Impact of urban land use on the bacterial phyllosphere of ivy (**Hedera sp.**)

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Wenke Smets[1], Karen Wuyts[1], Eline Oerlemans[1], Sander Wuyts[1,2], Siegfried Denys[3], Roeland Samson[1], Sarah Lebeer[1,$]

[1]University of Antwerp, Dept. Bioscience Engineering, Environmental Ecology and Applied Microbiology, Antwerp, Belgium

[2]Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

[3]University of Antwerp, Dept. Bioscience Engineering, Sustainable Energy, Air and Water Technology, Antwerp, Belgium

[$]corresponding author: Sarah Lebeer, University of Antwerp, Dep. Bioscience Engineering, Groenenborgerlaan 171, B-2020: sarah.lebeer@uantwerpen.be, Tel: +32 32653285

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Abstract

The surface of the aerial parts of the plant, also termed the phyllosphere, is a selective habitat for microbes. The bacterial composition of the phyllosphere depends on host plant species, leaf characteristics, season, climate, and geographic location of the host plant. In this study, we investigated the effect of an urban environment on the bacterial composition of phyllosphere communities. We performed a passive biomonitoring experiment in which leaves were sampled from ivy (*Hedera sp.*), a common evergreen climber species, in urban and non-urban locations. Exposure to traffic-generated particulate matter was estimated using leaf biomagnetic analyses. The bacterial community composition was determined using 16S rRNA gene sequencing on the Illumina MiSeq. The phyllosphere microbial communities of ivy differed greatly between urban and non-urban locations, as we observed a shift in several of the dominant taxa: *Beijerinckia* and *Methylocystaceae* were most abundant in the non-urban phyllosphere, whereas *Hymenobacter* and *Sphingomonadaceae* were dominating the urban ivy phyllosphere. The richness, diversity and composition of the communities showed greater variability in the urban than in the non-urban locations, where traffic-generated PM was lower. Interestingly, the relative abundances of eight of the ten most dominant taxa correlated well with leaf magnetism, be it positive or negative. The results of this study indicate that an urban environment can greatly affect the local phyllosphere community composition. Although other urban-related factors cannot be ruled out, the relative abundance of most of the dominant taxa was significantly correlated with exposure to traffic-generated PM.

1. Introduction

The leaf surface habitat, also known as the phyllosphere, supports diverse bacterial communities shaped by both plant factors and environmental conditions (Vorholt, 2012). In turn, the epiphytic bacteria can affect the host plant by, for example, preventing colonization of certain plant pathogens and encouraging plant growth (Vorholt, 2012). Furthermore, these bacteria are capable of degrading or detoxifying atmospheric pollutants, such as polycyclic aromatic hydrocarbons (PAH) (Yutthammo et al., 2010), and are considered an important source of airborne bacterial particles (Gandolfi et al., 2013; Lindemann et al., 1982).

Various culture-independent studies indicate that the host plant is an important factor for the composition of a phyllosphere community. In their study of 56 different tree species, Redford et al. (2010) showed that different tree species harbour distinct phyllosphere communities, since community variability between tree species exceeded the variability within one tree species. This principle was confirmed for trees in temperate and tropical climates and for Mediterranean perennials as well (Kembel et al., 2014; Kim et al., 2012; Laforest-Lapointe et al., 2016; Lambais et al., 2006; Vokou et al., 2012). However, the microbial composition within plant species can also be influenced by geographic location of the host plant (Finkel et al., 2012; Finkel et al., 2011; Knief et al., 2010; Qvit-Raz et al., 2012; Rastogi et al., 2012). This geographic effect may be caused by climatic differences (Finkel et al., 2011) or the limited dispersal of the colonizing taxa (Finkel et al., 2012; Qvit-Raz et al., 2012). Moreover, some studies indicate that there are important microbial community differences between urban and non-urban locations. For instance, Jumpponen and Jones (2010) have previously found significant differences in the culturable fraction of the fungal phyllosphere communities of urban and non-urban oak trees in the USA, but they did not include bacteria. Smaller studies, based on the culturing of phyllosphere bacteria, revealed important differences between one site with heavy traffic pollution and one less polluted site (Brighigna et al., 2000; Joshi et al., 2008; Khanna, 1986). However, these studies included a limited number of sample locations and were based on culture methods, which only allow a small part of the phyllosphere diversity to be studied (Whipps et al., 2008).

The aim of this study was to explore differences in the phyllosphere communities of a common and evergreen plant species between an urban and a non-urban environment, using a culture-independent method. To this end, we sampled the phyllosphere of *Hedera sp.* (ivy) plants at three different locations in the city of Antwerp (Belgium) and at three locations outside of the city. A culture-independent approach based on high-throughput *16S rRNA* amplicon sequencing was used to determine the bacterial community structure of the phyllosphere of this ubiquitous evergreen climber. Furthermore, we investigated the relation between the bacterial community composition and local atmospheric pollution for all sampled ivy plants, using leaf magnetism as a proxy for particulate matter (PM) originating from traffic.

