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Diversity and hydrocarbon-degrading potential of epiphytic microbial communities on *Platanus x acerifolia* leaves in an urban area

Authors: Isabella Gandolfi[a], Claudia Canedoli[a], Valeria Imperato[a], Ilario Tagliaferri[a], Panagiotis Gkorezis[b], Jaco Vangronsveld[b], Emilio Padoa Schioppa[a], Maddalena Papacchini[c], Giuseppina Bestetti[a], Andrea Franzetti[a]

[a]Dept. of Earth and Environmental Sciences, University of Milano-Bicocca, Milan, Italy

[b]Centre for Environmental Sciences – Hasselt University, Hasselt, Belgium

[c]INAIL, Dipartimento Innovazioni Tecnologiche e Sicurezza degli Impianti, Prodotti ed Insediamenti Antropici, Rome, Italy

Corresponding author: Isabella Gandolfi, Dept. of Earth and Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milan (Italy) – isabella.gandolfi@unimib.it

Abstract

Plants and their associated bacteria have been suggested to play a role in air pollution mitigation, especially in urban areas. Particularly, epiphytic bacteria might be able to degrade atmospheric hydrocarbons. However, phyllospheric bacterial communities are highly variable depending on several factors, e.g. tree species, leaf age and physiology, environmental conditions. In this work, bacterial communities hosted by urban *Platanus x acerifolia* leaves were taxonomically characterized using high throughput sequencing of 16S rRNA gene, and their temporal and spatial variability was assessed by comparing samples collected from different locations in the city of Milan (Italy) and in different months. The diversity of alkane hydroxylase (*alk*B) phylotypes harboured by phyllospheric bacteria associated to urban *Platanus* trees was also evaluated. Results revealed that temporal changes, which are related to seasonality, acted as a stronger driver both on *Platanus* phyllospheric community structure and on *alk*B phylotype diversity than sampling location. Biodiversity of bacterial communities decreased along the growing season, leading to a strong dominance by the genus *Stenotrophomonas*. On the contrary, diversity of hydrocarbon-degrading populations increased over the months, although it resulted lower than that reported for other habitats. It was therefore hypothesized that atmospheric hydrocarbons might play a key role in the selection of phyllospheric populations in urban areas.

Capsule: Increasing diversity of hydrocarbon-degrading bacterial populations hosted by urban *Platanus* leaves suggests that atmospheric hydrocarbons might select phyllospheric populations.

Keywords: phyllosphere, phylloremediation, hydrocarbons, alkane hydroxylase, air purification

Introduction

Air pollution is a matter of global concern, especially in urban areas, due to the harmful effects of atmospheric pollutants on human health and on the environment. Current emission reduction methods and mitigation strategies are not adequate to fully meet the World Health Organization (WHO) guidelines for air pollutants (Ali et al., 2012; Weyens et al., 2015). Since most of the environmental problems in urban areas are generated at local level, often one of the most effective ways to deal with them is through local solutions (Bolund and Hunhammer, 1999). Plants have been suggested to effectively contribute to reduce air pollution levels and offsetting greenhouse gas emissions in cities (Beckett et al., 1998; Dzierzanowski et al., 2011; McPherson et al., 1998; Nowak and Crane, 2002; Nowak et al., 2006; Paoletti, 2009; Redford et al., 2010; Yang et al., 2005; Zhao et al., 2010). In this context, the regulation of ecosystem services (the direct and indirect contributions of ecosystems to human well-being (TEEB, 2011)) provided by vegetation in urban areas is of great importance (Baró et al., 2014). Many studies, in fact, indicated that the management of urban forests to enhance ecosystem service supply can be a cost-effective strategy to meet specific environmental standards or policy targets (Escobedo et al., 2010, 2011). Furthermore, it has been recognized that also plant-associated bacteria can play a crucial role in air bioremediation processes (Glick, 2015; Weyens et al., 2015). Particularly, the aerial parts of terrestrial plants, mainly leaves (i.e. the phyllosphere) host huge amounts of bacteria. In fact, although phyllospheric microorganisms comprise also fungi, yeasts, algae, protozoa and nematodes, bacteria are by far the most abundant inhabitants of leaf surfaces (Lindow and Brandl, 2003). Since phyllospheric bacteria are often found at an average of 10^6-10^7 cells cm^{-2} of leaf surface (Lindow and Brandl, 2003), the planetary phyllospheric bacterial population has been estimated to be as large as 10^{26} cells (Morris and Kinkel, 2015). Among them, epiphytic bacteria, which primarily live on leaf surfaces, are directly positioned to the

interface with the atmosphere. Thus, they are exposed to several detrimental factors such as UV radiation, desiccation, severe temperature changes and, especially in urban areas, atmospheric pollutants (Lindow and Brandl, 2003). For this reason, they are expected to have developed metabolic abilities towards atmospheric hydrocarbons and therefore to play a potential role in air bioremediation processes. Indeed, several papers have already reported the ability of phyllospheric bacteria to degrade aliphatic (Al-Awadhi et al., 2012) and aromatic hydrocarbons, namely phenolic compounds, toluene, xylene and phenanthrene (De Kempeneer et al., 2004; Sandhu et al., 2007; Sangthong et al., 2016; Scheublin et al., 2014; Waight et al., 2007; Yutthammo et al., 2010).

