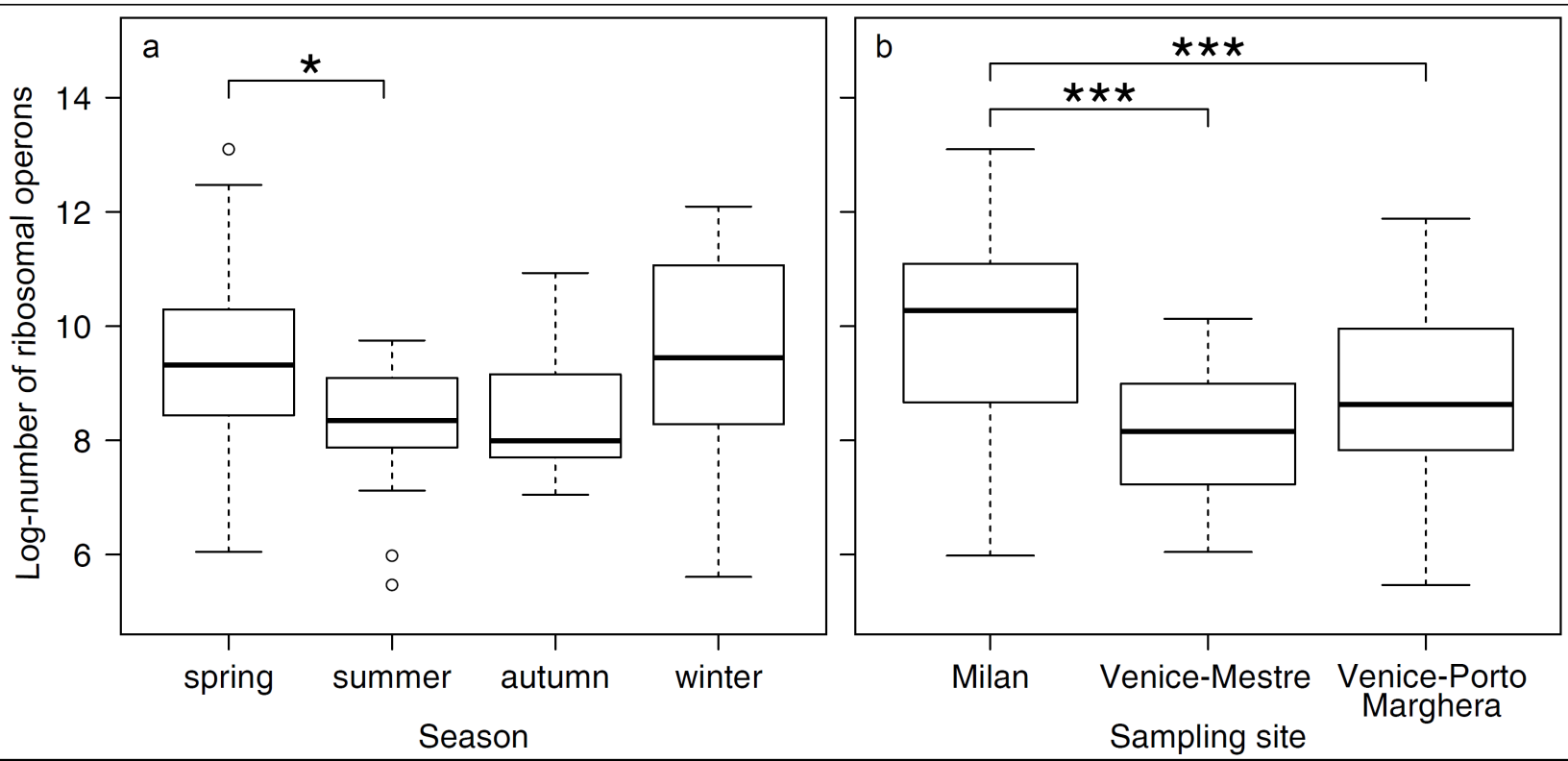

a
b
*
***
***
Log-number of ribosomal operons
14
12
10
8
6
spring
summer
autumn
winter
Season
Milan
Venice-Mestre
Venice-Porto Marghera
Sampling site

