Temporal variability and effect of environmental variables on airborne bacterial communities in an urban area of Northern Italy

Valentina Bertolini, Isabella Gandolfi, Roberto Ambrosini, Giuseppina Bestetti, Elena Innocente, Giancarlo Rampazzo, et al.

Applied Microbiology and Biotechnology

ISSN 0175-7598
Volume 97
Number 14

Appl Microbiol Biotechnol (2013)
97:6561-6570
DOI 10.1007/s00253-012-4450-0

Springer

Appl Microbiol Biotechnol (2013) 97:6561–6570
DOI 10.1007/s00253-012-4450-0

ENVIRONMENTAL BIOTECHNOLOGY

Temporal variability and effect of environmental variables on airborne bacterial communities in an urban area of Northern Italy

Valentina Bertolini · Isabella Gandolfi · Roberto Ambrosini · Giuseppina Bestetti · Elena Innocente · Giancarlo Rampazzo · Andrea Franzetti

Received: 16 August 2012 / Revised: 14 September 2012 / Accepted: 17 September 2012 / Published online: 5 October 2012

Abstract Despite airborne microorganisms representing a relevant fraction of atmospheric suspended particles, only a small amount of information is currently available on their abundance and diversity and very few studies have investigated the environmental factors influencing the structure of airborne bacterial communities. In this work, we used quantitative PCR and Illumina technology to provide a thorough description of airborne bacterial communities in the urban area of Milan (Italy). Forty samples were collected in 10-day sampling sessions, with one session per season. The mean bacterial abundance was about 10^4 ribosomal operons per m^3 of air and was lower in winter than in the other seasons. Communities were dominated by *Actinobacteridae*, *Clostridiales*, *Sphingobacteriales* and few proteobacterial orders (*Burkholderiales*, *Rhizobiales*, *Sphingomonadales* and *Pseudomonadales*). *Chloroplasts* were abundant in all samples. A higher abundance of *Actinobacteridae*, which are typical soil-inhabiting bacteria, and a lower abundance of chloroplasts in samples collected on cold days were observed. The variation in community composition observed within seasons was comparable to that observed between seasons, thus suggesting that airborne bacterial communities show large temporal variability, even between consecutive days. The structure of airborne bacterial communities therefore suggests that soil and plants are the sources which contribute most to the airborne communities of Milan atmosphere, but the structure of the bacterial community seems to depend mainly on the source of bacteria that predominates in a given period of time.

Keywords 16S rRNA · Airborne bacteria · Community structure · Illumina sequencing · Multivariate regression trees

Electronic supplementary material The online version of this article (doi:10.1007/s00253-012-4450-0) contains supplementary material, which is available to authorized users.

V. Bertolini · I. Gandolfi (✉) · G. Bestetti · A. Franzetti
POLARIS Research Centre, Department of Environmental Sciences (DISAT), University of Milano-Bicocca,
Piazza della Scienza 1,
20126 Milan, Italy
e-mail: isabella.gandolfi@unimib.it

R. Ambrosini
Department of Biotechnology and Biosciences,
University of Milano-Bicocca,
Piazza della Scienza 2,
20126 Milan, Italy

E. Innocente · G. Rampazzo
Department of Environmental Sciences, Informatics and Statistics,
University of Ca' Foscari,
Dorsoduro 2137,
30123 Venice, Italy

Introduction

Airborne particulate matter (PM) is a complex and dynamic mixture of components having different origins, both inorganic and biological, as well as different chemical and physical properties (Putaud et al. 2004). In the last decade, a large number of studies have investigated the risks that PM poses both to human health and ecosystem functions (Samet et al. 2006), but most of these studies have typically focused on the non-biological fraction of suspended particles (Colbeck and Lazaridis 2009). In recent years, interest has grown also in the biological fraction of PM (bioaerosols) (Jones and Harrison 2004), boosted by an increasing amount of evidence suggesting that this fraction may play a critical role in the effects of PM on biological systems (D'Amato 2002; Finnerty et al. 2007; Jaenicke 2005; Moorman et al. 2011). Bioaerosols have negative effects on human health and ecosystem functioning, by determining, for example, tissue inflammation (Camatini et al. 2010) and the spread of plant, livestock and human pathogens (Hirano and Upper 1983; Pillai and Ricke 2002). Such effects are exacerbated

in urban areas, where the atmosphere is enriched in PM. Interest has therefore grown in the study of atmospheric pollution of urban areas, and recent studies have provided evidence for a larger impact of urban air pollution on human health than previously hypothesised (Gouveia and Maisonet 2006).

Bioaerosols have a diverse composition. Fungal spores, pollens and dust mite allergens have been studied in detail (Beggs and Kerr 2000), while the diversity of airborne bacteria and their potential sources remain poorly investigated (Bowers et al. 2009, 2011; Maron et al. 2005, 2006; Womack et al. 2010). In addition, it has only recently been acknowledged that both the potential sources of PM and the possible dynamics of bacterial populations in the atmosphere may influence the spatial and temporal variation in airborne bacterial community structure (Franzetti et al. 2010). Therefore, there is a deep gap in the knowledge of the structure and dynamics of airborne bacterial communities and in the ecological factors that drive their structure. It is also worth noting that it is even unclear whether airborne bacteria are an actual ecological community, with the different populations growing and interacting in the atmosphere, or if they are simply a pool of organisms passively gathered together from different sources (Bowers et al. 2011). For this reason, in this paper, the term "bacterial community" is used to refer to the ensemble of bacterial groups present in the atmosphere, without necessarily implying the existence of dynamics and interactions between bacterial populations in this environment. Besides bacteria, PM of vegetable origin represents a considerable fraction of bioaerosols. *Chloroplasts* are revealed by the same analytical tools used for the analyses of airborne bacterial communities (e.g. massive parallel sequencing, see below), and their abundance provides information on the abundance of PM of vegetable origin. Plants, on the other hand, are one of the main sources of airborne bacteria, so that the relative abundance of chloroplast sequences among bacterial ones may provide information on the potential sources of airborne bacteria (Brodie et al. 2007). For this reason, in the present work, chloroplast abundance was always included in the analyses, and the term "bacterial community" was used to refer in short to the ensemble of bacteria and chloroplasts observed in a sample of PM, despite that a portion of the community that was analysed was not bacteria.