Despite their continuous exchange with airborne populations (Lighthart, 2006; Lymperopoulou et al., 2016; Whipps et al., 2008), phyllospheric bacteria are not random assemblages but they rather form actual communities. In fact, some bacterial taxa are recurrently retrieved from leaf-associated habitats, leading to the hypothesis that, after recruitment, they undergo some selection processes (Delmotte et al., 2009; Rastogi et al., 2013; Vorholt, 2012; Yang et al., 2001). The relative abundance of a specific bacterial taxon in phyllospheric communities, however, can vary considerably. The main drivers that were suggested to shape community structure include host plant species, leaf age and physiology, season, geographical location, and environmental factors, such as solar radiation, humidity and nutrient availability (Laforest-Lapointe et al., 2016; Müller and Ruppel, 2014; Peñuelas et al., 2012; Rastogi et al., 2012; Redford et al., 2010; Vokou et al., 2012). Interactions between these factors can also affect bacterial communities. For example, Wagner and colleagues (2016) suggested that the plant genotype-by-sampling site interaction was a stronger driver than plant genotype only. Moreover, the occurrence of a contribution from stochastic processes was also observed (Maignien et al., 2014). Therefore, due to the high variability of phyllospheric community structure, a more profound knowledge about bacterial

communities hosted by different plant species in different environments is needed to assess their potential contribution to air bioremediation. Among plant species that can be typically found in urban areas, *Platanus* trees are widespread in most cities of central and southern Europe. They are frequently planted along high traffic roads, since they are known to be considerably resistant to stresses caused by urban pollution (Yang et al., 2015). To the best of our knowledge, bacterial communities associated to *Platanus* leaves were characterized only by Zhang et al. (2015), who however limited their research to the assessment of functional diversity of the culturable fraction.

The aims of this work were: (i) a deep phylogenetic characterization of bacterial communities hosted by urban *Platanus x acerifolia* leaves using high-throughput sequencing (HTS) methods; (ii) an evaluation of the diversity of alkane hydroxylase (*alk*B) phylotypes harboured by phyllospheric bacteria associated to urban *P. x acerifolia* trees; (iii) the assessment of temporal and spatial variability of bacterial phyllospheric communities associated to *P. x acerifolia* trees located in different areas of the city of Milan (Italy) and sampled in different months.

Materials and Methods

Sampling

Leaves were collected from eight different *Platanus x acerifolia* trees in the city of Milan (Italy). Four of them were located in an urban park (Parco Nord), next to a low-traffic secondary road, and the other four were planted along a high-traffic road (Viale Fulvio Testi), which is one of the major arterial roads in the northern part of the city (Fig. S1). Meteorological conditions and atmospheric pollutant concentrations for this area are reported in Fig. S2. Sampling was performed at the beginning (April 17, 2014) and in the middle of the growing season (July 11, 2014). For each tree, samples were collected in triplicates, for a

total of 48 samples. Each sample was composed by three young leaves in April and by two mature leaves in July, collected at a height ranging approximately between 1.50 and 2.00 m. Leaves were handled with metal scissors and tweezers rinsed with ethanol and immediately put in sterile 120 mm Petri dishes to prevent DNA contamination from external sources.

DNA extraction

Total DNA of epiphytic bacteria was extracted with FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA). Leaves were thoroughly rinsed in sterile Petri dishes with approximately 4 mL of Sodium Phosphate Buffer supplied with the kit under a laminar flow hood. After rinsing, it was possible to recover approximately 2 mL of the used buffer. It was collected from the Petri dish with a micropipette and placed in the kit Lysing Matrix E Tube. Further steps were performed according to manufacturer's instructions.

Illumina sequencing

The V5-V6 hypervariable regions of the bacterial 16S rRNA gene were PCR-amplified using 783F and 1046R primers (Huber et al., 2007; Wang and Qian, 2009). For the characterization of *alk*B diversity, three different primer pairs were preliminarily tested on our samples (pairs (d), (e) and (f) of Jurelevicius et al. (2013)); detectable amplification was obtained with primer pair (f) only, which was therefore chosen for subsequent analyses. At the 5' end of each primer, a 6-bp barcode was included to allow sample pooling and sequence sorting. All amplicons were sequenced by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) with a 250 bp × 2 paired-end protocol. For each sample, 2 × 75 µL volume PCR reactions were performed with GoTaq® G2 Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 µM of each primer. The cycling conditions for the amplification of the 16S rRNA gene fragment were: initial denaturation at 94 °C for 4 min; 28 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. The cycling conditions for the amplification of the *alk*B fragment were: initial denaturation at 96 °C for 4 min; 40 cycles at 96 °C for 45 s, 47 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 5 min. The amplicons were purified with the Wizard® SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI, USA) and purified DNA was quantified using Qubit® (Life Technologies, Carlsbad, CA, USA). Groups of 9/12 amplicons bearing different barcode pairs were pooled together to build a single library. Further library preparation with the addition of standard Nextera indexes (Illumina, Inc., San Diego, CA, USA) and sequencing were carried out at Parco Tecnologico Padano (Lodi, Italy).

Sequence analysis

Reads from both 16S rRNA and *alk*B genes sequencing were demultiplexed according to the indexes. Uparse pipeline was used for the following elaborations (Edgar, 2013). In case of 16S rRNA genes, forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters. Conversely, since *alk*B reads were not overlapping, only one read was analysed. Suspected chimeras and singleton sequences (i.e. sequences appearing only once in the whole data set) were removed. Phylotypes were defined on the whole data set clustering the sequences at a 97% of similarity and defining a representative sequence for each cluster. Representative sequences of 16S rRNA gene phylotypes (Operational Taxonomic Units – OTUs) were classified using SINA with SILVA database (Pruesse et al., 2012) and sequences not classified as belonging to Bacteria domain (i.e. Archaea, chloroplasts and mitochondria) were discarded. Abundance of each OTU was estimated by mapping the sequences of each sample against the remaining OTU representative sequences at 97% of similarity. Representative sequences of *alk*B gene phylotypes were translated into aminoacid sequences considering the proper frame and annotated with Blastp (Altschul et al., 1990).

Sequences not annotated as *alk*B were discarded; sequences of each sample were then mapped against the remaining representative phylotype sequences at 97% of similarity.