2. Materials & Methods

2.1 Sample collection

The study was conducted in the province of Antwerp, Belgium. Six sampling locations were selected within a distance of 22 km of each other. We sampled at three different locations in the city of Antwerp ('urban locations') and at three locations in more rural areas ('non-urban locations') surrounding the city. The non-urban sampling locations were selected in quiet, green and parklike residential areas, at the edge of the nature reserve *Kalmthoutse Heide* (locations 1 and 2) and in the ancient forest *Zevenbergenbos* (location 3). All urban sampling locations (locations 4 to 6) were located in densely built-up areas and next to busy roads with medium to high traffic intensity (daytime average between 1050 and 2100 vehicles/h; SGS, 2010). Moreover, they were all within a distance of 200 to 370 m from a very traffic-intensive motorway (daytime average about 8000 vehicles/h; SGS, 2010). The mean atmospheric concentrations of NO_x, PM_{10} and $PM_{2.5}$ modelled by ATMOSYS (www.atmosys.eu) for 2013 (the most recent available data) were consistently lower at the non-urban locations compared to the urban locations (Table 1). The soil texture of the sampling locations was similar: sandy loam soils were found at all locations except for location two, where the soil was sandy (http://www.geopunt.be/). All locations were sampled on the same day (17 February 2015), to minimize the effect of other factors such as weather and season. All locations experience a maritime temperate climate, with lowest and highest mean temperatures of 3 °C in January and 18 °C in July, respectively, and annual mean rainfall of 900 mm (Royal Meteorological Institute, www.kmi.be).

Table 1: Modelled location-specific atmospheric concentrations according to ATMOSYS (annual mean)

Location	Coordinates	Land use	NO_x (µg/m^3)	PM_{10} (µg/m^3)	$PM_{2.5}$ (µg/m^3)
1	51.387427 N, 4.448910 E	Non-urban	16-20	21-25	13-15
2	51.356064 N, 4.419640 E	Non-urban	21-25	21-25	13-15
3	51.199092 N, 4.554420 E	Non-urban	21-25	21-25	16-20
4	51.208759 N, 4.437652 E	Urban	41-45	31-35	16-20
5	51.192955 N, 4.421895 E	Urban	46-50	31-35	16-20
6	51.190219 N, 4.397139 E	Urban	36-40	31-35	16-20

At each location, three *Hedera sp.* (*H. helix* or *H. hibernica*; common name: ivy) plants were selected from which healthy, mature, vegetative, undamaged leaves were sampled at 1.5-2 m height. Leaves were cut with scissors using gloves, which were sterilized on site with 70% ethanol. For microbiological analysis, at least 200 cm^2 of leaves of each plant were put in a sterile 50 mL falcon (VWR) and transported to the lab. A field blank consisted of a falcon

without leaves. In parallel, three leaf samples per plant were collected of about 100 cm² each for magnetic analysis and stored in paper envelopes for transport to the lab.

2.2 DNA extraction and 16S rRNA V4 amplicon sequencing

Phyllosphere microbes were extracted from the leaves upon arrival in the lab, 1 to 5 hours after the samples were taken, by adding 20 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) to each falcon tube. To suspend the phyllosphere bacteria in the TE buffer, the tubes were alternately vortexed for 15 s at maximum speed with the Vortex Genie® 2 (MoBio) and 15 s manually shaken, four times in total. The leaves were removed and the remaining TE buffer was centrifuged at 4000 g for 10 minutes and most of the supernatant was discarded. The pellet and remaining supernatant were centrifuged again in 2 mL tubes at 8000 g for 10 minutes. The remaining supernatant was removed and the pellet was resuspended in 400 µL of Bead Solution (PowerFecal DNA Isolation Kit, MoBio) and stored at -80°C for optimal preservation of DNA, until further processing.

DNA extraction of the samples was done with the PowerFecal DNA Isolation Kit, according to the manufacturer's instructions. DNA was also extracted from a falcon without leaves, but treated like the other falcons, to identify potential contaminants of the sampling procedure. Additionally, a DNA extraction was carried out solely with kit reagents to identify kit contaminants. To attain a DNA-sequence-based identification of the bacteria in the samples, a short suitable sequence was targeted and its numbers were increased to allow for Illumina sequencing. Hence, a PCR amplification of the V4 region of the 16S rRNA gene was done using barcoded primers (IDT) as described by Kozich *et al.* (2013). Primers with different barcodes (short artificial DNA sequences) were used for different samples, in order to attribute sequences to their original samples after sequencing. The Phusion High-Fidelity DNA polymerase (Thermo Scientific) was used to limit the number of errors introduced during PCR, as these errors may lead to misidentification of the bacteria. A PCR blank was included to confirm the absence of non-specific amplification. Each DNA extract was amplified in duplicate with different barcodes to assess technical variation. The resulting amplicons were purified with Agencourt AMPure XP PCR purification system (Beckman Coulter) and quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Based on these DNA concentrations, samples were pooled at equimolar concentrations into one tube and diluted to 2 nM. The pooled amplicons were sequenced at the Centre for Medical Genetics (Edegem, Belgium) with Illumina MiSeq, using the 500-cycles MiSeq Reagent Kit v2 (Illumina). The sequences obtained in this study are available in the European Nucleotide Archive database under study accession number PRJEB14262.

