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

3

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16 **Abstract**

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

34

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

38

39 **Keywords:** phyllosphere, phylloremediation, hydrocarbons, alkane hydroxylase, air  
40 purification

## 41 **Introduction**

42 Air pollution is a matter of global concern, especially in urban areas, due to the harmful  
43 effects of atmospheric pollutants on human health and on the environment. Current emission  
44 reduction methods and mitigation strategies are not adequate to fully meet the World Health  
45 Organization (WHO) guidelines for air pollutants (Ali et al., 2012; Weyens et al., 2015).  
46 Since most of the environmental problems in urban areas are generated at local level, often  
47 one of the most effective ways to deal with them is through local solutions (Bolund and  
48 Hunhammer, 1999). Plants have been suggested to effectively contribute to reduce air  
49 pollution levels and offsetting greenhouse gas emissions in cities (Beckett et al., 1998;  
50 Dzierzanowski et al., 2011; McPherson et al., 1998; Nowak and Crane, 2002; Nowak et al.,  
51 2006; Paoletti, 2009; Redford et al., 2010; Yang et al., 2005; Zhao et al., 2010). In this  
52 context, the regulation of ecosystem services (the direct and indirect contributions of  
53 ecosystems to human well-being (TEEB, 2011)) provided by vegetation in urban areas is of  
54 great importance (Baró et al., 2014). Many studies, in fact, indicated that the management of  
55 urban forests to enhance ecosystem service supply can be a cost-effective strategy to meet  
56 specific environmental standards or policy targets (Escobedo et al., 2010, 2011).  
57 Furthermore, it has been recognized that also plant-associated bacteria can play a crucial role  
58 in air bioremediation processes (Glick, 2015; Weyens et al., 2015). Particularly, the aerial  
59 parts of terrestrial plants, mainly leaves (i.e. the phyllosphere) host huge amounts of bacteria.  
60 In fact, although phyllospheric microorganisms comprise also fungi, yeasts, algae, protozoa  
61 and nematodes, bacteria are by far the most abundant inhabitants of leaf surfaces (Lindow  
62 and Brandl, 2003). Since phyllospheric bacteria are often found at an average of  $10^6$ - $10^7$  cells  
63  $\text{cm}^{-2}$  of leaf surface (Lindow and Brandl, 2003), the planetary phyllospheric bacterial  
64 population has been estimated to be as large as  $10^{26}$  cells (Morris and Kinkel, 2015). Among  
65 them, epiphytic bacteria, which primarily live on leaf surfaces, are directly positioned to the

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

75 Despite their continuous exchange with airborne populations (Lighthart, 2006;  
76 Lympelopoulou et al., 2016; Whipps et al., 2008), phyllospheric bacteria are not random  
77 assemblages but they rather form actual communities. In fact, some bacterial taxa are  
78 recurrently retrieved from leaf-associated habitats, leading to the hypothesis that, after  
79 recruitment, they undergo some selection processes (Delmotte et al., 2009; Rastogi et al.,  
80 2013; Vorholt, 2012; Yang et al., 2001). The relative abundance of a specific bacterial taxon  
81 in phyllospheric communities, however, can vary considerably. The main drivers that were  
82 suggested to shape community structure include host plant species, leaf age and physiology,  
83 season, geographical location, and environmental factors, such as solar radiation, humidity  
84 and nutrient availability (Laforest-Lapointe et al., 2016; Müller and Ruppel, 2014; Peñuelas  
85 et al., 2012; Rastogi et al., 2012; Redford et al., 2010; Vokou et al., 2012). Interactions  
86 between these factors can also affect bacterial communities. For example, Wagner and  
87 colleagues (2016) suggested that the plant genotype-by-sampling site interaction was a  
88 stronger driver than plant genotype only. Moreover, the occurrence of a contribution from  
89 stochastic processes was also observed (Maignien et al., 2014). Therefore, due to the high  
90 variability of phyllospheric community structure, a more profound knowledge about bacterial

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

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

106

## 107 **Materials and Methods**

### 108 *Sampling*

109 Leaves were collected from eight different *Platanus x acerifolia* trees in the city of Milan  
110 (Italy). Four of them were located in an urban park (Parco Nord), next to a low-traffic  
111 secondary road, and the other four were planted along a high-traffic road (Viale Fulvio Testi),  
112 which is one of the major arterial roads in the northern part of the city (Fig. S1).