Very little information is available regarding the total amount of bacteria present in the atmosphere at a given place and time because this datum is technically difficult to determine, mainly due to limitations of microscopy techniques (Bowers et al. 2010, 2011). Culture-based microbiological methods have been the standard investigation procedure for a long time (Fang et al. 2007; Lighthart and Shaffer 1995a, b), despite the fact that it was well-recognised that they can capture only a small portion of the total microbial diversity (Pace 1997; Peccia and Hernandez 2006). As a result, the number of studies which have applied culture-independent techniques to the study of airborne bacteria is still limited. For example, such methods were used to assess the diversity of bacteria associated with small-sized particles during dust events (Polymenakou et al. 2008) and to characterise the spatial and temporal variations of bioaerosols in different geographical localities and/or different land uses (Angenent et al. 2005; Bowers et al. 2009, 2010, 2011; Brodie et al. 2007; Franzetti et al. 2010; Frohlich-Nowoisky et al. 2009). However, the potential of culture-independent molecular methods, such as quantitative PCR (qPCR), rRNA gene sequencing and hybridisation (i.e. PhyloChip) and massive parallel sequencing technologies, for quantifying and describing the total diversity and composition of microbial communities has not yet been fully exploited (Bowers et al. 2010; Rinsoz et al. 2008). Among these methods, the Illumina technology can reveal unprecedented diversity from even the most complex microbial environments (Claesson et al. 2010), and it is therefore a promising tool to investigate airborne bacterial communities and to contribute filling the gaps in the current knowledge of their structure and dynamics.

In this work, the Illumina technology and qPCR were used to provide a thorough description of airborne microbial communities in the urban area of Milan (Northern Italy). Samples were taken in 10-day sessions equally distributed across the different seasons in order to describe both short- and long-term variations in bacterial community structure and composition. The aims of the present study are therefore threefold: (1) to deeply characterise the abundance and diversity of airborne microbial populations in this urban area, (2) to disentangle short-term (days) and seasonal variation of microbial communities and (3) to identify the potential sources of the bacteria present in the atmosphere of this urban area.

Materials and methods

Sample collection and DNA extraction

Total suspended particulate matter (TSP) was sampled on quartz fibre filters (Whatman, Maidstone, England) with a high-volume sampler (ECHO HiVol, TCR TECORA, Milan, Italy) and a flux speed of 250 $L min^{-1}$. The sampling campaigns were carried out in 2010 in 10-day periods in the four seasons as follows: 22 February–4 March, 24 May–3 June, 23 August–3 September, and 24 November–4 December. Each collection period lasted 24 h. The sampler was located in Milan, Italy (45°31′20″ N, 9°12′45″ E), in a typical urban area in the northern part of the city near a high-traffic road. The characteristics of the sampling site have been previously described (Gandolfi et al. 2011). The sampler was placed approximately 20 m from the nearest

roads and 50 m from the nearest traffic lights. TSP sampling was performed at approximately 1.5 m from the ground.

Total bacterial DNA was extracted directly from a quarter of each filter using the FastDNA Spin for Soil Kit (MP Biomedicals, Solon, OH, USA). Individual filter portions were cut into small pieces and loaded into the bead tube of the DNA extraction kit, after adding 1 M $CaCO_3$ in order to increase the pH, and then shaken at 200 rpm for 60 min. The remaining steps of the DNA extraction were performed according to the manufacturer's instructions.

Quantitative PCR

The abundance of airborne bacteria was estimated by the quantification of the number of copies of the gene 16S rRNA. This parameter is not directly related to cell number due to the presence of multiple ribosomal operons in the bacterial genomes; however, it can be used to look at relative shifts in microbial biomass as the bias in copy number is likely constant across samples. A 466-bp fragment of the bacterial 16S rDNA (331–797 according to *Escherichia coli* position, V3 and V4 hypervariable regions) was PCR-amplified with a universal primer set (Nadkarni et al. 2002). The PCR was performed in a total volume of 20 μL using the FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 μM (final concentration) forward and reverse primers. One microlitre out of the 85 μL extracted from the filter was used as the template. The amplification was carried out under the following conditions: 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, with acquisition of the fluorescence at the end of each 72 °C elongation step. Genomic bacterial DNA used for standard concentration curves was extracted from pure cultures of *E. coli* K-12 substr. DH10B. The strain contains seven copies of the gene 16S rRNA (accession number: NC_010473) (Durfee et al. 2008). The amount of extracted standard DNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The standards and the samples were included in triplicate in each run.

16S rRNA fragment libraries by Illumina Genome Analyser IIx

The V5–V6 hypervariable regions of the 16S rRNA gene were PCR-amplified for Genome Analyser IIx sequencing. The PCR was performed in 3× 75-μL volume reactions with @Taq® Hot start Taq polymerase (Euroclone, Pero, Italy), 4 mM $MgCl_2$, 0.2 mM dNTPs mix and 1 μM of each primer. The 783F and 1046R primers were used (Huber et al. 2007; Wang and Qian 2009) and the cycling conditions were as follows: initial denaturation at 94 °C for 5 min; 29 cycles at 94 °C for 50 s, 47 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. At the 5′ end of the 783F primer, one of eight 6-bp barcodes was also included to allow sample pooling and subsequent sequence sorting (see below and Table S1). The amplified products of 288 bp were purified with the Wizard SV PCR Purification Kit (Promega Corporation, Madison, WI, USA) and DNA quantity and purity was evaluated spectrophotometrically using NanoDrop™ (Thermo Scientific, USA). Multiplexed samples were prepared with the Illumina Multiplexing Sample Preparation Oligonucleotide Kit, which provides 12 index oligos for pooling up to 12 samples per lane. Therefore, our customised tagging system with eight barcodes at the 5′ end of the 783F primer allowed pooling up to 96 samples per lane. Sequencing of all the pooled samples was performed on a single Illumina GA-IIx lane, using a paired-end 76-bp protocol and the 4.0 sequencing chemistry. Illumina Real Time Analysis software version 1.8.7 was used to perform the cluster extraction and base-calling processing analyses. The Illumina GA-IIx sequencing was carried out at the Department of Clinical and Preventive Medicine, University of Milano-Bicocca, Italy.

Sequence analyses

Each sequence was assigned to its original sample according to its index oligo and barcode. After sorting, the reverse read of each paired-end sequence was reverse-complemented and merged with the corresponding forward read, inserting 20 Ns in between (Claesson et al. 2010). A quality cutoff was applied in order to remove sequences (1) that did not contain the barcode and (2) with an average base quality value (Q) lower than 30. The barcode was removed from sequences before further processing. The source code used for sequence processing is available upon request to the authors.