2.3 Bioinformatic analysis

The UPARSE pipeline (Edgar, 2013) was used to assemble the paired reads, conduct quality filtering, cluster sequences into operational taxonomic units, and assign taxonomy. Assembly of the paired end reads was set to a maximum of 24 differences in the overlap zone and the minimum length of the overlap of 150 base pairs. A maximum per sequence expected error frequency value of 0.5 was used to quality-filter sequences and global singletons were removed. The remaining sequences were clustered into OTUs (operational taxonomic units) at 97% similarity using a *de novo* clustering approach. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) trained on the Greengenes database (version 13_08). Mitochondrial and chloroplast OTUs were removed and the data were rarefied to 16323 reads. Technical PCR repeats were included. The community compositions of these technical repeats were consistently very similar (Fig A.1 and A.2). The technical repeats of each sample were therefore merged into one representative community per plant. An OTU table was generated for further statistical analyses.

2.4 Magnetic analysis

As PM is (at least partially) deposited on leaves, the biomagnetic signal of the leaves was measured to estimate local PM pollution. The one-sided surface area of the leaf samples for magnetic analysis was determined using a leaf area meter (LI-3100C, LI-COR), after which the samples were ovendried (60 °C). The saturation isothermal remanent magnetisation (SIRM) was determined of each leaf sample, as a proxy for exposure to traffic- and industry-derived PM (Hansard et al., 2011; Kardel et al., 2012b; Mitchell and Maher, 2009; Muxworthy et al., 2003). Given that metal emitting industries were sufficiently distant from all sampling locations, we can consider SIRM as a proxy for traffic-derived PM in this study. Following the protocol described by Kardel et al. (2011), the samples were tightly packed in small sample pot using cling film and magnetized in a pulse DC magnetic field of 1 using a Molspin pulse magnetizer (Molspin Ltd, UK) and, immediately after, the remanent magnetisation was measured using a Minispin magnetometer (Molspin) with high sensitivity (~0.1×10^{-10} A m^2). The leaf area-normalized SIRM (in A) was obtained by normalizing the magnetic signal by the volume of the sample pot (10 cm^3) and the sample leaf area.

2.5 Statistical analyses

Statistical analyses were done in R (R Core Team, 2016). A significance level of 0.05 was used for all statistical tests. The OTU richness (cfr. species richness) was calculated as the total number of different OTUs in a sample and true OTU diversity (cfr. true species diversity) as the exponential function of the Shannon index of a sample. These measures of alpha

diversity were compared between the urban and non-urban samples using the Mann-Whitney-Wilcoxon test.

A Bray-Curtis dissimilarity matrix of the Hellinger transformed data of the OTU table was calculated, using the vegan package (Oksanen et al., 2013). Nonmetric multidimensional scaling (NMDS) was applied to these Bray-Curtis dissimilarities to visualize the data in two dimensions. The effect of land-use type and location on bacterial community composition was determined with a permutational multivariate analysis of variance or PERMANOVA using the Bray-Curtis distance matrix. The Kruskal-Wallis test was used to determine which taxa showed a significantly different abundance between the urban and non-urban samples.

The SIRM values per plant were determined as the mean of three measurements. To obtain normally-distributed data, a logarithmic transformation was applied to these mean SIRM values by taking their natural logarithm. The transformed SIRM values of urban and non-urban plants were compared using a one-sided t-test. Spearman rank correlations were determined for OTU richness, true OTU diversity and the abundances of the most abundant taxa (>1% in all samples combined) with the transformed leaf SIRM values.

3. Results

3.1 Bacterial community composition differs between urban and non-urban locations

We determined the microbial community composition of the phyllosphere of the ivy samples by 16S rRNA amplicon sequencing. After sequence assembly and filtering, a total of 2 164 859 partial 16S rRNA gene sequences were generated from the 18 phyllosphere samples and their technical repeats. After rarefaction and merging of technical repeats, a total of 277 491 reads in 17 samples remained for further analyses.

The bacterial richness, based on the total number of OTUs (Fig. 1A), was 760 ± 70 OTUs (always expressed as mean ± SE) per sample. The true OTU diversity based on the Shannon index (Fig. 1B) was 110 ± 20 OTUs per sample. The richness and true diversity did not significantly differ between the urban and non-urban bacterial phyllosphere communities ($P = 0.54$ and $P = 0.67$, respectively).

Subsequently, we analysed the bacterial composition of the different samples. To compare general community differences, pairwise dissimilarities of the communities were visualized. Dimensional scaling of the Bray-Curtis dissimilarity matrix to two dimensions (Fig. 1C) revealed that the communities clustered into an urban and a non-urban cluster. The stress value of this NMDS, an indication of the variation that is not visualised in this two-dimensional space, was low (0.062). PERMANOVA revealed that land-use type was in fact a significant factor for the phyllosphere community composition ($P < 0.001$, $R^2 = 0.38$), while the sampling location was not ($P = 0.35$).