113 Meteorological conditions and atmospheric pollutant concentrations for this area are reported  
114 in Fig. S2. Sampling was performed at the beginning (April 17, 2014) and in the middle of  
115 the growing season (July 11, 2014). For each tree, samples were collected in triplicates, for a

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

120

#### 121 *DNA extraction*

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

128

#### 129 *Illumina sequencing*

130 The V5-V6 hypervariable regions of the bacterial 16S rRNA gene were PCR-amplified using  
131 783F and 1046R primers (Huber et al., 2007; Wang and Qian, 2009). For the characterization  
132 of *alkB* diversity, three different primer pairs were preliminarily tested on our samples (pairs  
133 (d), (e) and (f) of Jurelevicius et al. (2013)); detectable amplification was obtained with  
134 primer pair (f) only, which was therefore chosen for subsequent analyses. At the 5' end of  
135 each primer, a 6-bp barcode was included to allow sample pooling and sequence sorting. All  
136 amplicons were sequenced by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) with a  
137 250 bp  $\times$  2 paired-end protocol. For each sample, 2  $\times$  75  $\mu$ L volume PCR reactions were  
138 performed with GoTaq® G2 Green Master Mix (Promega Corporation, Madison, WI, USA)  
139 and 1  $\mu$ M of each primer. The cycling conditions for the amplification of the 16S rRNA gene  
140 fragment were: initial denaturation at 94 °C for 4 min; 28 cycles at 94 °C for 50 s, 47 °C for

141 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. The cycling conditions for  
142 the amplification of the *alkB* fragment were: initial denaturation at 96 °C for 4 min; 40 cycles  
143 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.  
144 The amplicons were purified with the Wizard® SV Gel and PCR Clean-up System (Promega  
145 Corporation, Madison, WI, USA) and purified DNA was quantified using Qubit® (Life  
146 Technologies, Carlsbad, CA, USA). Groups of 9/12 amplicons bearing different barcode  
147 pairs were pooled together to build a single library. Further library preparation with the  
148 addition of standard Nextera indexes (Illumina, Inc., San Diego, CA, USA) and sequencing  
149 were carried out at Parco Tecnologico Padano (Lodi, Italy).

150

#### 151 *Sequence analysis*

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



166 Sequences not annotated as *alkB* were discarded; sequences of each sample were then mapped  
167 against the remaining representative phylotype sequences at 97% of similarity.  
168 To assess the spatial and temporal variability both of the structure of phyllospheric bacterial  
169 communities hosted by *Platanus* leaves and of *alkB* phylotypes, samples were grouped  
170 according to their sampling location (urban park or high-traffic road) and to sampling month  
171 (April or July). Non-metric Multidimensional Scaling (NMDS) analyses based on Hellinger  
172 distances were performed using R (Vegan package) (Oksanen et al., 2009). Differences in  
173 abundance of the most abundant genera ( $\geq 2\%$ ) between months (April and July) or locations  
174 (park and road) were tested by t-tests. P-values were corrected for multiple testing according  
175 to the False Discovery Rate (FDR) procedure (Benjamini and Hochberg, 1995) using the  
176 MULTTEST package in R.