The taxonomic attribution of filtered sequences was carried out using the stand-alone version of RDP Bayesian Classifier, using a 50 % confidence level as suggested for sequences shorter than 200 bp (Claesson et al. 2009; Wang et al. 2007). Operational taxonomic units (OTUs) were defined by the RDP classifier, considering the fourth taxonomic level, which in most cases corresponded to Order. Recent papers demonstrated that short sequences of hypervariable regions of 16S rRNA gene provide enough variability to capture the differences in the structure of bacterial communities (Caporaso et al. 2012; Claesson et al. 2009; Jeraldo et al. 2011).

Environmental factors

Meteorological data and particulate matter concentration

Daily average temperature, wind speed at ground level and relative humidity for Milan in the nearest meteorological station in the city, as well as the concentrations of PM smaller than 10 μm (PM10) and smaller than 2.5 μm (PM2.5) in three

different urban stations during sampling periods were retrieved from the Agenzia Regionale per la Protezione dell'Ambiente (Regional Agency of Environmental Protection, ARPA Lombardia) and are available at http://ita.arpalombardia.it/meteo/dati/richiesta.asp.

Analysis of inorganic ions

A portion of the filter used to collect PM was dissolved in MilliQ® water in an ultrasonic bath and analysed by ion chromatography (Dionex DX500 system) to determine the water-soluble inorganic ion concentration (Na^+, NH_4^+, Mg^{2+}, Ca^{2+}, F^-, Cl^-, NO_3^- and SO_4^{2-}) as described by Squizzato et al. (2012).

Statistical analyses

The concentrations of PM10 measured on the same day at three different weather stations in Milan were highly repeatable (r=0.953, $F_{39,72}$=57.70, P<0.001) and were therefore averaged within each day. Missing data on the concentration of PM2.5 were imputed by regressing PM2.5 concentrations on PM10 concentrations (see SI Section 1.1.1 for further details).

A hierarchical cluster analysis of the relative abundance of OTUs was performed with the HCLUST procedure in R 2.8.1 (R Development Core Team 2008) with the complete linkage method on the Hellinger distance between sites. This distance depends on the difference in the proportion of OTUs between samples, decreases the importance of OTU abundance over occurrence and avoids the double-zero problem when comparing species composition between samples (De Cáceres et al. 2010; Legendre and Legendre 1998).

Principal component analysis (PCA) on Hellinger-transformed community data (Legendre and Gallagher 2001) was used to visualise the changes in airborne bacterial community among seasons. Similarly, changes in meteorological data and ion and PM concentrations in the atmosphere (hereafter "air and PM factors" for brevity) were visualised by means of PCA on normalised variables. PCA analyses were performed with the BIODIVERSITYR package (Kindt and Coe 2005) in R.

The analysis of similarity (ANOSIM) was used to assess the difference in OTU composition between samples collected in different seasons. Pairwise ANOSIM was used to investigate differences in airborne microbial communities between seasons. P values of pairwise ANOSIM were corrected according to the Bonferroni procedure to account for multiple statistical tests. Similarly, differences in air and PM factors between seasons were assessed with ANOSIM tests on the Euclidean distance between normalised variables. ANOSIM was performed with the VEGAN package (Oksanen et al. 2009) in R. See SI Section 1.1.2 for further information about ANOSIM.

Hellinger-transformed OTU relative abundances were related to air and PM factors by multivariate regression tree (MRT) analysis using the MVPART package (Therneau et al. 2007) in R. The best tree was chosen as the smallest tree whose cross-validated relative error (CVRE) was within 1 standard error of the CVRE of the best tree (Borchard et al. 2011). The difference in OTU relative abundance among groups identified by the MRT analysis was tested by ANOVA. P values were corrected for multiple testing according to the false discovery rate (FDR) procedure (Benjamini and Yekutieli 2001) using the MULTTEST package in R.

Short-term variation in the structure of bacterial communities and in air and PM factors was investigated on one hand by Mantel tests and Mantel correlograms between the matrix of the Hellinger distances of bacterial communities or by the matrix of the Euclidean distances between normalised air and PM factors, and on the other hand by the difference in days between samples taken during each 10-day period (see SI Section 1.1.3 for further details). Goodness-of-fit Mantel tests (sensu Legendre and Legendre 1998) were used to compare variation in bacterial community structure among samples taken in the same season (within-season variation) and among samples taken in different seasons (between-season variation). Mantel tests were performed using the ECODIST package (Goslee and Urban 2007) in R, and P values were corrected according to the FDR procedure.

Sequence data

De-multiplexed fastq-formatted DNA sequences are available on Sequence Read Archive, with the study accession number ERP001381 (http://www.ebi.ac.uk/ena/data/view/ERP001381).

Results

Quantification of airborne bacteria

The mean number of copies of the 16S rRNA gene, which was used here as a measure of microbial biomass (see the "Materials and methods"), was 1.7 (0.3 SE)×10^5 ribosomal operons per m^3 of sampled air. An ANOVA model disclosed significant differences in microbial biomass among seasons ($F_{3,26}$=5.11, P=0.005; Fig. 1), and a post hoc test (Tukey method) revealed that winter samples contained significantly less bacterial biomass than spring (t_{36}=−2.91, P=0.030) and summer (t_{36}=−3.70, P=0.004) samples.

Microbial community structures

A total of 271,587 sequences that passed the quality filter were obtained from sequencing. The number of sequences of each sample ranged from 765 to 26,187. A total of 107

OTUs were detected across the 40 collected samples. Among them, 16 OTUs represented more than 1 % of the total number of the obtained sequences in at least one sample (most abundant OTUs). In each sample, 26.7 to 39.0 % of the sequences could not be classified and were discarded from subsequent analyses.

The taxonomic classification of bacteria based on the fourth taxonomic level is shown in Fig. 2. Each community was dominated by few taxa throughout the year, with a marked prevalence of *Actinobacteridae*, *Clostridiales*, *Sphingobacteriales* and a few proteobacterial orders (*Burkholderiales*, *Rhizobiales*, *Sphingomonadales* and *Pseudomonadales*). A relevant fraction of chloroplasts were also detected in all samples.

On average, there was no significant difference either in the number of OTUs detected in different seasons ($F_{3,36}$= 0.05, P=0.99) or in the Shannon–Weaver diversity index calculated from the OTU number detected at each sample ($F_{3,36}$=1.41, P=0.26) (Table 1).