To assess the spatial and temporal variability both of the structure of phyllospheric bacterial communities hosted by *Platanus* leaves and of *alk*B phylotypes, samples were grouped according to their sampling location (urban park or high-traffic road) and to sampling month (April or July). Non-metric Multidimensional Scaling (NMDS) analyses based on Hellinger distances were performed using R (Vegan package) (Oksanen et al., 2009). Differences in abundance of the most abundant genera (≥ 2%) between months (April and July) or locations (park and road) were tested by t-tests. P-values were corrected for multiple testing according to the False Discovery Rate (FDR) procedure (Benjamini and Hochberg, 1995) using the MULTTEST package in R.

Results and Discussion

Phylogenetic diversity

From NMDS analysis, two main groups could be identified, corresponding to the April and July samples respectively (Fig. 1). Within each of the two sampling months, samples from the urban park and from the high-traffic road were close but clearly distinguishable. Therefore, it can be hypothesized that temporal changes, which are in turn related to seasonality, acted as a stronger driver on the *Platanus* phyllospheric community structure than sampling location. This is in agreement with the observations of several authors, e.g. Copeland et al. (2015), Rastogi et al. (2012) and Peñuelas et al. (2012), which identified seasonal changes as a major factor shaping bacterial phyllospheric communities associated to different plant species. Furthermore, environmental conditions and atmospheric pollutant concentrations are known to be substantially homogeneous in the Po Valley, where Milan is located; therefore, this area as a whole is generally considered as a pollutant hot-spot

(Marcazzan et al, 2002; Maurizi et al., 2013; Vecchi and Valli, 1999). For this reason, it can be hypothesized that environmental variables may have been not sufficiently different at the two sites, which are approximately 2 km from each other, to cause appreciable dissimilarities in bacterial community composition.

The relative abundance of the main bacterial phyllospheric populations at the taxonomic levels of Class and Genus is shown in Fig. 2 (a and b, respectively). Overall, the most abundant classes were *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Bacilli* and *Actinobacteria.* They have already been described by several authors as common classes in phyllospheric bacterial communities associated with different plant species (Dees et al., 2015; Rastogi et al., 2012; Vorholt, 2012; Whipps et al., 2008), although in some cases they were reported with very different relative abundances (Redford et al., 2010). In April, the *Platanus* phyllospheric communities were not clearly dominated by any class or genus. On the contrary, July communities exhibited a large prevalence of *Gammaproteobacteria*, with a relative abundance of approximately 50%. Within this class, most sequences belonged to the genus *Stenotrophomonas* (approximately 42% of total bacteria). This genus has already been reported to be one of the major genera commonly detected in phyllospheric communities, although at much lower percentages (Vorholt, 2012). Particularly, *Stenotrophomonas* has been generally described as a member of endophytic, rather than epiphytic, bacterial communities of different plant species (Ferrando and Fernández Scavino, 2015; Kgomotso et al., 2015; Mastretta et al., 2009; Romero et al., 2014; Taghavi et al., 2009). Several isolates belonging to this genus were demonstrated to possess plant-growth promoting properties (Calciolari and Silva, 2013; Islam et al., 2015). The same abilities were observed for a rhizospheric *S. maltophilia* strain and confirmed through genome sequencing (Wu et al., 2015). Furthermore, some plant-associated *Stenotrophomonas* strains were reported as able to degrade oil hydrocarbons (Ali et al., 2012) and phenanthrene (Muratova et

al., 2015). In a culture-independent study on endophytic communities of *Cucurbita pepo*, members of genera *Stenotrophomonas* and *Sphingomonas* showed a significantly higher abundance in the presence of DDE, the most common and persistent degradation product of the pesticide DDT, than in the absence of the molecule (Eevers et al., 2016). Thus, it can be hypothesized that the genus *Stenotrophomonas* may play a key role also in the ecology of phyllospheric communities associated to urban *Platanus* leaves. Table S1 reports the results of multiple t-tests on abundant genera that significantly varied between months. The genus *Hymenobacter* was identified as significantly more abundant in July phyllospheric communities, with average relative abundances of 11.1% and 4.3% in park and road samples, respectively. Some members of this genus have been described as radiation tolerant (Kim et al., 2016; Lee et al., 2014; Su et al., 2014) and psychrophilic or psychrotolerant (Klassen and Foght, 2011; Mi et al., 2014). Due to these features, it can be hypothesized that these bacteria may undergo a selection process, throughout the growing season, by the harsh conditions of the phyllospheric environment. Given the continuous exchange of bacterial populations between leaf surface and air, and the shared characteristics of high UV radiation and low temperature of the two environments, it is not surprising that the genus *Hymenobacter* was also reported in outdoor airborne communities (Fahlgren et al., 2011; Yooseph et al., 2013). The other genus identified as significantly more abundant in July samples was *Massilia* (Table S1). Members of this genus have already been described as commonly retrieved in phyllospheric epiphytic communities (Rastogi et al., 2013, 2012), as well as endophytes (Croes et al., 2015; Thijs et al., 2014). Therefore, it may have been enriched over time due to the selective conditions of the phyllospheric environment, which could favour it over other genera.

