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## Urban greenness influences airborne bacterial community composition



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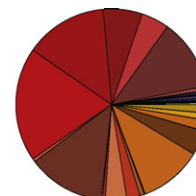
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### HIGHLIGHTS

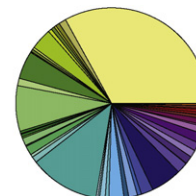
- We compared airborne bacterial samples from parks and parking lots with 16S sequencing.
- Bacterial communities from parks and parking lots were compositionally distinct.
- Proportion of vegetated area within 50 m explained 15% of the variation among samples.
- Parking lots had similar bacterial signatures, but parks tended to be unique.
- Passive and active collection methods gave comparable results.

### GRAPHICAL ABSTRACT

#### Parking Lot



#### Park



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### ABSTRACT

Urban green space provides health benefits for city dwellers, and new evidence suggests that microorganisms associated with soil and vegetation could play a role. While airborne microorganisms are ubiquitous in urban areas, the influence of nearby vegetation on airborne microbial communities remains poorly understood. We examined airborne microbial communities in parks and parking lots in Eugene, Oregon, using high-throughput sequencing of the bacterial 16S rRNA gene on the Illumina MiSeq platform to identify bacterial taxa, and GIS to measure vegetation cover in buffer zones of different diameters. Our goal was to explore variation among highly vegetated (parks) versus non-vegetated (parking lots) urban environments. A secondary objective was to evaluate passive versus active collection methods for outdoor airborne microbial sampling. Airborne bacterial communities from five parks were different from those of five parking lots ( $p = 0.023$ ), although alpha diversity was similar. Direct gradient analysis showed that the proportion of vegetated area within a 50 m radius of the sampling station explained 15% of the variation in bacterial community composition. A number of key taxa, including several *Acidobacteriaceae* were substantially more abundant in parks, while parking lots had higher relative abundance of *Acetobacteraceae*. Parks had greater beta diversity than parking lots, i.e. individual parks were characterized by unique bacterial signatures, whereas parking lot communities tended to be similar to each other. Although

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parks and parking lots were selected to form pairs of nearby sites, spatial proximity did not appear to affect compositional similarity. Our results also showed that passive and active collection methods gave comparable results, indicating the “settling dish” method is effective for outdoor airborne sampling. This work sets a foundation for understanding how urban vegetation may impact microbial communities, with potential implications for designing neighborhoods and open space systems that foster better human health.

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## 1. Introduction

Human well-being in urban areas is linked to the abundance and degree of access to nearby green space (e.g., Maas 2006; Maas et al. 2009; Villeneuve et al., 2012; Mitchell and Popham 2007; Davvand et al. 2012; Donovan et al. 2013). However, the specific mechanisms linking health and green space are not well understood. New evidence indicates that exposure to microbial diversity, especially from soil, plants and some animals, is an understudied pathway through which health benefits may arise (Von Hertzen and Haahtela 2006; Hanski et al. 2012; Fall et al. 2015).

Humans evolved under constant exposure to air, water, and soil containing a diversity of environmental microbes. However, over the past few centuries our lifestyles have shifted dramatically (indoor living, antibiotic use, processed food, chemical treatment of water, etc.) and, in the process, this has altered the abundance, diversity, and composition of the microbial communities to which we are exposed on a daily basis (Blaser and Falkow 2009). While these shifts in lifestyle have been associated with reduced incidence of many diseases, greater longevity, and other benefits, it is also now widely recognized that early life immunological experiences, including exposures to various environmental substances as well as the lack of exposures, are associated with the development of later life immune-mediated disease, such as asthma, allergy and other inflammatory disorders (Russell et al. 2012; Rook, 2013; Ege et al. 2012).