177

## 178 **Results and Discussion**

### 179 *Phylogenetic diversity*

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

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

195 The relative abundance of the main bacterial phyllospheric populations at the taxonomic  
196 levels of Class and Genus is shown in Fig. 2 (a and b, respectively). Overall, the most  
197 abundant classes were *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*,  
198 *Bacilli* and *Actinobacteria*. They have already been described by several authors as common  
199 classes in phyllospheric bacterial communities associated with different plant species (Dees  
200 et al., 2015; Rastogi et al., 2012; Vorholt, 2012; Whipps et al., 2008), although in some cases  
201 they were reported with very different relative abundances (Redford et al., 2010). In April,  
202 the *Platanus* phyllospheric communities were not clearly dominated by any class or genus.  
203 On the contrary, July communities exhibited a large prevalence of *Gammaproteobacteria*,  
204 with a relative abundance of approximately 50%. Within this class, most sequences belonged  
205 to the genus *Stenotrophomonas* (approximately 42% of total bacteria). This genus has already  
206 been reported to be one of the major genera commonly detected in phyllospheric  
207 communities, although at much lower percentages (Vorholt, 2012). Particularly,  
208 *Stenotrophomonas* has been generally described as a member of endophytic, rather than  
209 epiphytic, bacterial communities of different plant species (Ferrando and Fernández Scavino,  
210 2015; Kgomotso et al., 2015; Mastretta et al., 2009; Romero et al., 2014; Taghavi et al.,  
211 2009). Several isolates belonging to this genus were demonstrated to possess plant-growth  
212 promoting properties (Calciolari and Silva, 2013; Islam et al., 2015). The same abilities were  
213 observed for a rhizospheric *S. maltophilia* strain and confirmed through genome sequencing  
214 (Wu et al., 2015). Furthermore, some plant-associated *Stenotrophomonas* strains were  
215 reported as able to degrade oil hydrocarbons (Ali et al., 2012) and phenanthrene (Muratova et

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

239 The genus *Buttiauxella* was the only one to be recognized as significantly more abundant in  
240 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

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

281

### 282 *Diversity of alkB phylotypes*

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

291 bacterial communities. For this reason, it would be also necessary to explore a range of  
292 suitable marker genes in phyllospheric metagenomes. Up to now, only *chiA*, encoding a  
293 chitinase, was extensively studied through amplicon HTS (Cretoiu et al., 2012). More  
294 comprehensive approaches were chosen instead, in order to identify the main metabolic  
295 adaptations to phyllospheric life: shotgun metagenomic sequencing was applied to bacterial  
296 communities hosted by *Tamarix aphylla* leaves (Finkel et al., 2016) while metaproteomics  
297 was used on soybean, clover and *Arabidopsis thaliana* communities (Delmotte et al., 2009).  
298 In this work, alkane hydroxylase (*alkB*) was selected as reference gene to roughly estimate  
299 the diversity of *Platanus* phyllospheric bacteria possessing the potential ability to degrade  
300 alkanes. Diversity of alkane hydroxylases has already been studied in the rhizosphere of  
301 different tree and grass species, both in isolates (Fatima et al., 2015; Tesar et al., 2002;  
302 Yousaf et al., 2010) and in whole bacterial communities through culture-independent  
303 methods (Mukherjee et al., 2015; Tsuboi et al., 2015). However, a characterization of the  
304 diversity of *alkB* phylotypes in phyllospheric communities is still lacking. From NMDS  
305 analysis, April and July samples were clearly distinguishable (Fig. 3).  
306 Moreover, while samples from the two sampling locations formed two separate groups in  
307 April, in July they showed a high overlapping. Therefore, although *alkB* phylotypes were  
308 different in the two sampling locations at the beginning of the growing season, they became  
309 highly similar over time. As already observed for phylogenetic diversity, it can be  
310 hypothesized that both environmental conditions such as temperature, humidity and solar  
311 exposure, and pollution levels were probably similar at the two locations. Thus, not only  
312 bacterial communities considered as a whole, but also hydrocarbon-degrading populations  
313 could have been subjected to the same selection drivers regardless the sampling location.  
314 However, in contrast with what observed for phylogenetic biodiversity, the number of *alkB*  
315 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 *alkB* phylotypes was 3036. A phylogenetic tree was built  
320 with the 51 phylotypes with a total abundance  $\geq 0.3\%$  (Fig. 4). Most of these phylotypes  
321 clustered together, and showed high similarities with *alkB* 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 *alkB* 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 *alkB* 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 *alkB* 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

341 effectiveness of urban plants in air quality improvement is still affected by several  
342 uncertainties. Therefore, we need at least to be able to estimate the actual involvement of  
343 plants, and of plant-phyllospheric bacteria associations, in air pollutant removal.  
344 On *Platanus x acerifolia* leaves, biodiversity of bacterial communities decreased along the  
345 growing season, while the diversity of hydrocarbon-degrading populations increased. This  
346 phenomenon might indicate that, in the phyllosphere of urban plants, selection effects on  
347 bacteria are driven more strongly by atmospheric hydrocarbons than by other environmental  
348 factors, such as temperature, humidity or solar radiation. However, the actual ability of  
349 phyllospheric bacterial communities to degrade hydrocarbons *in situ* still needs to be  
350 confirmed. Therefore, future research should be aimed at the quantification of the actual  
351 contribution of bacteria in air pollutant removal per unit of leaf weight or leaf area under  
352 different environmental conditions, and at the evaluation of the efficiency of different plant-  
353 bacteria systems in air quality improvement.