The genus *Buttiauxella* was the only one to be recognized as significantly more abundant in park samples, with average abundances of 8.3% and 2.0% in April and July samples,

241 respectively (Table S2). It is not reported to be one of the most common genera among
242 phyllospheric bacteria (Bulgarelli et al., 2013; Vorholt, 2012). However, some *Buttiauxella*
243 sp. strains were previously cultivated from atmospheric particulate matter (Fang et al., 2007;
244 Gandolfi et al., 2011). On the contrary, the only genus identified as significantly more
245 abundant in road communities was *Aeribacillus* (Table S2). Members of this genus have been
246 often described as thermophilic bacteria, isolated from hot springs, geothermal reservoirs and
247 different environments of sub-tropical areas (Aanniz et al., 2015; Filippidou et al., 2015;
248 Yanmis and Adiguzel, 2014). Moreover, some strains can produce exo-polysaccharides as a
249 way to survive high temperatures (Radchenkova et al., 2013; Zheng et al., 2012). These
250 features can possibly be also useful to deal with locally very high temperatures on leaf
251 surfaces exposed to solar radiation.
252 Among the other most abundant genera, as reported in Fig. 2b, *Sphingomonas*, *Arthrobacter*,
253 *Methylobacterium*, *Pseudomonas*, *Pantoea*, *Rhodococcus* and *Flavobacterium* have already
254 been retrieved in phyllospheric environments (Delmotte et al., 2009; Maignien et al., 2014;
255 Rastogi et al., 2013, 2012; Vorholt, 2012). Thus, a "core" of phyllospheric bacterial
256 communities appears to exist (Laforest-Lapointe et al., 2016), although the relative
257 abundance of each genus can show high variability both in different plant species and in
258 different individuals of the same plant species (Bulgarelli et al., 2013).
259 The average number of OTUs detected in April samples was significantly higher than that in
260 July samples (Fig. S3). Moreover, genera that were less abundant than 2% in all the four
261 sample groups, indicated as "Others" in Fig. 2b, together constituted approximately 61% and
262 26% of April and July communities, respectively. Thus, the diversity of bacterial
263 communities of young leaves appeared to be higher than that of the communities hosted by
264 older leaves, as already observed by several authors (Copeland et al., 2015; Dees et al., 2015;
265 Lindow and Brandl, 2003). This phenomenon is generally explained by a selection effect on

biodiversity, which is due both to harsh environmental conditions typical of the phyllospheric habitat and to the plant characteristics determined by its genotype (Whipps et al., 2008). It has also been suggested that seasonality and/or leaf maturation may determine a progressive decrease of nutrient availability (Dees et al., 2015), thus decreasing the number of bacterial populations that can be sustained. Nevertheless, this trend, although widespread, can not be considered to be the general rule, since in some cases phyllospheric communities remained stable over time (Delmotte et al., 2009), or even an increase in the richness of epiphytic bacteria was observed with increasing time of colonization (Peñuelas et al., 2012). Moreover, Laforest-Lapointe and colleagues (2016) observed that phyllospheric communities of five tree species in Canada underwent a succession during the growing season, although plant species was a stronger driver on bacterial diversity than sampling time. Therefore, more research is needed in order to better describe time-dependent shifts in phyllospheric community structures of an extensive range of plant species. This is particularly important for perennial plants, which can undergo a wide variability of climatic conditions throughout the year, especially in temperate areas.

Diversity of alkB phylotypes

In addition to the phylogenetic-based community structure, knowledge about potential metabolic abilities of phyllospheric bacteria and their functional diversity are of critical importance to assess their possible contribution to air remediation. Zhang et al. (2015) evaluated the carbon substrate utilization pattern through the BIOLOG method, in order to estimate the functional diversity of bacteria associated to leaves of urban trees in China, including a species of *Platanus* (*P. orientalis*). They found that phyllospheric communities associated with different trees significantly differed in their metabolic abilities. However, this method relies on laboratory cultivation. Thus, results are limited to the culturable fraction of

bacterial communities. For this reason, it would be also necessary to explore a range of suitable marker genes in phyllospheric metagenomes. Up to now, only *chi*A, encoding a chitinase, was extensively studied through amplicon HTS (Cretoiu et al., 2012). More comprehensive approaches were chosen instead, in order to identify the main metabolic adaptations to phyllospheric life: shotgun metagenomic sequencing was applied to bacterial communities hosted by *Tamarix aphylla* leaves (Finkel et al., 2016) while metaproteomics was used on soybean, clover and *Arabidopsis thaliana* communities (Delmotte et al., 2009). In this work, alkane hydroxylase (*alk*B) was selected as reference gene to roughly estimate the diversity of *Platanus* phyllospheric bacteria possessing the potential ability to degrade alkanes. Diversity of alkane hydroxylases has already been studied in the rhizosphere of different tree and grass species, both in isolates (Fatima et al., 2015; Tesar et al., 2002; Yousaf et al., 2010) and in whole bacterial communities through culture-independent methods (Mukherjee et al., 2015; Tsuboi et al., 2015). However, a characterization of the diversity of *alk*B phylotypes in phyllospheric communities is still lacking. From NMDS analysis, April and July samples were clearly distinguishable (Fig. 3).

Moreover, while samples from the two sampling locations formed two separate groups in April, in July they showed a high overlapping. Therefore, although *alk*B phylotypes were different in the two sampling locations at the beginning of the growing season, they became highly similar over time. As already observed for phylogenetic diversity, it can be hypothesized that both environmental conditions such as temperature, humidity and solar exposure, and pollution levels were probably similar at the two locations. Thus, not only bacterial communities considered as a whole, but also hydrocarbon-degrading populations could have been subjected to the same selection drivers regardless the sampling location. However, in contrast with what observed for phylogenetic biodiversity, the number of *alk*B phylotypes was significantly higher in July (Fig. S4). This led to put forward the hypothesis