There is evidence that airborne microbial communities vary across major land use types, e.g. forest, agricultural land and urban areas (Bowers et al. 2010; Shaffer and Lighthart 1997; Burrows et al. 2009). At local scales, spatial proximity is an important predictor of microbial community similarity among outdoor samples (Adams et al. 2013; Adams et al. 2014), although long-range transport is also known to play a significant role in shaping airborne bioaerosol composition (Barberán et al. 2015). Little is known, however, about how airborne microbial composition varies within an urban area, nor what factors influence its variation. Beginning to describe fine-scale biogeographic patterns, such as distance-decay relationships (i.e. the spatial distance at which similarity of microbial community composition breaks down) at the scale of urban blocks and neighborhoods, would be a valuable contribution to the scientific knowledge base (Womack et al. 2010).

Vegetation structure and composition could play a role in the localized variability of microbial communities. Plants are important sources of airborne microorganisms (Lindemann and Upper 1985; Lindemann et al. 1982; Bowers et al. 2011). It is estimated that leaf surfaces comprise the largest biological surface type on the planet – over a billion  $\text{km}^2$  – and may host up to  $10^8$  bacteria per  $\text{cm}^2$  (Delmotte et al., 2009; Vorholt 2012; Peñuelas and Terradas, 2014). These leaf-inhabiting microorganisms become airborne during plant processes, like evapotranspiration, as well as by meteorological processes, such as rain splash, wind gusts and thermal plumes (Lighthart et al. 2009; Whipps et al. 2008). Different species of plants are associated with different compositions and emission rates of microbes (Lambais et al. 2014; Jumpponen and Jones 2010; Lindow and Brandl 2003; Kembel et al., 2014; Vokou et al. 2012). Although culture-based investigations of the population dynamics of leaf-surface microbes concluded that, “nearby vegetation strongly influences the atmospheric microbial concentration and composition at a given location” (Kinkel 1997), there have been few studies

using modern molecular techniques. A notable exception is the recent work by Lympelopoulou et al. (2016) investigating the abundance and composition of airborne microbes in relation to the sample’s proximity to vegetated versus non-vegetated areas. Their results showed that local vegetation could contribute up to half of the airborne bacteria found at a distance of 50 m downwind.

The primary goal of this study was to explore how urban vegetation, spatial proximity of sample sites, and other factors influence the composition of airborne microbial communities, specifically focusing on bacteria (fungal analysis to follow in a separate paper). We hypothesized that locations with large amounts of vegetation would have different airborne bacterial communities than areas with little or no vegetation, and that spatial proximity would have less influence on composition than the amount of vegetation. In the longer term, this course of investigation has the potential to substantially change our understanding of how to design healthy urban neighborhoods.