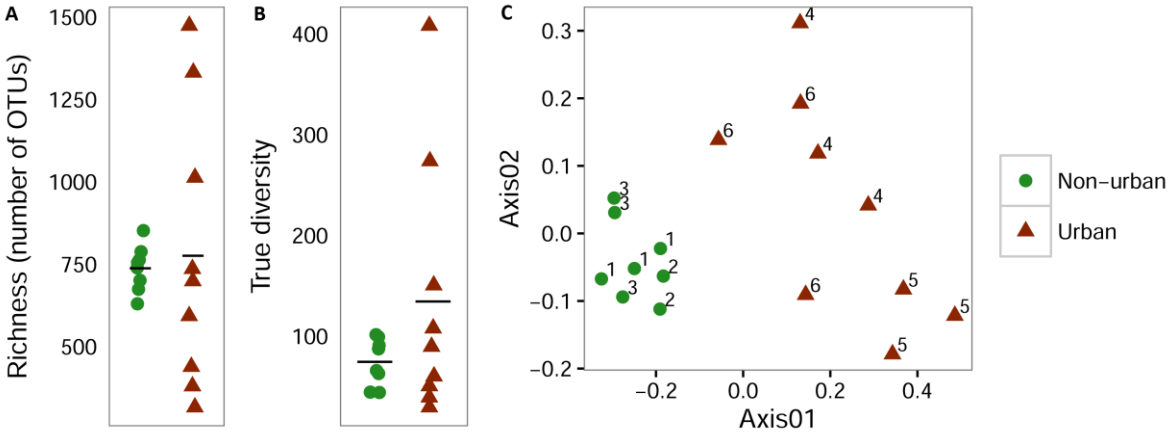

Figure 1: Comparison of the phyllosphere communities at the urban and non-urban sampling locations. The distributions and means of the OTU richness (A) and true OTU diversity (B) are shown (N=17). In the NMDS plot (C), every point represents the bacterial community composition of one sample and the distance between these points refers to the dissimilarities between the communities. The number labels indicate different samples within a sampling location (Table 1).

The most abundant OTUs (>1% relative abundance in all samples combined) belonged to the *Alphaproteobacteria* (*Beijerinckia, Sphingomonadaceae, Acetobacteraceae, Methylocystaceae, Polaromonas* and *Methylobacterium*), the Bacteroidetes (*Hymenobacter* and *Sphingobacteriaceae*), the *Gammaproteobacteria* (*Pseudomonas*) and the *Acidobacteria* (*Edaphobacter*) (Fig. 2A). Eight of these ten dominant families and genera showed significantly different abundances between the urban and non-urban samples (Fig. 2B). The two taxa that were not different between the land-use types were *Pseudomonas* and *Acetobacteraceae*. The most abundant sequence of the OTU that caused the dominant presence of *Pseudomonas* in one plant at a non-urban location, was blasted (Zhang et al., 2000) against the EMBL and GenBank accessions and showed 100% identity (of the amplified region) with several *Pseudomonas* species, among which *Pseudomonas syringae* and *Pseudomonas cannabina*, which are known plant pathogens. The *Acetobacteraceae* OTU could not be identified beyond family level, but seemed to be a consistent ivy phyllosphere bacterium at non-urban locations, whereas its abundance was very variable in the urban environment.