316 that atmospheric hydrocarbons might play a key role in the selection of phyllospheric
317 populations in urban areas. In fact, the selective pressure they exert would cause a decrease in
318 phylogenetic diversity while increasing the diversity of hydrocarbon-degrading populations.
319 The overall number of detected *alk*B phylotypes was 3036. A phylogenetic tree was built
320 with the 51 phylotypes with a total abundance ≥ 0.3% (Fig. 4). Most of these phylotypes
321 clustered together, and showed high similarities with *alk*B from different species of the genus
322 *Rhodococcus*, particularly with *R. aetherivorans*, and with *Mycobacterium smegmatis*. Other
323 18 out of 51 phylotypes, which formed a separate cluster, revealed their best similarity with
324 uncultured bacteria from various molecular studies. Although there are no indications on the
325 taxonomy of these uncultured bacteria, the cluster to which they belong appears to be nearer
326 to that including *Rhodococcus* sequences than to other reference strains. However, when
327 comparing this cluster with sequences reported in a comprehensive *alk*B tree that was
328 recently published, it was not possible to clearly identify its position on it (Nie et al., 2014).
329 Conversely, only one of the considered phylotypes was highly similar to *alk*B belonging to a
330 Gammaproteobacteria genus, i.e. *Shewanella*. The high prevalence of sequences from
331 Actinobacteria suggests the mainly terrestrial origin of potential alkane-degrading bacteria
332 (Nie et al., 2014). However, the overall diversity of *alk*B phylotypes in bacterial communities
333 hosted by *Platanus* leaves, although increased over time as observed above, appears to be still
334 lower than that reported for other habitats (Nie et al., 2014). This may be possibly due to the
335 harsher conditions in the phyllospheric environment than in other environments, which limit
336 biodiversity.
337
338 **Conclusions**
339 A proper management of vegetation has been suggested to be a promising strategy to
340 decrease air pollution in urban areas. However, our understanding of the potential

effectiveness of urban plants in air quality improvement is still affected by several uncertainties. Therefore, we need at least to be able to estimate the actual involvement of plants, and of plant-phyllospheric bacteria associations, in air pollutant removal.

On *Platanus x acerifolia* leaves, biodiversity of bacterial communities decreased along the growing season, while the diversity of hydrocarbon-degrading populations increased. This phenomenon might indicate that, in the phyllosphere of urban plants, selection effects on bacteria are driven more strongly by atmospheric hydrocarbons than by other environmental factors, such as temperature, humidity or solar radiation. However, the actual ability of phyllospheric bacterial communities to degrade hydrocarbons *in situ* still needs to be confirmed. Therefore, future research should be aimed at the quantification of the actual contribution of bacteria in air pollutant removal per unit of leaf weight or leaf area under different environmental conditions, and at the evaluation of the efficiency of different plant-bacteria systems in air quality improvement.

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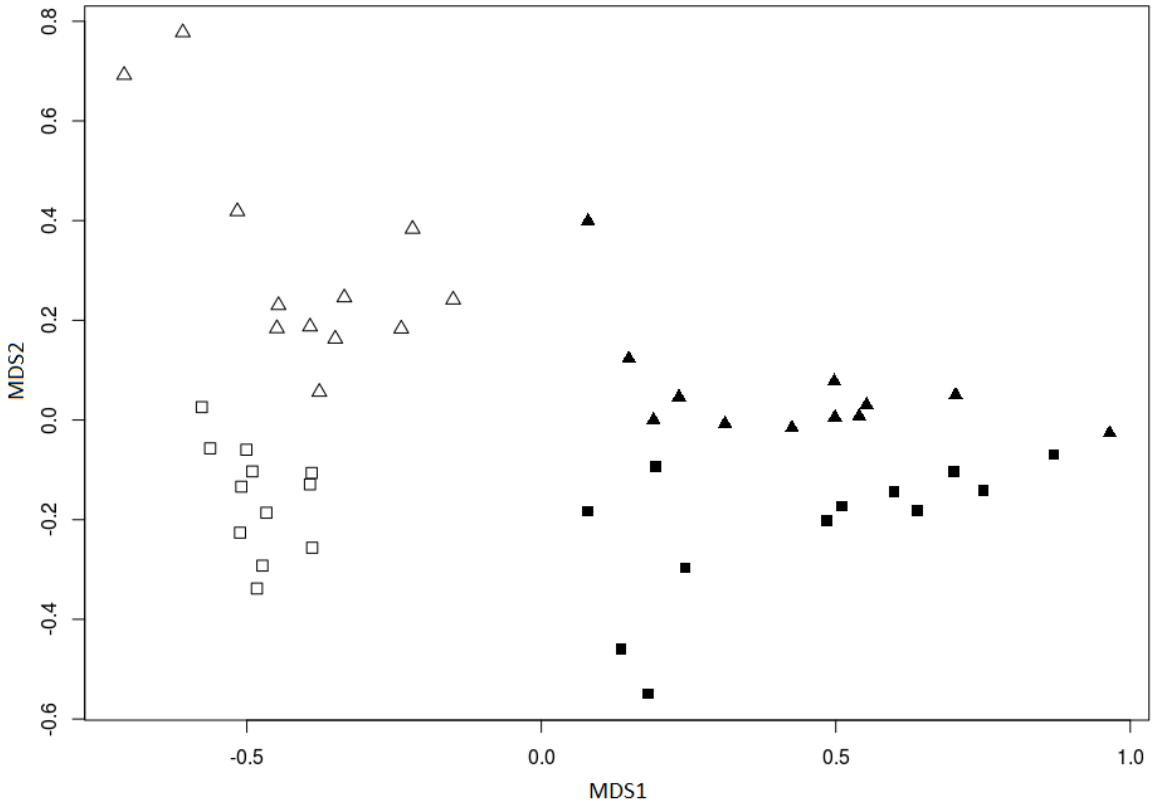
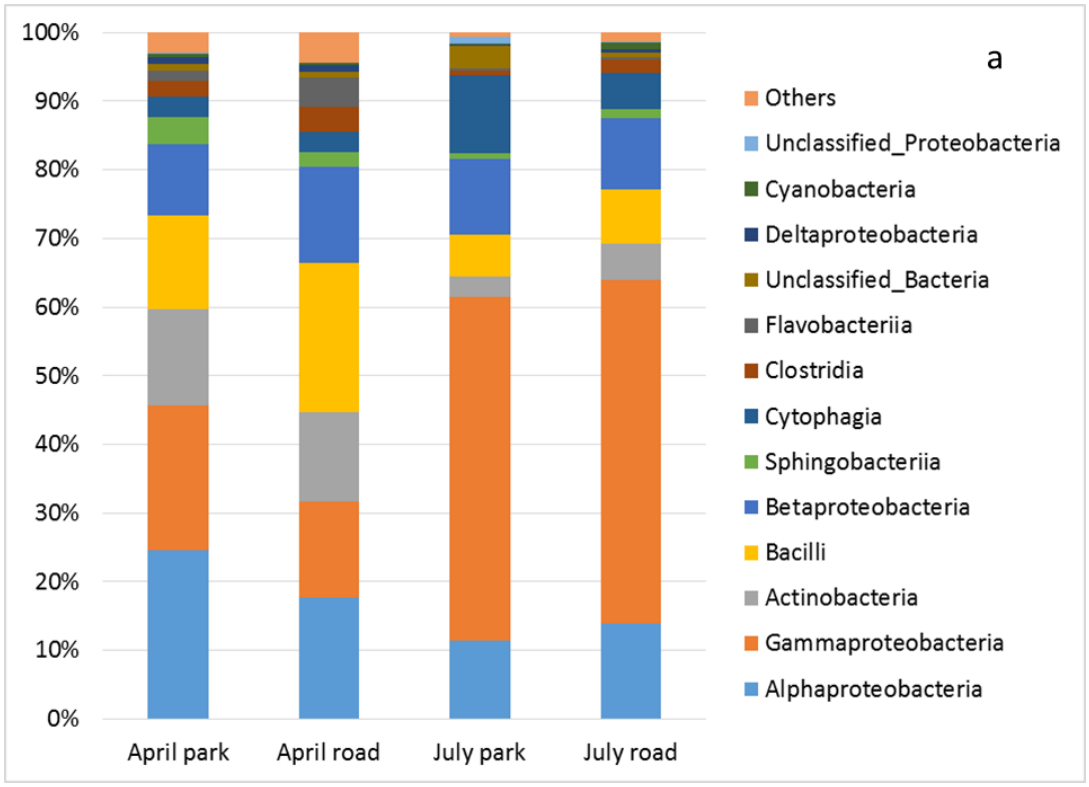