The clustering analysis based on the relative abundance of the OTUs showed two main clusters (Fig. S1). Qualitatively similar results could be obtained from an analysis restricted to the most abundant OTUs (Fig. S2). The OTU clustering separated the spring (cluster A—Fig. S2) and autumn samples (cluster B—Fig. S2) into different clusters, while the winter and summer samples were scattered in the two different clusters.

On the PCA plot, the points representing the microbial communities of autumn and spring samples formed clearly separated clusters, whereas those of winter and summer samples largely overlapped with each other and with both the clusters of autumn and spring samples (Fig. 3a). Almost identical results were obtained from the PCA restricted to the most abundant OTUs (Fig. S3).

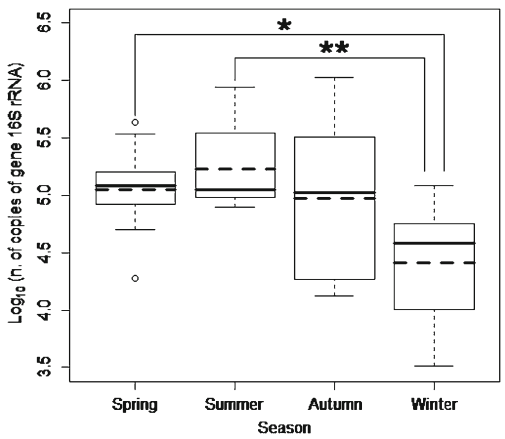

Fig. 1 Box plot diagram of the TSP-associated number of copies of gene 16S rRNA, obtained by qPCR in the four seasons. *Solid lines within boxes* represent median values; *dashed lines* mean values. *Lines with asterisks* highlight significant difference between seasons (*P<0.05; **P<0.01)

The ANOSIM showed overlapping, albeit significantly different, microbial communities between the four seasons, both in the analysis including all of the OTUs (global R= 0.255, P<0.001) and in that restricted to the most abundant OTUs (global R=0.283, P<0.001). Pairwise tests showed that autumn communities were different, albeit with some overlap from spring communities (R=0.669, P_{Bonf}=0.001), and overlap but were still separable, from summer and winter communities (R≥0.361, P_{Bonf}≤0.002). Conversely, spring, summer and winter communities were practically inseparable (R≤0.116, P_{Bonf}≥0.284). Identical results were obtained in post hoc tests conducted on the analysis restricted to the most abundant OTUs (details not shown).

In general, all physical characteristics of air and PM differed between seasons (Table 1). The PCA plot based on standardised air and PM factors showed that the autumn and winter samples were well separated from the spring and summer samples. This interpretation was supported by ANOSIM (R=0.692, P=0.001). In fact, no significant difference in air and PM factors was found between the winter and autumn samples (R=0.252, P_{Bonf}=0.056) or between the spring and summer ones (R=0.153, P_{Bonf}=0.256). All other comparisons showed clearly separated groups (R≥ 0.718, P_{Bonf}≤0.001).

MRT analysis showed that airborne microbial communities were separated mainly by the average daily temperatures. Samples collected on days with an average temperature <+6.32 °C formed a separate cluster with respect to those collected on days with temperature ≥+6.32 °C. This classification separated all of the autumn samples and three of the winter samples (1, 2 and 7; Table S2) from the others. The MRT analysis restricted to the most abundant OTUs consistently showed separation of samples according to a mean daily temperature higher or lower than +6.32 °C (Figs. 4 and S4).

In particular, the abundance of two OTUs significantly differed between samples collected on days with temperature higher or lower than +6.32 °C (P_{FDR}=0.008, both in the analysis conducted on all of the OTUs and in that restricted to the most abundant ones): *Actinobacteridae* were significantly more abundant in samples collected on cold days, while chloroplasts were significantly more abundant in samples collected on warm days (Fig. 5).

Short-term temporal variation of community structure

Mantel tests revealed no significant correlation of Hellinger distances between communities and the number of days between samplings within the same season (r_M≤0.215, P_{FDR}≥0.199). Similarly, Mantel correlograms disclosed no significant temporal autocorrelation between samples taken at different time lags within the same season ($|r_M|$≤0.441, P_{FDR}≥0.067). The same analyses conducted for Euclidean

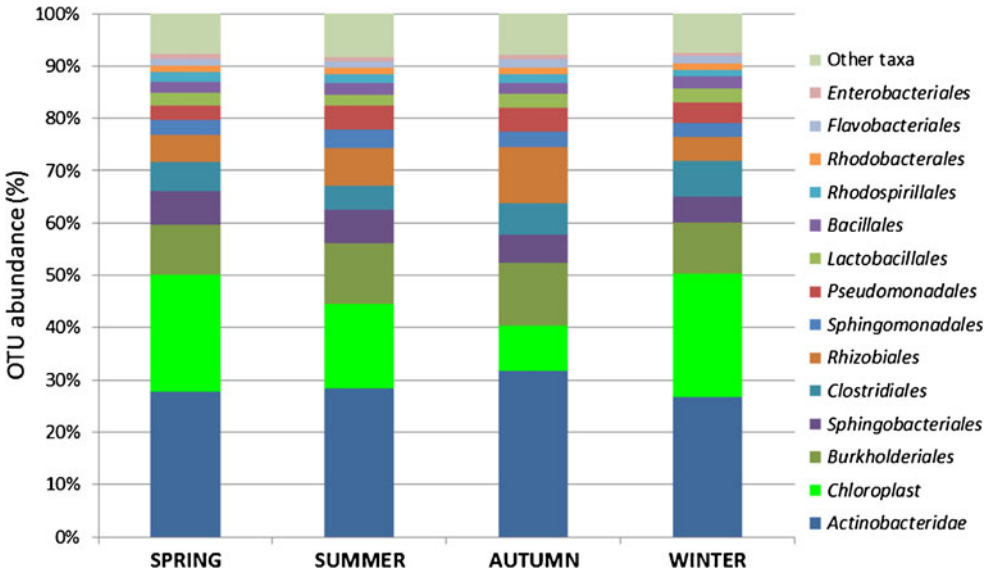

Fig. 2 Taxonomic classification of the sequences using an RDP Bayesian classifier (50 % confidence) at the fourth taxonomic level. Unclassified sequences were omitted from the figure

distances between normalised air and PM factors revealed no significant correlation with the number of days between samplings ($r_M \leq 0.197$, $P_{FDR} \geq 0.223$) nor significant temporal autocorrelation at any time lag ($|r_M| \leq 0.367$, $P_{FDR} \geq 0.275$).