354

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357

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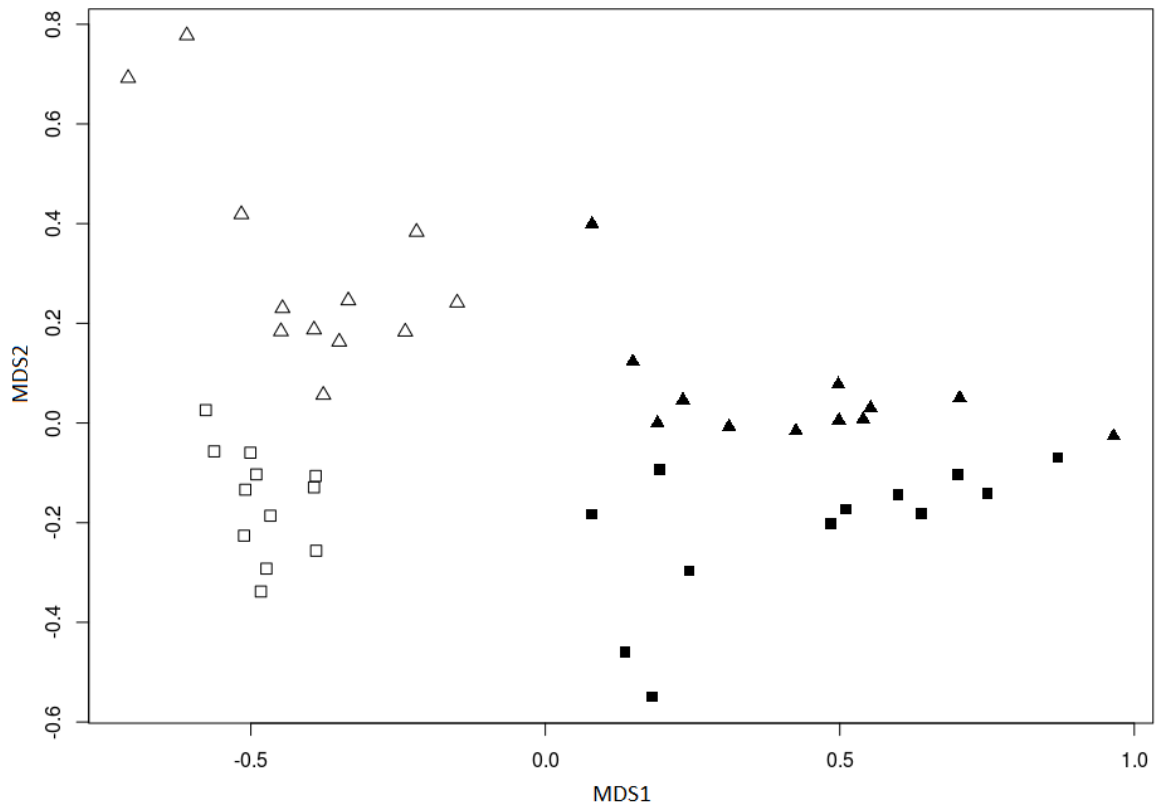
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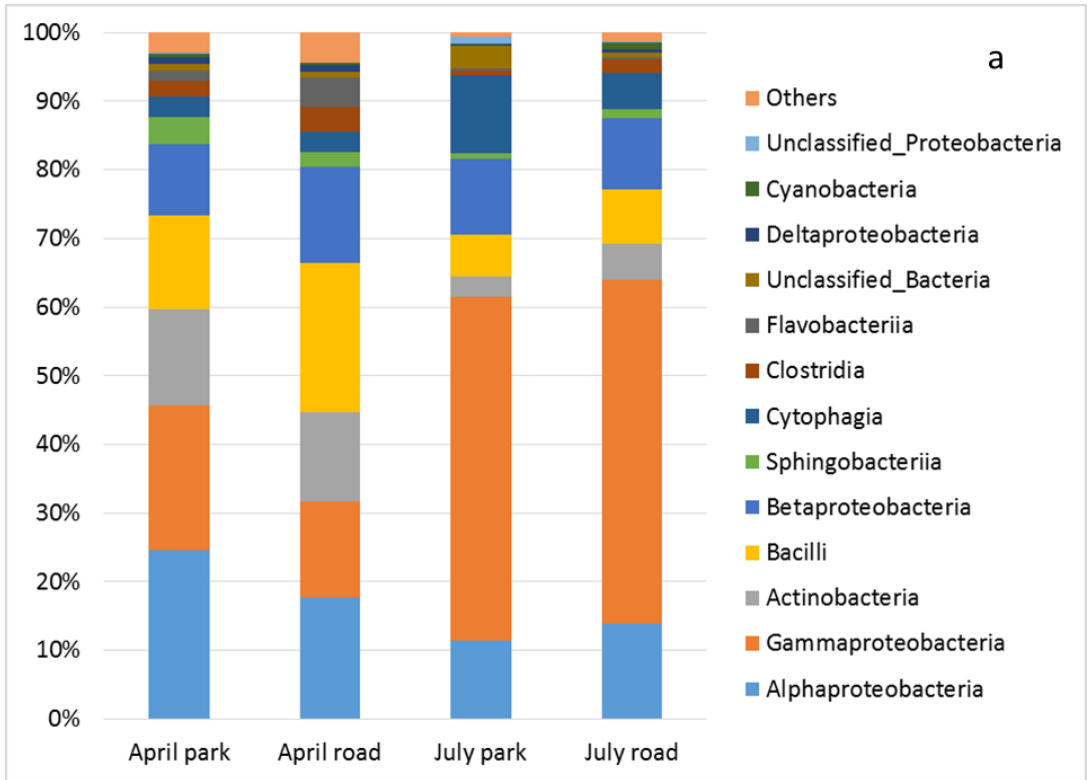
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