644

645 Fig. 1 – NMDS analysis of bacterial phylogenetic diversity. Hellinger distances among
646 samples were calculated on the basis of presence and abundance of OTUs. Empty symbols:
647 April; filled symbols: July; squares: park; triangles: road.

648


a
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
April park
April road
July park
July road
Others
Unclassified_Proteobacteria
Cyanobacteria
Deltaproteobacteria
Unclassified_Bacteria
Flavobacteriia
Clostridia
Cytophagia
Sphingobacteriia
Betaproteobacteria
Bacilli
Actinobacteria
Gammaproteobacteria
Alphaproteobacteria

649

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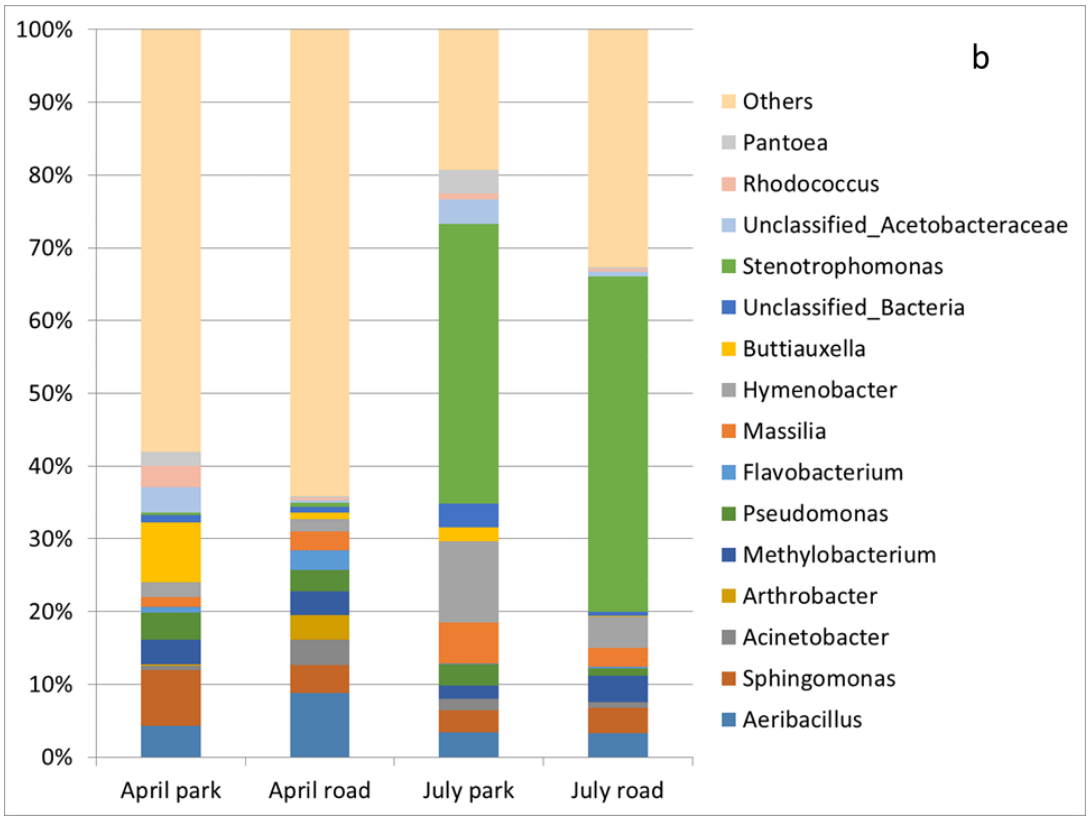
b
100%
90%
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70%
60%
50%
40%
30%
20%
10%
0%
April park
April road
July park
July road
Others
Pantoea
Rhodococcus
Unclassified_Acetobacteraceae
Stenotrophomonas
Unclassified_Bacteria
Buttiauxella
Hymenobacter
Massilia
Flavobacterium
Pseudomonas
Methylobacterium
Arthrobacter
Acinetobacter
Sphingomonas
Aeribacillus