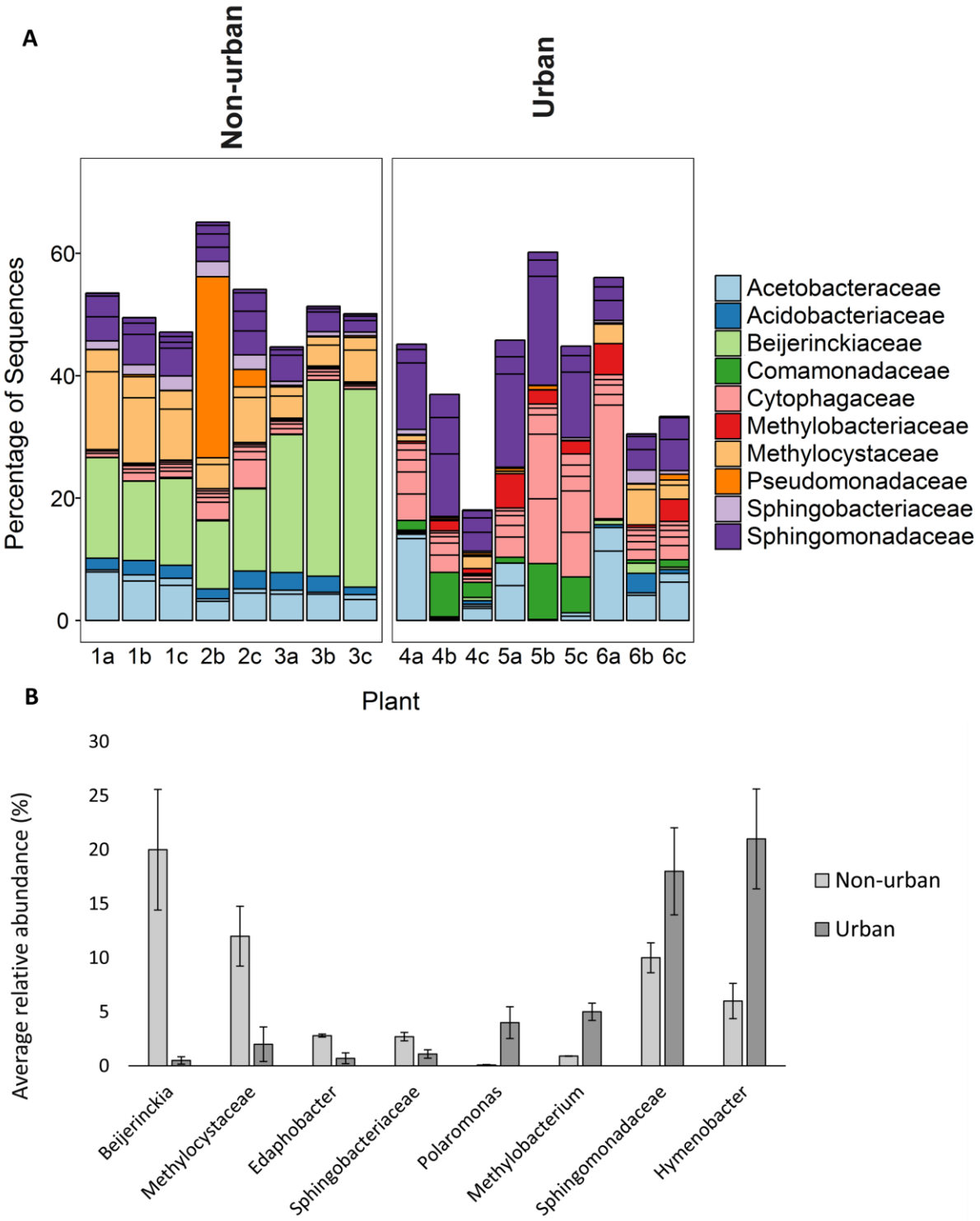

Figure 2: Relative abundances of the 20 most abundant OTUs in the different plants (three plants a, b and c per location) at each of the non-urban locations 1, 2 and 3 and urban locations 4, 5 and 6 (A). Different colours denote different families. Mean relative abundances and their SE of the taxa that occurred at significantly different abundances between the urban and non-urban plants (B).

3.2 Leaf SIRM is higher at the urban locations

The mean SIRM signal of the leaves, a proxy for traffic-generated PM exposure, was 31 ± 3 µA in the non-urban area and 200 ± 30 µA in the urban area (Figure 3). The log-transformed means differed significantly between the land-use types ($P < 0.0001$).

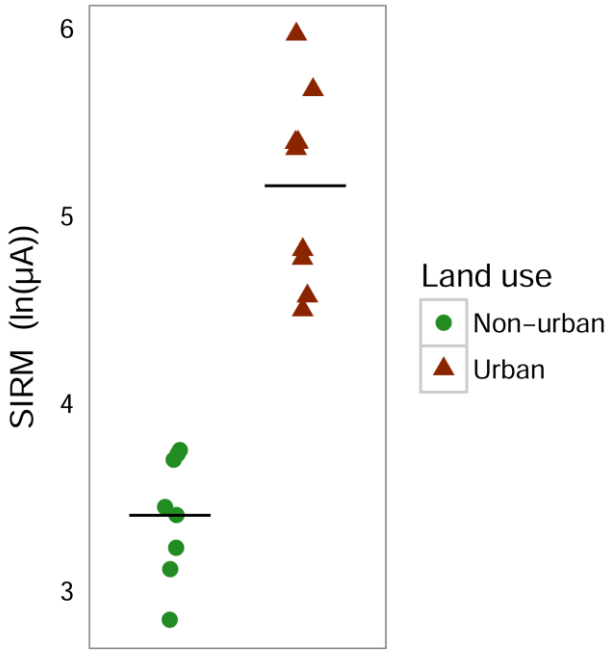

Figure 3: Log-transformed SIRM values and their means of the plants at the urban and non-urban locations (N=17)

3.3 Relationship between bacterial community and SIRM

The OTU richness and true OTU diversity of the phyllosphere bacterial communities did not significantly correlate with their corresponding log-transformed leaf SIRM values ($P = 0.16$ and $P = 0.77$, respectively). However, according to a PERMANOVA, the SIRM values correlated well with the bacterial community composition of the ivy phyllosphere ($P < 0.001$, $R^2 = 0.33$). Moreover, the abundances of the most dominant and land-use-dependent taxa were shown to be significantly correlated with the local SIRM values (Fig. 4).