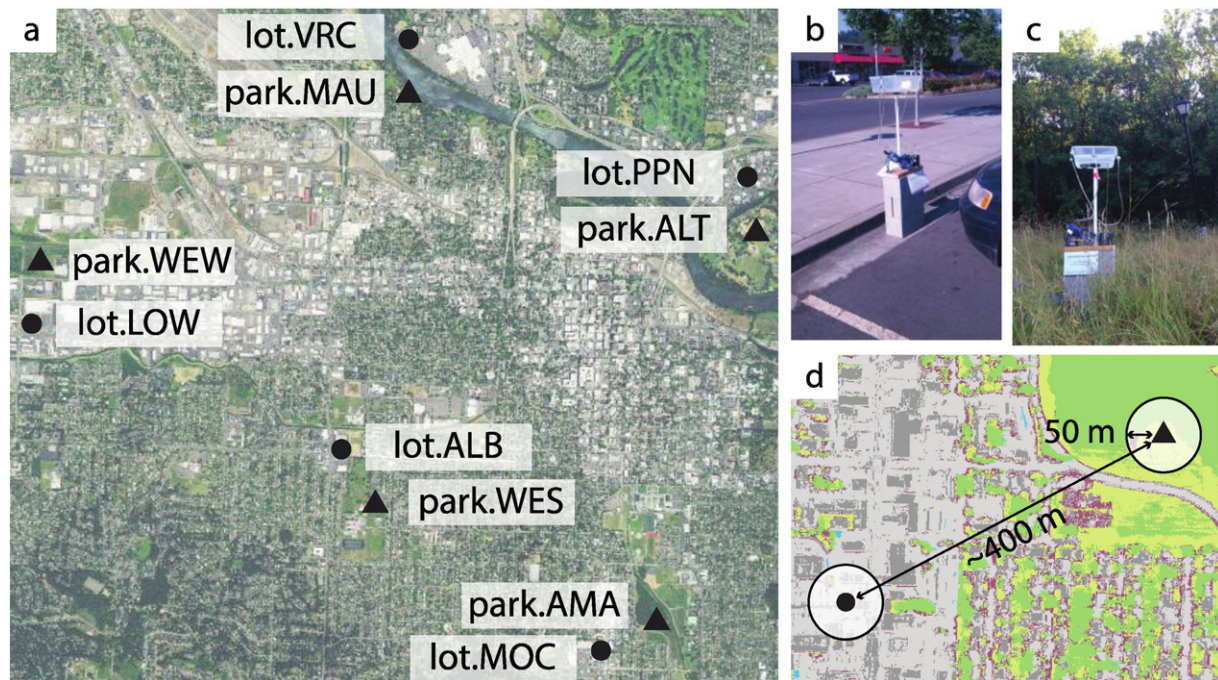
## 2. Materials & methods

### 2.1. Field sampling

We collected air samples for an eight-hour period on July 24th, 2013, beginning at 0800. Six samples from each site were collected simultaneously at five pairs of parks and nearby parking lots in Eugene, Oregon (Fig. 1a) for a total of 60 samples. The sampling station consisted of a custom tray (sterilized prior to use with 99% isopropyl alcohol) containing 3 passive settling dishes with their lids and 3 vacuum pump-powered button filters attached to the sides of the tray (Fig. 1b–1c) placed approximately 2 m above ground level in a relatively open area (i.e. not directly underneath tree canopy or other obstruction). SKC Button Samplers and SKC AirChex XR5000 Pumps (SKC Inc., Eighty Four, PA, USA) were set to draw 4 l/min (~1920 l total for the sampling period) through 25 mm-diameter cellulose ester filters (1.4  $\mu\text{m}$  pore diameter; autoclaved prior to sample collection), and HOBO U52 dataloggers (Onset Corporation) were used to measure temperature and relative humidity at 1-minute intervals. Technicians were present at each site to monitor the sampling equipment and perform hourly wind speed and direction measurements. All air samples were frozen at  $-80^\circ\text{C}$  immediately following sampling and stored frozen until processing.

### 2.2. GIS data

Urban environmental characteristics were measured using ArcGIS (Esri Inc., 2013) with geospatial data accessed from the Lane Council of Governments and the National Agriculture Imagery Program. All data layers were imported into a new geodatabase and re-projected to the NAD 1983 HARN StatePlane Oregon South (Feet Intl) Coordinate System, based on a Lambert Conformal Conic Projection. Six primary land cover types were identified (built, paved, dirt, grass, trees and shrubs, and water) using supervised maximum likelihood classification of aerial 4-band orthoimagery at 1-meter resolution. To assess the amount of surrounding vegetation, buffer zones of 50 m, 100 m, 200 m, 400 m, and 800 m radii were created around each sampling point and the proportion of vegetated area (grass + trees and shrubs) within each buffer zone was calculated using the classified raster image (Fig. 1d and Table SI in Supplementary material).



**Fig. 1.** a) Map of paired park and parking lot sites; b) photo of sampling station in parking lot; c) photo of sampling station in park; and d) classification image showing 50-m buffer zones and approximate distance between park-lot pairs.