651

652 Fig. 2 – Relative abundance of phyllospheric bacterial taxa at Class (a) and Genus (b) level.
653 Only taxa with an abundance ≥ 1% (Class) or ≥ 2% (Genus) in at least one of the four groups
654 of samples are shown. Samples are grouped according to month and sampling location.
655

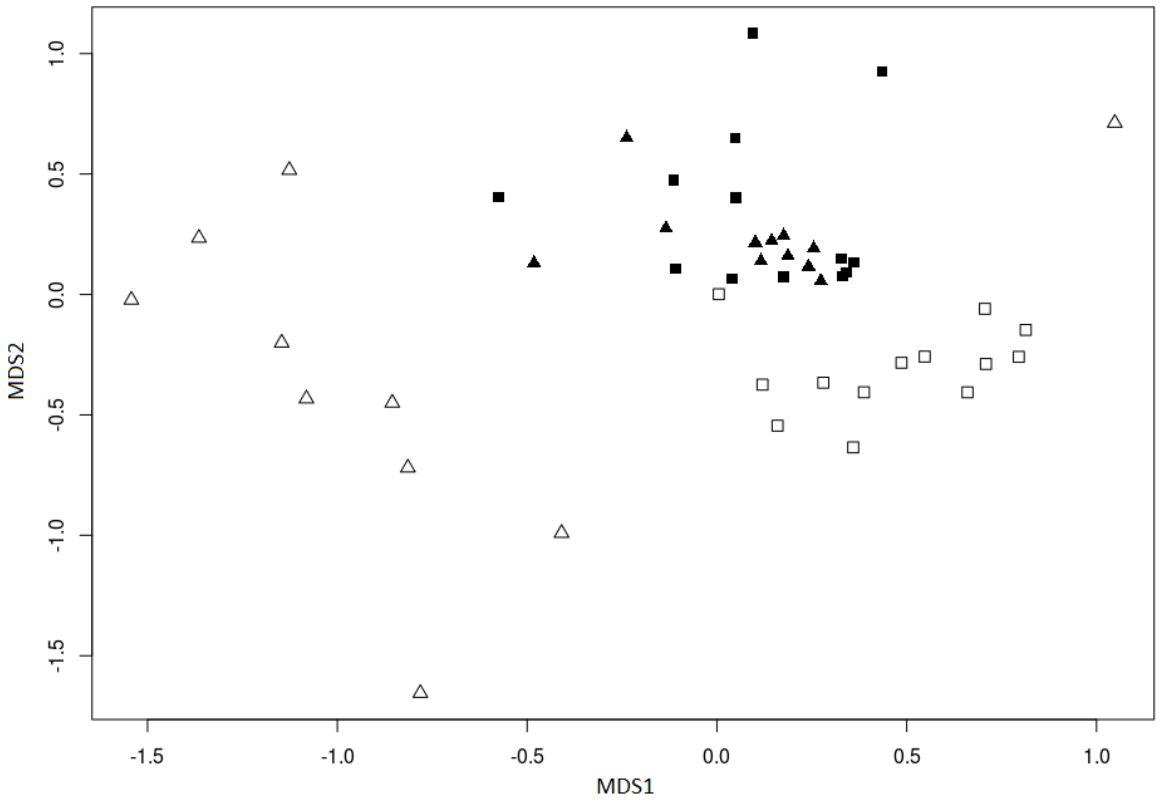
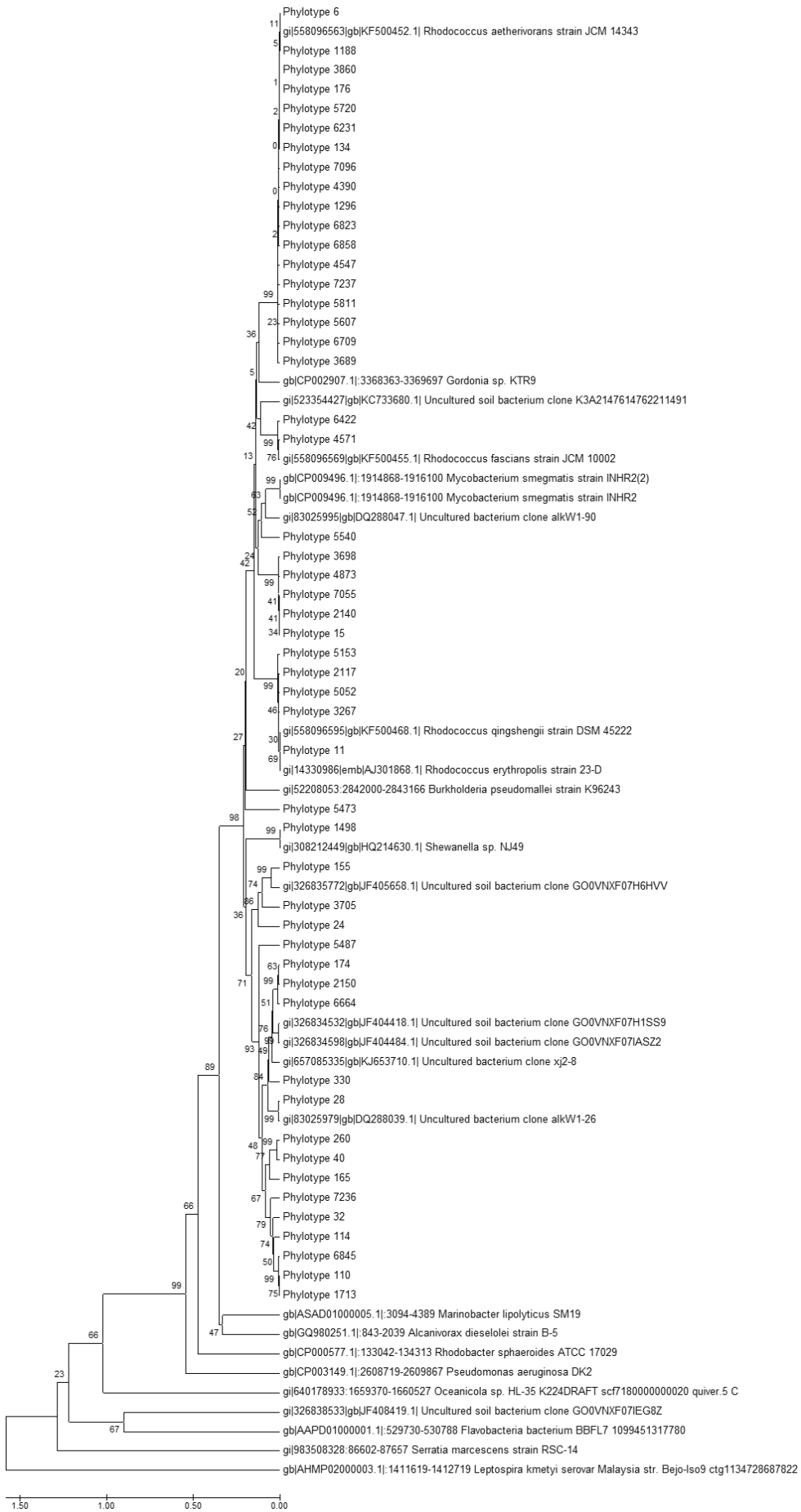