Figure 2

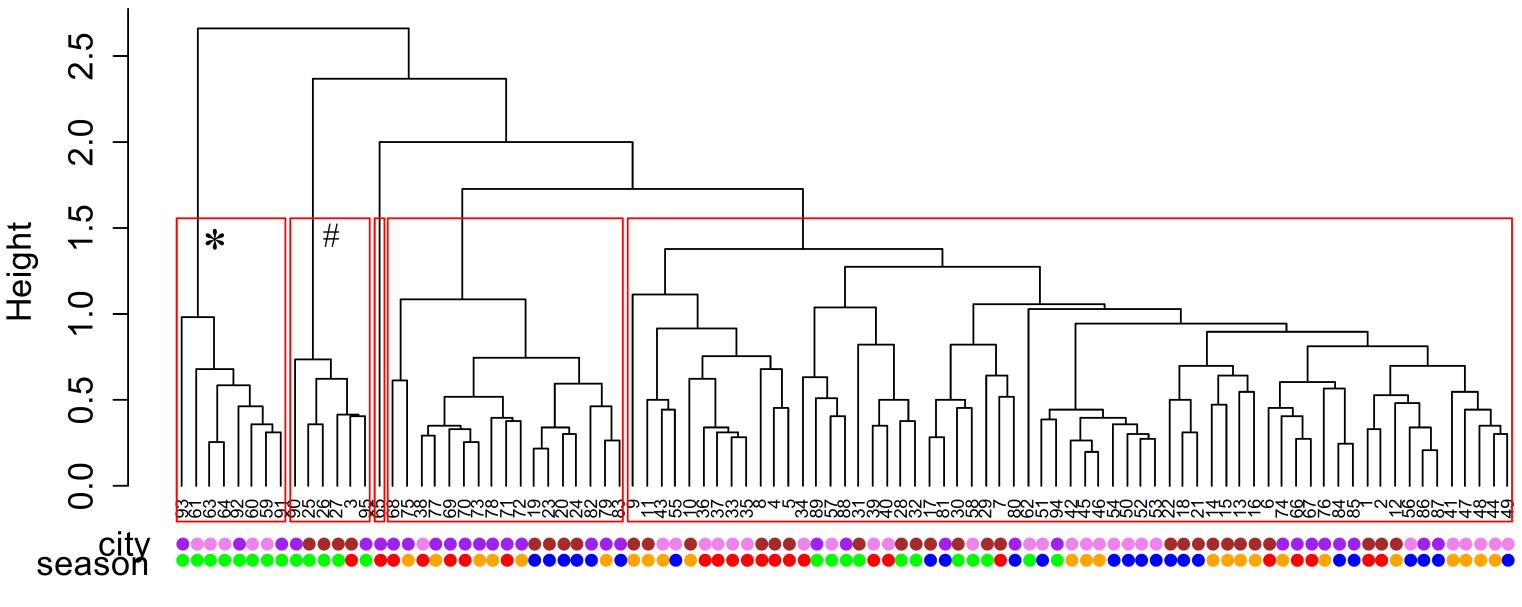
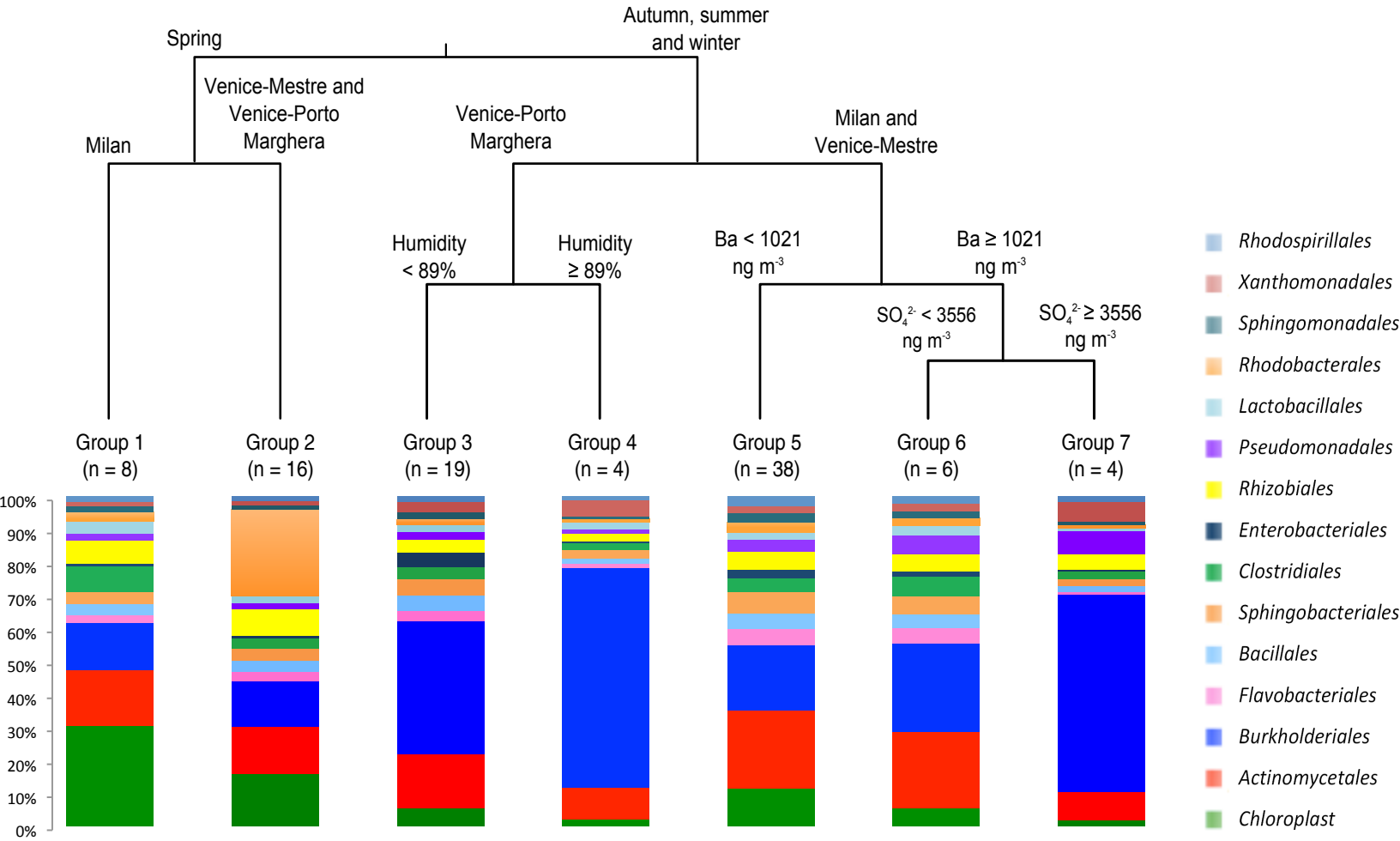


Figure 3




Error = 0.33; CV Error = 0.41; SE = 0.054

Supplementary Material

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