A goodness-of-fit Mantel test indicated that bacterial communities within a single season were significantly more similar (less distant) than those sampled in different seasons ($r_M = -0.206$, $P < 0.001$). In particular, goodness-of-fit Mantel tests comparing the distance between communities sampled within each season and those sampled in all the others indicated that significance of the overall test arose because summer communities were significantly more similar to each other than to those sampled in the other seasons (summer: $r_M = -0.225$, $P_{FDR} < 0.001$). Conversely, communities sampled during the other seasons were equally dissimilar to those collected during the same or in other seasons ($|r_M| \leq 0.123$, $P_{FDR} \geq 0.119$).

Discussion

Seasonal variability of airborne bacterial abundance

In this study, we determined the abundance of airborne bacteria in an urban area of Northern Italy across the four seasons by a culture-independent technique, namely qPCR. The observed values ranged from 3.3×10^3 to 1.0×10^6 ribosomal operons per m^3 (Fig. 1), with a significantly lower mean abundance in winter than in spring and summer. These values are much larger than those reported in the only other

Table 1 Mean (SE) value of the quantitative environmental variables in the different seasons

Variable	Winter	Spring	Summer	Autumn
No. of OTUs	52.0 (4.68)	51.8 (4.26)	50.0 (4.31)	50.8 (4.10)
Shannon–Weaver diversity index	2.30 (0.05)	2.31 (0.06)	2.40 (0.04)	2.39 (0.03)
Daily average temperature (°C)	7.57 (0.56)	20.21 (0.52)	21.81 (0.87)	2.33 (0.38)
Wind speed (ms^{-1})	1.51 (0.16)	2.28 (0.28)	2.22 (0.36)	1.38 (0.11)
Relative humidity (%)	83.14 (4.16)	56.15 (5.16)	61.66 (5.4)	88.85 (2.97)
PM_{10} concentration (μgm^{-3})	45.03 (6.87)	25.58 (2.94)	22.18 (2.29)	38.48 (3.96)
$PM_{2.5}$ concentration (μgm^{-3})	33.88 (6.53)[a]	13.80 (2.48)	9.60 (1.47)	22.00 (2.73)
F^- concentration (μgm^{-3})	0.24 (0.16)	0.06 (0.02)	0.03 (0.01)	0.07 (0.02)
Cl^- concentration (μgm^{-3})	0.63 (0.09)	0.11 (0.05)	0.24 (0.08)	1.07 (0.31)
NO_3^- concentration (μgm^{-3})	11.43 (2.37)	9.03 (2.65)	1.28 (0.2)	10.02 (3.8)
SO_4^{2-} concentration (μgm^{-3})	1.46 (0.19)	2.29 (0.46)	2.21 (0.57)	1.56 (0.21)
Na^+ concentration (μgm^{-3})	0.43 (0.03)	0.49 (0.1)	0.58 (0.09)	0.92 (0.29)
NH_4^+ concentration (μgm^{-3})	3.85 (0.60)	2.11 (0.69)	1.00 (0.30)	2.23 (0.48)
K^+ concentration (μgm^{-3})	0.30 (0.04)	0.16 (0.02)	0.13 (0.01)	0.37 (0.06)
Mg^{2+} concentration (μgm^{-3})	0.21 (0.04)	0.15 (0.02)	0.18 (0.02)	0.12 (0.03)
Ca^{2+} concentration (μgm^{-3})	2.66 (0.45)	2.16 (0.26)	2.43 (0.15)	1.94 (0.49)

[a]Two missing data

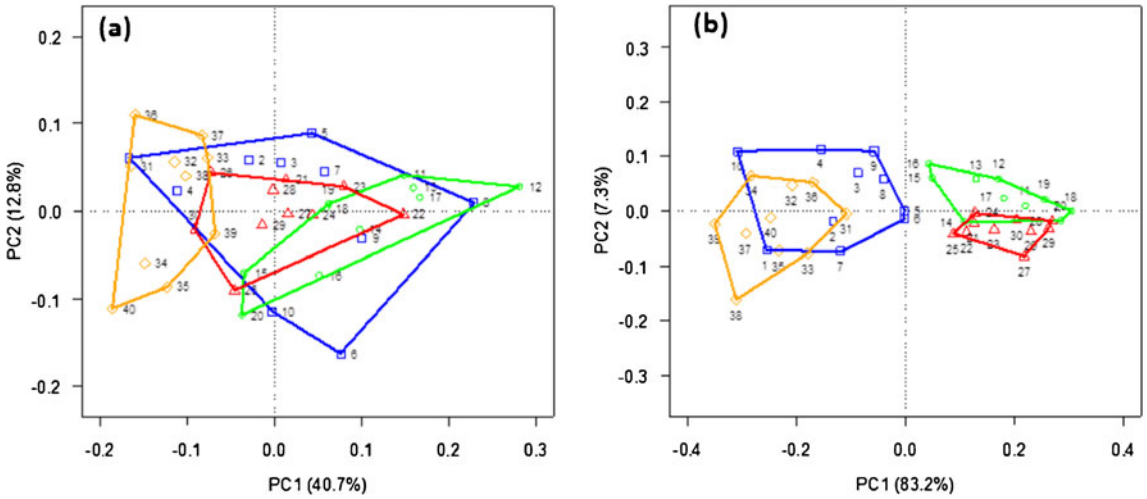

Fig. 3 Two-dimensional PCA plot of **a** microbial communities and **b** air and PM factors at different seasons (*squares and blue line* winter, *circles and green line* spring, *triangles and red line* summer, *diamonds and orange line* autumn). The percentage of variance explained by each axis is shown

study that used qPCR technique on the same PM dimensional fraction by Lee et al. (2010) in Seoul (South Korea) and in previous culture-based reports from urban areas (Bovallius et al. 1978; Fang et al. 2007). However, these latter values may have been underestimated due to cultivation biases (Peccia and Hernandez 2006). In fact, higher values of bacterial concentration were obtained in more recent studies that applied culture-independent methods (Bowers et al. 2009, 2010, 2011; Lee et al. 2010; Maron et al. 2005). However, the limited number of these studies, the use of different techniques (qPCR or direct microscopic count) and the different PM fractions investigated hampered direct comparisons with the results from the present study. In addition, in those studies, no