Fig. 3 – NMDS analysis of *alk*B diversity. Hellinger distances among samples were calculated on the basis of presence and abundance of OTUs. Empty symbols: April; filled symbols: July; squares: park; triangles: road.


Phylotype 6
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Phylotype 1188
Phylotype 3860
Phylotype 176
Phylotype 5720
Phylotype 6231
Phylotype 134
Phylotype 7096
Phylotype 4390
Phylotype 1296
Phylotype 6823
Phylotype 6858
Phylotype 4547
Phylotype 7237
Phylotype 5811
Phylotype 5607
Phylotype 6709
Phylotype 3689
gb|CP002907.1|:3368363-3369697 Gordonia sp. KTR9
gi|523354427|gb|KC733680.1| Uncultured soil bacterium clone K3A2147614762211491
Phylotype 6422
Phylotype 4571
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Phylotype 5540
Phylotype 3698
Phylotype 4873
Phylotype 7055
Phylotype 2140
Phylotype 15
Phylotype 5153
Phylotype 2117
Phylotype 5052
Phylotype 3267
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Phylotype 11
gi|14330986|emb|AJ301868.1| Rhodococcus erythropolis strain 23-D
gi|52208053:2842000-2843166 Burkholderia pseudomallei strain K96243
Phylotype 5473
Phylotype 1498
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Phylotype 155
gi|326835772|gb|JF405658.1| Uncultured soil bacterium clone GO0VNXF07H6HVV
Phylotype 3705
Phylotype 24
Phylotype 5487
Phylotype 174
Phylotype 2150
Phylotype 6664
gi|326834532|gb|JF404418.1| Uncultured soil bacterium clone GO0VNXF07H1SS9
gi|326834598|gb|JF404484.1| Uncultured soil bacterium clone GO0VNXF07IASZ2
gi|657085335|gb|KJ653710.1| Uncultured bacterium clone xj2-8
Phylotype 330
Phylotype 28
gi|83025979|gb|DQ288039.1| Uncultured bacterium clone alkW1-26
Phylotype 260
Phylotype 40
Phylotype 165
Phylotype 7236
Phylotype 32
Phylotype 114
Phylotype 6845
Phylotype 110
Phylotype 1713
gb|ASAD01000005.1|:3094-4389 Marinobacter lipolyticus SM19
gb|GQ980251.1|:843-2039 Alcanivorax dieselolei strain B-5
gb|CP000577.1|:133042-134313 Rhodobacter sphaeroides ATCC 17029
gb|CP003149.1|:2608719-2609867 Pseudomonas aeruginosa DK2
gi|640178933:1659370-1660527 Oceanicola sp. HL-35 K224DRAFT scf7180000000020 quiver.5 C
gi|326838533|gb|JF408419.1| Uncultured soil bacterium clone GO0VNXF07IEG8Z
gb|AAPD01000001.1|:529730-530788 Flavobacteria bacterium BBFL7 1099451317780
gi|983508328:86602-87657 Serratia marcescens strain RSC-14
gb|AHMP02000003.1|:1411619-1412719 Leptospira kmetyi serovar Malaysia str. Bejo-Iso9 ctg1134728687822
1.50
1.00
0.50
0.00

661

Fig. 4 – Phylogenetic tree of *alk*B phylotypes based on nucleotide sequence. Only phylotypes with a total abundance ≥ 0.3% were included. Sequences of *alk*B from some reference strains and from uncultured bacteria having a high similarity with phylotypes of this work were also included for comparison. The tree was built with the UPGMA method using MEGA7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.