### 2.3. DNA amplification and sequencing

The petri dishes and their lids were swabbed with nylon flocked swabs (copanusa.com; 552C), and DNA was extracted directly from the swabs and filter samples using the MO BIO PowerWater DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to manufacturer's instructions. Protocols followed those of Meadow et al. (2013), and negative controls were included at each step to evaluate potential contamination. We amplified the V4 region of the bacterial 16S rRNA gene using F515/R806 primers (5' -GTGCCAGCMGCCGCGG- 3', 5' -TACNVGGTATCTAATCC- 3') (Caporaso et al., 2012; Claesson et al. 2010). The samples were sequenced as paired-end reads at the Dana-Farber/Harvard Cancer Center DNA Resource Core (Boston, MA, USA; dnaseq.med.harvard.edu) using the Illumina MiSeq platform.

### 2.4. Analyses

Raw sequences were processed using the FastX Toolkit and QIIME pipeline (Caporaso et al. 2010). After recombining the barcodes from paired-end reads, forward reads were used for analysis due to lower quality reverse reads. In quality filtering, sequences that did not meet a 30 quality score over at least 75% of the read, or had ambiguous bases, or more than one primer mismatch, were removed. Sequence read lengths were trimmed to 250 bp, and taxonomy assignment was performed on a reference set of high-quality sequences using the open-reference OTU picking function in QIIME, which uses UCLUST (Edgar 2010). OTU clusters with a 97% similarity were identified using the Greengenes 13.5 database (DeSantis et al. 2006). All sequence files and metadata can be found in the FigShare data repository (FigShare DOI: 10.6084/m9.figshare.3362344).

### 2.5. Statistical analyses

Plant and mitochondrial sequences, sequences occurring fewer than three times, and the top three most abundant potential contaminants observed in our negative control samples were removed prior to statistical analyses. The potential contaminants that were excluded were

*Alicyclobacillus* sp., *Bradyrhizobium* sp., and *Shewanella* algae, altogether comprising slightly more than 60% of the sequences recovered from negative controls. It should be noted that the negative controls contained approximately 1% of the number of sequences that the study samples contained, therefore it is unlikely that any remaining contamination skewed our results. Three of the actively collected samples (two from the same park and one from a parking lot) did not meet the minimum criteria of 25,000 reads and were also eliminated from the analyses. Statistical analyses were implemented in R (R Development Core Team 2010) using chiefly the DESeq2, phyloseq, and vegan packages (Love et al. 2014; McMurdie and Holmes 2013; Oksanen et al., 2015). The variance-stabilizing transformation function in the DESeq2 package was used to adjust for unequal sample library sizes. The three samples for each site and sampling method were pooled before executing statistical analyses. We performed a Mantel test using Spearman rank correlation to test for spatial autocorrelation among sampling sites. The Morisita-Horn dissimilarity index was employed for beta diversity calculations because it has been shown to perform well when there is variability in sampling depth and when under-sampling is suspected (Huse et al., 2012). We used constrained analysis of principal coordinates (CAP), which requires the cloud of sample points to be plotted along orthogonal vector(s) that directly represent the explanatory variable(s). This method can uncover significant environmental effects on compositional differences even in “noisy” data (Erd-Downward et al. 2012). The adonis function from the vegan package, which performs Permutational Multivariate Analysis of Variance (PERMANOVA), was used to examine the statistical significance of compositional differences between parks and parking lots. Testing for differentially abundant taxa was achieved using the DESeq function in the DESeq2 package, which adjusts for testing multiple hypotheses by applying the Benjamini-Hochberg method (Benjamini and Hochberg 1995). This function automatically filters out data that is unlikely to have statistical significance and does so independently of the factors being studied (Love et al. 2014). All differentially abundant taxa that distinguish parks from parking lots were further identified in the NCBI 16S isolate database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain putative species assignments.