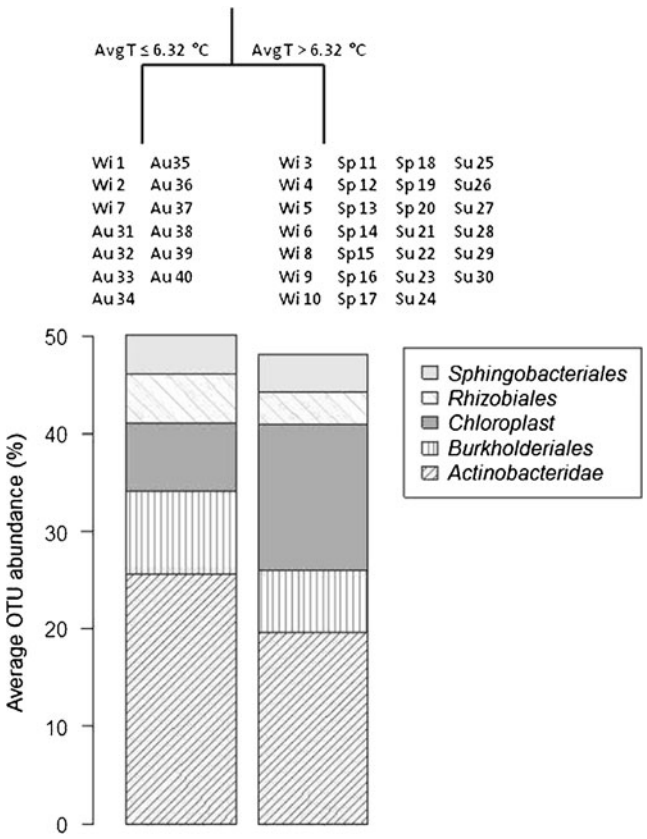

Fig. 4 MRT analysis of OTU abundance and air and PM factors. *Bar plots* show the average abundance of the five most common OTUs in samples, which accounts for more than 3 % of sequences, collected on days with average temperature lower (*left bar*) or higher (*right bar*) than +6.32 °C. Bar plots showing the average abundance of all the common OTUs are shown in Fig. S3

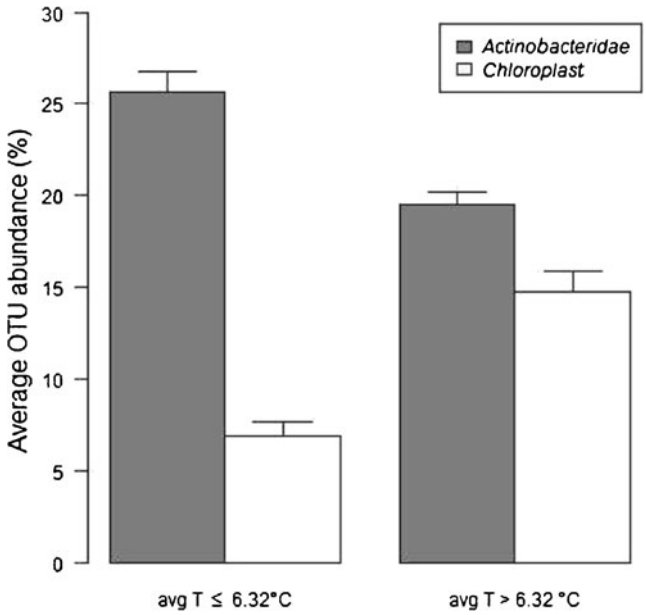

Fig. 5 Average relative abundance of the OTUs that significantly differed between the clusters of samples identified by MRT analysis. *Bars* represent standard errors

relationship between bacterial abundance, season and general features of the investigated sites was observed. Only recently, Bowers et al. (2011) observed a significantly higher abundance of bacteria in summer than in winter for PM2.5 in Detroit and Cleveland (USA), where the annual variation in temperature is generally similar to that in Milan. It could be speculated that the lower abundance of bacteria in winter might therefore be due to a reduced release from plant, soil and water sources, and to climatic conditions that are less favourable for bacterial growth during the winter season both in Milan and in the US Midwestern cities.

Seasonal and short-term variability of community structure

Differences were found in the structure of bacterial communities sampled across different seasons. In particular, microbial communities were similar during the winter, spring and summer, while the autumn communities were different from those observed in the other seasons. In fact, the ANOSIM clearly separated the communities of samples collected in the coldest period (autumn, 24 November–4 December) from those of other seasons. In addition, MRT analysis disclosed a significant difference in the structure of microbial communities sampled on days when the mean temperature was above or below +6.32 °C, with more *Actinobacteridae* and less chloroplasts in samples collected on days colder than +6.32 °C. It is worth noting that all of the autumn days in this dataset had a lower mean temperature than +6.32 °C. These findings are in agreement with the results of most of the previous studies conducted in different parts of the world, which consistently showed seasonal variability in bacterial communities (Bowers et al. 2010, 2011; Brodie et al. 2007; Fierer et al. 2008; Polymenakou et al. 2008). However, very few studies were able to identify a single meteorological factor that affects the structure of microbial communities (see, e.g. Brodie et al. 2007; Maron et al. 2006). This suggests that the seasonal variability in bacterial communities is probably due to the combined effects of differences in several meteorological factors, the chemical composition of particulate matter and the relative importance of the main sources of the air particles, rather than to temperature per se.

Over a shorter timescale, no indication of temporal autocorrelation was found in the structure of bacterial communities. In addition, only summer communities differed from each other less than those collected in other seasons, while communities sampled during the other seasons were equally dissimilar to those collected during the same or in other seasons. The similar structure of summer communities might be due to the stability of the air and PM factors in this season, which also appears to be true from the PCA plot. Indeed, in this plot, which explained more than 90 % of the total variance, summer samples were closer to each other than those of the other seasons (Fig. 3b). The large variation in bacterial community composition even within a few days observed in the other season is, however, not surprising, as previous studies reported an even larger variability both intra-day and intra-week (Fierer et al. 2008; Maron et al. 2006). Airborne bacterial communities seem therefore to be influenced mainly by the predominant source of bacteria rather than by an intrinsic ecological dynamic, as they do not seem to show an ecological succession with time. Alternatively, temporal variations in community structure, if any, may occur over longer time periods than the 10 days investigated in the present study.

Potential sources of bacteria and differences in the structure of bacterial communities associated with different PM size fractions

The observed seasonal differences in bacterial communities and a general knowledge of the ecology of the dominant bacterial taxa allowed speculation about the relative importance of different potential sources of airborne bacteria in different seasons (Fierer et al. 2008; Franzetti et al. 2010). Plants are probably the main source of bacteria found in the atmosphere in warm seasons, as suggested by the abundance of plant-associated bacteria, such as *Sphingomonadales* and *Rhizobiales*, and the high number of chloroplast sequences retrieved in samples collected in warm periods. Conversely, soil-inhabiting bacteria, such as *Actinobacteriales*, *Pseudomonadales* and *Burkholderiales*, prevailed in the atmosphere during cold seasons.