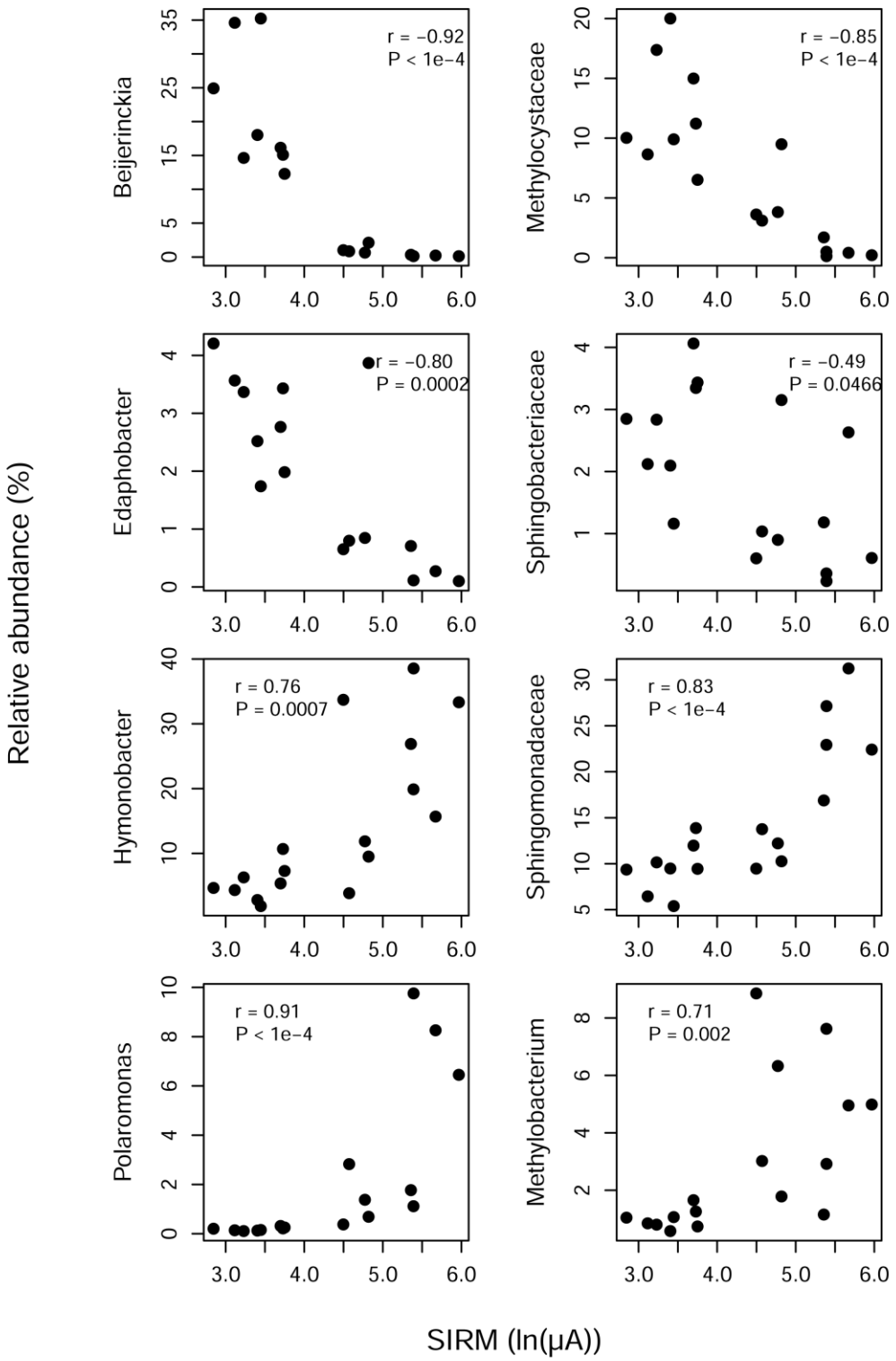

Figure 4: Correlation between log-transformed leaf SIRM and relative abundance of the most dominant taxa that differed significantly between the urban and non-urban locations, with "r" the Spearman's rank correlation coefficient and "P" the corresponding *p*-value. One data point represents one sample.

4. Discussion

The main purpose of this study was to investigate how an urban environment and its associated air pollution may affect the bacterial phyllosphere community of ivy. As knowledge on this topic is biased towards culturable bacteria, this study provides new insights through culture-independent techniques.

Previous sequencing efforts of the phyllosphere of different host plant species have found that the *Alphaproteobacteria* often make up the most abundant class of the bacterial phyllosphere, with *Methylobacteriaceae* and *Sphingomonadaceae* as important families (Delmotte et al., 2009; Vorholt, 2012). In addition, the *Beijerinckia* have been identified as the most abundant OTU in the phyllosphere of a neotropical forest (Kembel et al., 2014). Our results of the ivy phyllosphere are in accordance with these results, but also bring forward other dominant phyllosphere bacteria that may be specific for ivy or for this geographic region, such as unidentified members of the clades *Acetobacteraceae*, *Methylocystaceae* and *Hymenonbacter*.

The bacterial phyllosphere communities at the urban locations differed from those in the non-urban environment, as was observed in previous studies with culturable bacteria (Brighigna et al., 2000; Joshi et al., 2008; Khanna, 1986). Despite their equal mean richness and diversity, the communities in our study showed a dramatic shift in many of the dominant taxa. *Methylocystaceae*, which utilize only methane or other C1 compounds as a substrate (Bowman, 2006), and *Beijerinckia*, which are known to fix nitrogen and release plant growth regulators (Ruinen, 1965; Thuler et al., 2003), were dominant taxa at the non-urban locations. Furthermore, *Spingobacteriaceae* and *Edaphobacter modestus*, a member of the *Acidobacteria*, were typical for the non-urban phyllosphere. All of these taxa were found to significantly decrease with increasing leaf SIRM values. The *Beijerinckia* were not only most dominant at the non-urban locations but also showed the strongest negative correlation with leaf SIRM. *Methylobacterium*, a part of the community that is known for its degradation of methanol released by the leaves (Delmotte et al., 2009), *Spingomonadaceae*, abundant phyllosphere inhabitants (Delmotte et al., 2009), and *Hymenobacter* were significantly more abundant in the urban phyllosphere. In addition, these taxa were positively correlated with leaf SIRM. Furthermore, *Polaromonas* showed the strongest positive correlation with leaf SIRM. Leaf SIRM is shown to be a good proxy for source-specific atmospheric PM_{10} and $PM_{2.5}$ concentrations and the mass of particles deposited on leaves (Hofman et al., 2014; Kardel et al., 2011; Mitchell and Maher, 2009). Therefore, our results suggest that exposure of the phyllosphere to traffic-induced PM may play a crucial role in phyllosphere community composition.