### 3. Results

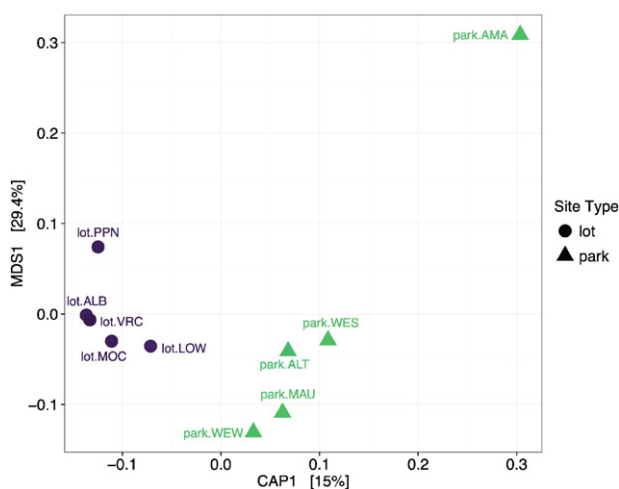
We collected air samples from five pairs of parks and parking lots to assess whether nearby vegetation influences airborne bacterial composition. After quality control and initial filtering there were 5,762,173 total reads, representing 16,633 operational taxonomic units (OTUs) from 40 unique bacterial phyla, based on 97% sequence similarity. The number of sequences recovered from each site ranged from 379,687 to 721,208. One OTU (*Sphingomonas* spp.) dominated the samples, comprising almost a quarter of all observed sequences. *Hymenobacter*, *Pedobacter*, *Agrobacterium*, and *Rhodococcus* spp. were also in the top ten most abundant OTUs and are common soil-associated bacteria (Oren 2006; Steyn et al. 1998; Matthyse 2006; Bell et al. 1998). About 83% of taxa were found in both parks and parking lots, and there was no significant difference in alpha-diversity as measured by the Shannon–Weaver index (Fig. S1 in Supplementary materials; lot mean = 4.76, park mean = 4.90,  $t = -1.66$ ,  $df = 15.1$ ,  $p = 0.12$ ).

#### 3.1. Passive and active sampling methods give comparable results

At each site we collected three active and three passive samples to verify whether the passive settling dish method gives satisfactory results. Active samples had slightly higher alpha diversity than passive samples (active mean = 4.94, passive mean = 4.72,  $p = 0.0086$ ), but composition was quite similar (Fig. S2 in Supplementary materials). To further evaluate the correspondence of active versus passive collection, we used PERMANOVA to test the null hypothesis that sampling method does influence composition for the top 50 OTUs, representing over 68% of sequences. Results of this test showed that sampling method explains nearly 0% of observed variation ( $R^2 = 0.001$ ,  $p = 0.89$ ). Since the two methods were found to be comparable, all further analyses were performed on passive samples only. We chose to analyze the passive samples instead of active samples because: a) three of the active samples failed; b) passive samples had higher biomass; and c) passive sampling is more cost-effective and easier to deploy for future studies.

#### 3.2. Nearby vegetation influences community composition

Airborne bacterial communities from parks and parking lots were significantly different ( $R^2 = 0.148$ ,  $p = 0.032$ ) in our PERMANOVA analysis. When we ran the same analysis using vegetation cover within 50 m of the sampling station instead of the site type, the model gave a similar result ( $R^2 = 0.15$ ,  $p = 0.023$ ). A constrained PCoA shows this result in



**Fig. 2.** Constrained ordination of samples from parks (green triangles) and parking lots (purple circles), where the horizontal axis uses proportion of vegetated area within 50 m as a constraint.

ordination space (Fig. 2), where the x-axis is constrained by the 50 m vegetation cover gradient. No other buffer zone radius (100 m, 200 m, 400 m, or 800 m) improved the model fit and, in fact, we found significant negative linear relationships between buffer radius and both  $R^2$  and  $p$ -value (Fig. 3 and Table SII in Supplementary materials). The potential model improvement using the 50 m radius vegetation was strongly constrained because all parking lots were close to 0% vegetated area and all parks were close to 100% as a result of the sampling design (Table SI).