The observed variability in the composition of bacterial communities was lower than that reported in a previous study on the finer PM fractions (Franzetti et al. 2010). In fact, a strong seasonal shift in bacterial community composition had been observed on PM10 and PM2.5 in the same sampling location in Milan (Franzetti et al. 2010). In the present study, which in contrast is focused on TSP, the seasonal differences were less exacerbated, thus suggesting that airborne bacterial communities associated with different dimensional fractions of PM may differ. In fact, in the present study, which was focused on TSP, *Actinobacteridae* were the most abundant taxon and *Burkholderiales* were among the dominant taxa throughout the whole year (Fig. 2). On the contrary, in the previous study, which focused on PM10 and PM2.5, *Actinobacteridae* were the dominant taxon only in the winter and were observed at a very low abundance in the summer. In addition, only a few *Burkholderiales* were found in both seasons, while *Sphingomonadales*, *Sphingobacteriales* and *Rhizobiales* were the most abundant taxa in summer. Hence, analyses of TSP and finer PM fractions (<10 μm) showed different seasonal patterns of variation in the most abundant bacterial taxa. This suggests that the bacteria associated with particles larger than 10 μm, which are usually excluded from the analyses in all the works that consider PM10 only, significantly contribute to the structure of the entire airborne bacterial community. *Sphingomonadales*

and *Rhizobiales* are typical plant-associated bacteria, while *Actinobacteridae* and *Burkholderiales* are typically found in soil. It can therefore be supposed that the relative contribution of soil and plant sources differs for bacteria associated with particles larger than 10 μm with respect to those associated with PM10. Admittedly, these speculations are only qualitative and a quantitative assessment of source contribution to the airborne communities as well as a precise knowledge of the quantitative distribution of bacteria between fine (<10 μm) and coarse (>10 μm) particles would have allowed a more detailed interpretation of these findings. Unfortunately, this information was unavailable in the present study.

In conclusion, in recent years, encouraging progress in next-generation sequencing techniques has led to an increase in our comprehension of the structure and diversity of airborne bacterial communities. The results of the present study confirmed that airborne bacterial communities showed a large variability even between consecutive days, thus suggesting that their structure mainly depends on the source of bacteria that predominates in a given period of time. In addition, a comparison of the results of the present and of a previous study conducted in the same area indicates that different bacterial communities could be found in different PM fractions, thus suggesting that a detailed investigation of the structure, temporal variability and potential sources of bacterial communities associated to PM of different sizes may disclose further insights into the ecology of bioaerosols.

Acknowledgements This work was partially supported by Cariplo Foundation (Milan, Italy) in the frame of the project TOSCA (Toxicity of particulate matter and molecular markers of risk). The authors thank Rocco Piazza and Alessandra Pirola for the DNA sequencing and bioinformatics support.

References

Angenent LT, Kelley ST, St Amand A, Pace NR, Hernandez MT (2005) Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc Natl Acad Sci U S A 102:4860–4865

Beggs CB, Kerr KG (2000) The threat posed by airborne microorganisms. Indoor Built Environ 9:241–245

Benjamini Y, Yekutieli (2001) The control of the false discovery rate in multiple testing under dependency. Ann Stat 29:1165–1188

Borchard D, Gillet F, Legendre F (2011) Numerical ecology with R. Springer, New York. doi:10.1007/978-1-4419-7976-6

Bovallius A, Bucht B, Roffey R, Anas P (1978) Three-year investigation of the natural airborne bacterial flora at four localities in Sweden. Appl Environ Microb 35:847–852

Bowers RM, Lauber CL, Wiedinmyer C, Hamady M, Haller AG, Fall R, Knight R, Fierer N (2009) Characterization of airborne microbial communities at a high-elevation site and their potential to act as atmospheric ice nuclei. Appl Environ Microb 38:6029–6041

Bowers RM, McLetchie S, Knight R, Fierer N (2010) Spatial variability in airborne bacterial communities across land-use types and their relationship to the bacterial communities of potential source environments. ISME J 5:601–612

Bowers RM, Sullivan AP, Costello EK, Collet JL, Knight R, Fierer N (2011) Source of bacteria in outdoor air across cities in the Midwestern United States. Appl Environ Microbiol 77:6350–6356

Brodie EL, DeSantis TZ, Parker JPM, Zubietta IX, Piceno YM, Andersen GL (2007) Urban aerosols harbour diverse and dynamic bacterial populations. Proc Natl Acad Sci U S A 104:299–304

Camatini M, Corvaja V, Pezzolato E, Mantecca P, Gualtieri M (2010) PM10-biogenic fraction drives the seasonal variation of pro-inflammatory response in A549 cells. Environ Toxicol 27:63–73

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621–1624

Claesson MJ, O'Sullivan O, Wang Q, Nikkila J, Marchesi JR, Smidt H, de Vos WM, Ross RP, O'Toole PW (2009) Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4:e6669

Claesson MJ, Wang QO, O'Sullivan O, Greene-Diniz R, ColeR JR, Ross P, O'Toole PW (2010) Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res 38:e200

Colbeck I, Lazaridis M (2009) Aerosols and environmental pollution. Naturwissenschaften 97:117–131

D'Amato G (2002) Environmental urban factors (air pollution and allergens) and the rising trends in allergic respiratory diseases. Allergy 57:30–33

De Cáceres M, Legendre P, Moretti M (2010) Improving indicator species analysis by combining groups of sites. Oikos 119:1674–1684

Durfee T, Nelson R, Baldwin S, Plunkett G III, Burland V, Mau B, Petrosino JF, Qin X, Muzny DM, Ayele M, Gibbs RA, Csörgo B, Pósfai G, Weinstock GM, Blattner FR (2008) The complete genome sequence of *Escherichia coli* DH10B: insights into the biology of a laboratory workhorse. J Bacteriol 190:2597–2606

Fang ZG, Ouyang ZY, Zheng H, Wang XK, Hu LF (2007) Culturable airborne bacteria in outdoor environments in Beijing, China. Microb Ecol 54:487–496