The explanation of the relation between phyllosphere bacterial composition and leaf SIRM by PM is not incontrovertible. Increased leaf SIRM values and urban land use are indirectly associated with other factors such as other traffic-related, co-emitted air pollutants (e.g. NO_x

and organic pollutants) and environmental conditions. The environmental conditions of an urban environment include higher temperature, lower relative humidity and lower wind speed (Arnfield, 2003). Environmental conditions are known to affect phyllosphere bacterial community composition (Vorholt 2012), either directly or indirectly. It is known that a warmer and dryer (urban) environment alters leaf characteristics and plant biochemistry (Balasooriya et al., 2009; Della Torre et al., 1998; Eleftheriou, 1987; Mitrovic et al., 2006; Mudd, 2012). In this case, methane and methanol are potentially important drivers, as they are known to be released by plants (Keppler et al., 2006; MacDonald and Fall, 1993) and drive the *Methylobacterium* and *Methylocystaceae* abundances. Also certain leaf characteristics, such as the cuticular wax composition and morphology, and stomatal density are known to be affected by the environment and local air pollution (Honour et al., 2009; Kardel et al., 2012a; Kardel et al., 2010; Rai et al., 2010; Shepherd and Griffiths, 2006; Wuytack et al., 2013) and may affect members of the phyllosphere community like the *Sphingomonas* (Bodenhausen et al., 2014; Reisberg et al., 2013). Other leaf characteristics, including epidermal cell wall junctions, have also been found to affect the phyllosphere community composition (Hunter et al., 2010). Hence, air pollution and other factors associated with an urban environment may affect the phyllosphere community indirectly, through the host plant.

Besides environmental variation, genetic differentiation between the host plants may affect phyllosphere composition (Bodenhausen et al., 2014; Redford et al., 2010). Host genotype or the host plant history was not assessed in this study, so we cannot exclude the existence of relevant host genotype differences between the urban and non-urban locations. Furthermore, the observed differences in phyllosphere communities may also be caused by the location-specific bacterial colonizers, as suggested by Knief et al. (2010). There are indeed city-specific bacterial sources, as illustrated by research of atmospheric bacterial communities. The community composition of airborne bacteria has been found to strongly depend on land-use type (Bowers et al., 2011), which indicates that the phyllosphere of different land-use types is exposed to different colonizers.

We acknowledge that this study encompasses a limited number of locations (six) and sampled plants (eighteen) and that we could not yet take multiple spatiotemporal factors into account, such as traffic intensity, population density, weather conditions etc. However, leaf SIRM is a time-integrated measure (PM deposition) and it is correlated with the abundances of dominant phyllosphere bacteria, implying the phyllosphere structure is also rather stable. Moreover, the differences and the strength of the correlations in this study indicate that land use and atmospheric pollution are important factors to take into account when studying the phyllosphere.

5. Conclusion

In this work, culture-independent analysis showed that OTU richness and diversity of the phyllosphere bacterial community of ivy are alike at urban and non-urban locations, but their OTU composition differs significantly. Many of the dominant non-urban taxa are replaced by other taxa in an urban environment. In addition, we were able to relate the relative abundances of eight of the ten most dominant taxa with the leaf magnetism (SIRM, a proxy for traffic-derived atmospheric PM), which suggests that the atmospheric PM exposure is an important driver of the community composition. Nonetheless, other factors might also directly or indirectly contribute to the shift in phyllosphere community composition between urban and non-urban locations, such as temperature, humidity and other atmospheric pollutants than PM. As such, care has to be taken when interpreting the relationships with leaf SIRM, as the effects of PM and other urban environmental conditions could not be disentangled in this study. It is clear that further study is needed to disentangle the driving forces for the urban influence on phyllosphere bacterial community composition. We can expect that the observed urban land-use effect on phyllosphere community composition affects plant health and the role of the phyllosphere as source of airborne bacterial particles. Moreover, our results shed a new light on the potential of phyllosphere bacteria for atmospheric pollutants bioremediation.