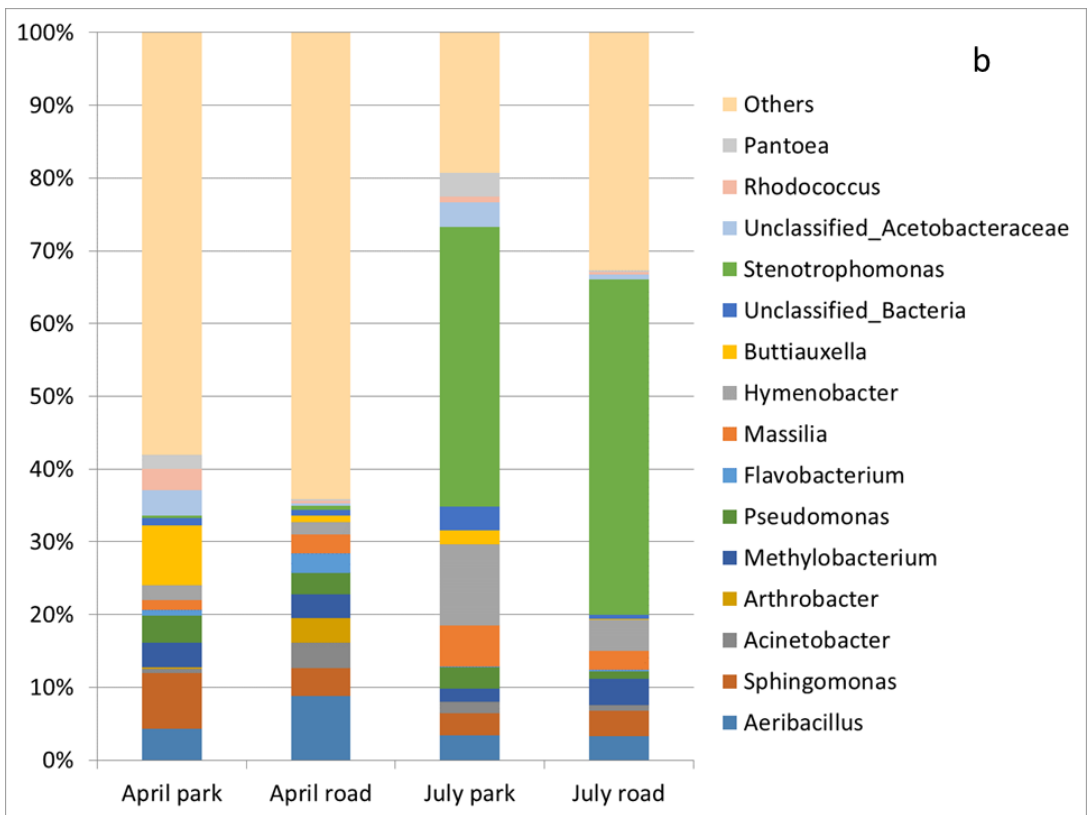
645 Fig. 1 – NMDS analysis of bacterial phylogenetic diversity. Hellinger distances among  
 646 samples were calculated on the basis of presence and abundance of OTUs. Empty symbols:  
 647 April; filled symbols: July; squares: park; triangles: road.