Fierer N, Liu Z, Rodriguez-Hernandez M, Knight R, Henn M, Hernandez MT (2008) Short-term temporal variability in airborne bacterial and fungal populations. Appl Environ Microb 74:200–207

Finnerty K, Choi JE, Lau A, Davis-Gorman G, Diven C, Seaver N, Linak WP, Witten M, McDonagh PF (2007) Instillation of coarse ash particulate matter and lipopolysaccharide produces a systemic inflammatory response in mice. J Toxicol Environ Health A 70:1957–1966

Franzetti A, Gandolfi I, Gaspari E, Ambrosini R, Bestetti G (2010) Seasonal variability of bacteria in fine and coarse urban air particulate matter. Appl Microbiol Biotechnol 90:745–753

Frohlich-Nowoisky J, Pickersgill DA, Despres VR, Poschl U (2009) High diversity of fungi in air particulate matter. Proc Natl Acad Sci USA 106:12814–12819

Gandolfi I, Franzetti A, Bertolini V, Gaspari E, Bestetti G (2011) Antibiotic resistance in bacteria associated to coarse atmospheric particulate matter in an urban area. J Appl Microbiol 110:1612–1620

Goslee SC, Urban DL (2007) The ecodist package for dissimilarity-based analysis of ecological data. J Stat Soft 22:1–19

Gouveia NC, Maisonet M (2006) Health effects of air pollution: an overview. In: air quality guidelines: global update 2005. World Health Organization, Copenhagen, pp 87–109

Hirano SS, Upper CD (1983) Ecology and epidemiology of foliar bacterial plant pathogens. Annu Rev Phytopathol 21:243–269

Huber J, Welch DM, Morrison H, Huse S, Neal P, Butterfield D, Sogin M (2007) Microbial population structures in the deep marine biosphere. Science 318:97–100

Jaenicke R (2005) Abundance of cellular material and proteins in the atmosphere. Science 308:73

Jeraldo P, Chia N, Goldenfeld N (2011) On the suitability of short reads of 16S rRNA for phylogeny-based analyses in environmental surveys. Environ Microbiol 13:3000–3009

Jones AM, Harrison RM (2004) The effects of meteorological factors on atmospheric bioaerosol concentrations—a review. Sci Total Environ 326:151–180

Kindt R, Coe R (2005) Tree diversity analysis. A manual and software for common statistical methods for ecological and biodiversity studies. World Agroforestry Centre (ICRAF), Nairobi

Lee SH, Lee HJ, Kim SJ, Lee HM, Kang H, Kim YP (2010) Identification of airborne bacterial and fungal community structures in an urban area by T-RFLP analysis and quantitative real-time PCR. Sci Total Environ 408:1349–1357

Legendre P, Gallagher ED (2001) Ecologically meaningful transformations for ordination of species data. Oecologia 129:271–280

Legendre P, Legendre L (1998) Numerical ecology, 2nd edn. Elsevier, Amsterdam

Lighthart B, Shaffer BT (1995a) Viable bacterial aerosol particle size distributions in the midsummer atmosphere at an isolated location in the high desert chaparral. Aerobiologia 11:19–25

Lighthart B, Shaffer BT (1995b) Airborne bacteria in the atmosphere surface layer: temporal distribution above a grass seed field. Appl Environ Microbiol 61:1492–1496

Maron PA, Lejon DPH, Carvalho E, Bizet K, Lemanceau P, Ranjard L, Mougel C (2005) Assessing genetic structure and diversity of airborne bacterial communities by DNA fingerprinting and 16S rDNA clone library. Atmos Environ 39:3687–3695

Maron PA, Mougel C, Lejon DPH, Carvalho E, Bizet K, Marck G, Cubito N, Lemanceau P, Ranjard L (2006) Temporal variability of airborne bacterial community structure in an urban area. Atmos Environ 40:8074–8080

Moorman JE, Zahran H, Truman BI, Molla MT (2011) Current asthma prevalence—United States, 2006–2008. MMWR Surveill Summ 60((Suppl)):84–86

Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257–266

Oksanen J, Kindt R, Legendre P, O'Hara B, Simpson GL, Solymos P, Stevens MHH, Wagner H (2009) Vegan: community ecology package. R package version 1.15-3. Available at http://cran.r-project.org/, http://vegan.r-forge.r-project.org/

Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science 276:734–740

Peccia J, Hernandez M (2006) Incorporating polymerase chain reaction-based identification, population characterization and quantification of microorganisms into aerosol science: a review. Atmos Environ 40:3941–3961

Pillai SD, Ricke SC (2002) Bioaerosols from municipal and animal wastes: background and contemporary issues. Can J Microbiol 48:681–696

Polymenakou PN, Mandalakis M, Stephanou EG, Tselepides A (2008) Particle size distribution of airborne microorganisms and pathogens during an intense African dust event in the Eastern Mediterranean. Environ Health Perspect 116:292–296

Putaud J, Raes F, Van Dingenen R, Brüggemann E, Facchini M, Decesari S, Fuzzi S, Gehrig R, Hüglin C, Laj P, Lorbeer G, Maenhaut W, Mihalopoulos N, Müller K, Querol X, Rodriguez S, Schneider J, Spindler G, Ten Brink H, Tørseth K, Wiedensohler A (2004) A European aerosol phenomenology—2: chemical characteristics of particulate matter at kerbside, urban, rural and background sites in Europe. Atmos Environ 38 (16):2579–2595

R Development Core Team (2008) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rinsoz T, Duquenne P, Greff-Mirguet G, Oppliger A (2008) Application of real-time PCR for total airborne bacterial assessment: comparison with epifluorescence microscopy and culture-dependent methods. Atmos Environ 42:6767–6774

Samet JM, Brauer M, Schlesinger R (2006) Particulate matter. In: Air quality guidelines: global update 2005. World Health Organization, Copenhagen, pp 217–305

Squizzato S, Masiol M, Innocente E, Pecorari E, Rampazzo G, Pavoni B (2012) A procedure to assess local and long-range transport contributions to PM2.5 and secondary inorganic aerosol. Aerosol Science 46:64–76

Therneau TM, Atkinson B, Ripley B, Oksanen J, De'ath G (2007) mvpart: multivariate partitioning. R package version 1.2-6. Available at http://cran.r-project.org/web/packages/mvpart/index.html

Wang Y, Qian P (2009) Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One 4:e7401

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microb 73:5261–5267

Womack AM, Bohannan BJM, Green JL (2010) Biodiversity and biogeography of the atmosphere. Philos Trans R Soc B Biol Sci 365:3645–3653