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Appendix A

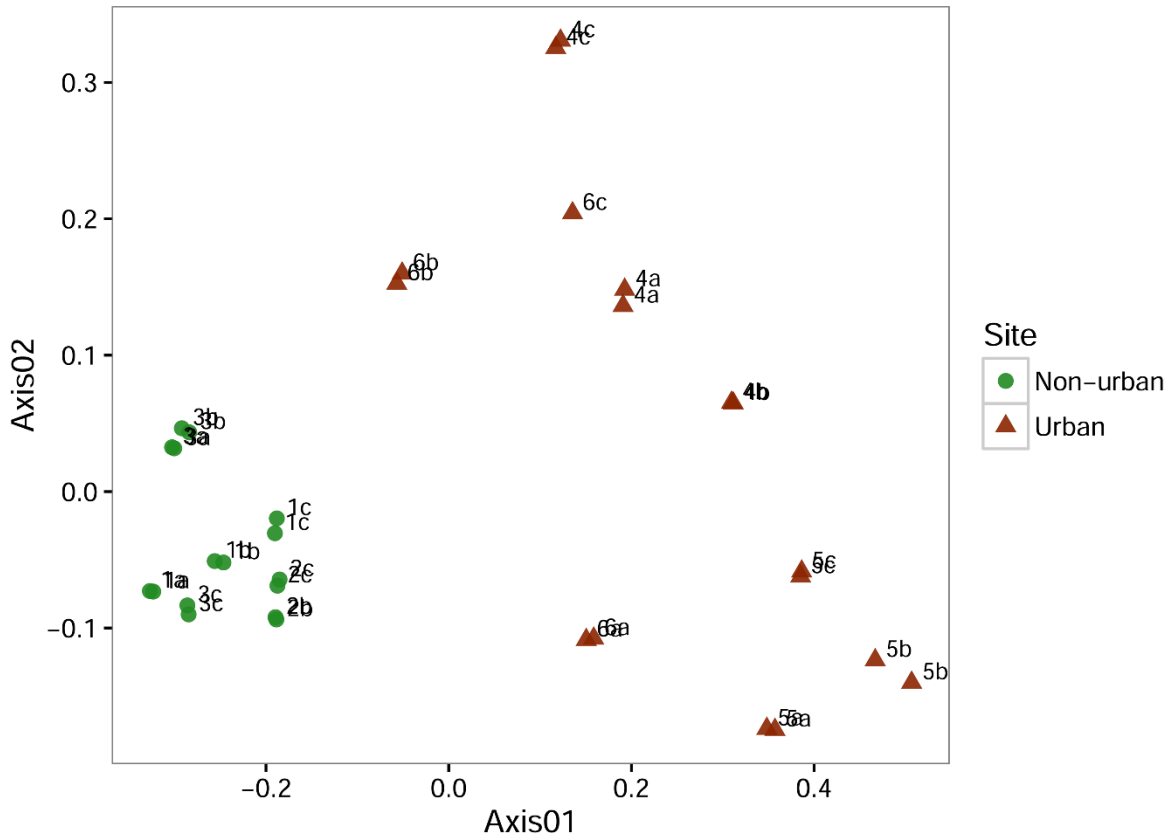

Figure A.1: The NMDS plot where every point represents the bacterial community composition of one of the technical repeats. The distance between these points refers to the dissimilarities between the communities. The label number indicates the location of the sample and the label letter refers to the sample. Samples with the same labels are technical repeats (DNA extracts of samples were amplified in duplicate).

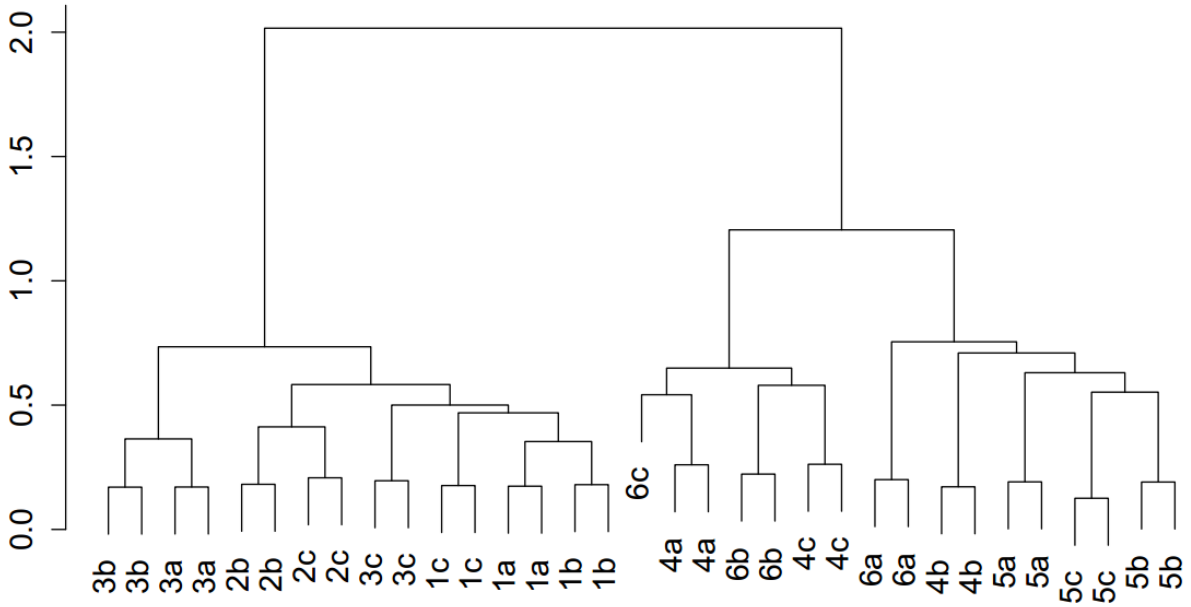

Figure A.2: Based on community composition, a cluster dendrogram using Ward's criterion was generated before merging technical repeats, after rarefaction. Samples with identical labels are technical repeats.