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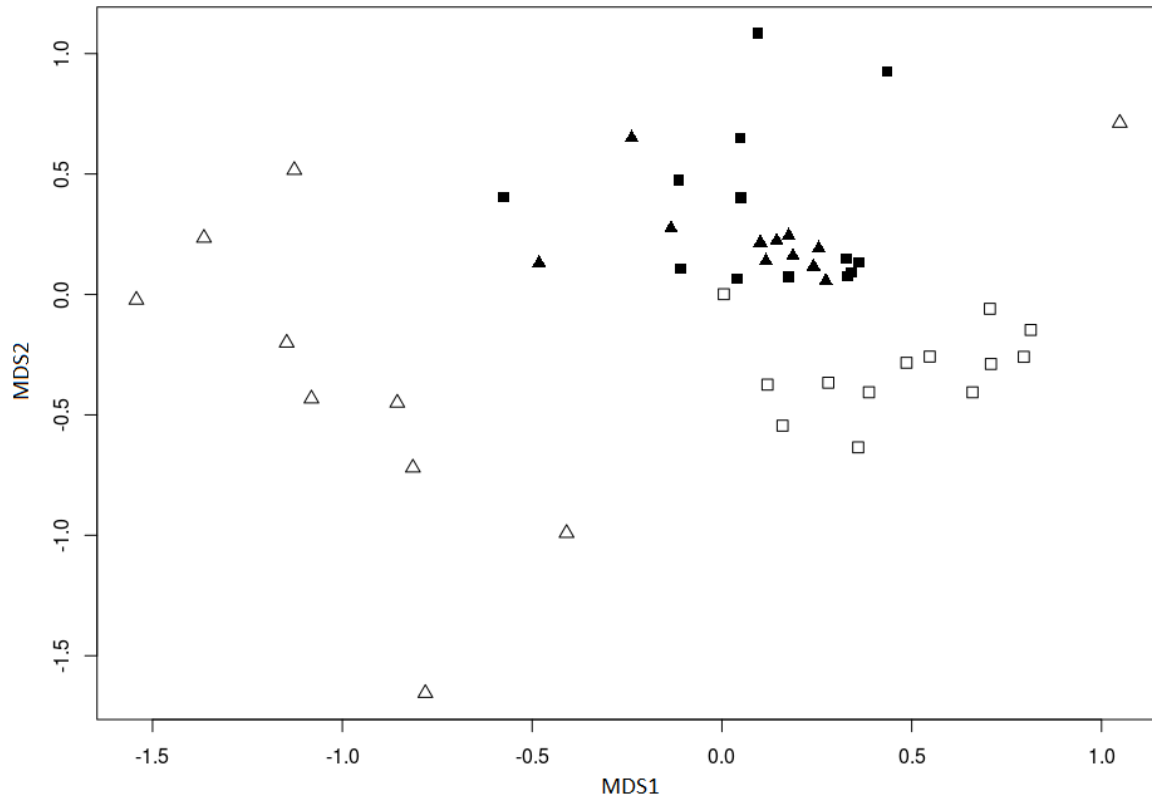
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652 Fig. 2 – Relative abundance of phyllospheric bacterial taxa at Class (a) and Genus (b) level.  
653 Only taxa with an abundance  $\geq 1\%$  (Class) or  $\geq 2\%$  (Genus) in at least one of the four groups  
654 of samples are shown. Samples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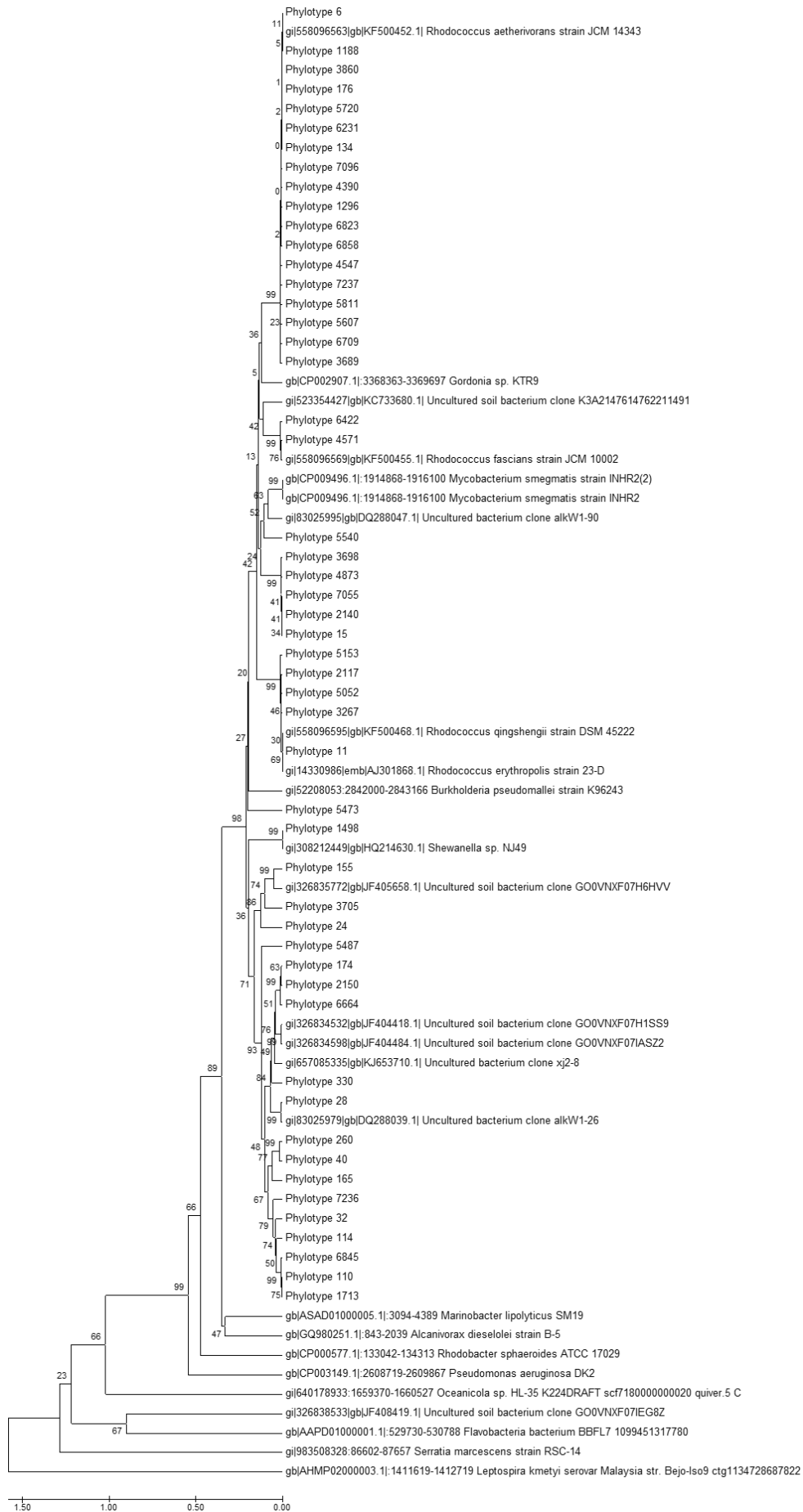


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657 Fig. 3 – NMDS analysis of *alkB* diversity. Hellinger distances among samples were  
 658 calculated on the basis of presence and abundance of OTUs. Empty symbols: April; filled  
 659 symbols: July; squares: park; triangles: road.

660





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