#### 3.3. Key bacterial families differentiate parks from parking lots

Several key bacterial taxa were identified as differentially abundant either in parks or parking lots using a generalized linear model based on the negative binomial distribution. There were 23 OTUs identified as significantly more abundant in parking lots, seven of which were *Acetobacteraceae*, and 44 OTUs that were significantly more abundant in parks, 15 of which were *Acidobacteriaceae* (Fig. 4 and Table SIII in Supplementary materials; note that some OTUs were unable to be matched precisely in the NCBI database and therefore taxa names may occur more than once). Altogether, despite accounting for only 0.4% of all OTUs, these 67 differentially abundant OTUs for both parks and parking lots comprised 13.6% of all the sequences recovered from passive sampling. Individual OTUs in this group ranged from 0.0087%–1.62% relative abundance, which may be rare in comparison to the most abundant OTUs but are not inconsequential, especially given the fact that microbial communities tend to be typified by having a handful of highly abundant taxa and an extremely long ‘tail’ of low-abundance taxa (Shade et al. 2014). Parking lots tended to have similar compositions of these differentially abundant OTUs, whereas parks were more variable (Fig. 5). It should be noted that the OTUs shown in Fig. 5 are only those that were found to be differentially abundant and do not include the entire community. The fact that the samples were paired spatially did not significantly influence community composition (Spearman correlation = 0.013, significance = 0.497), suggesting that the distance to which the influence of any site-scale characteristics might extend is less than ~400 m (the average distance separating park-parking lot pairs).

### 4. Discussion

We investigated the heterogeneity of airborne bacterial communities within an urban area and the role of vegetation as a potential driver of variation. We found that the most prevalent taxa were highly abundant at all sites and were primarily comprised of plant and soil-associated bacteria. The consistent abundance of this large suite of common taxa may have been influenced by our decision to sample only in open areas with short herbaceous vegetation but no shrub layer or overhanging tree canopy to influence air movement. In these open areas, it is likely that the larger air mass moving through the region has a stronger influence on the composition of urban airborne microbial communities than individual site characteristics. For example, the most abundant taxon observed in this study, *Sphingomonas* spp., comprised almost a quarter of all sequences collected. Sphingomonads are commonly present in soil and on plant surfaces; they are considered ubiquitous across numerous species of higher plants (Kim et al. 1998; Innerebner et al. 2011). A BLAST search identified this OTU as either *S. faeni* or *S. aurantiaca*, both of which had been previously observed from hay dust (Busse, 2003). Our sampling period coincided with prime grass harvesting season in the region and Linn County, colloquially known as “the grass seed capital of the world,” is immediately north of Eugene. As the dominant wind direction is from the north during the time of year in which we sampled it is perhaps likely that airborne microbial community composition at our sampling locations was influenced by upwind regional agricultural activities. In fact, earlier researchers estimated that grass harvesting in Linn County may contribute up to

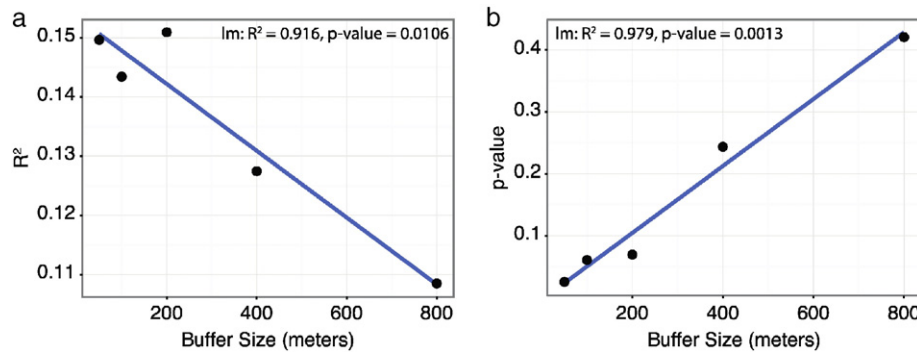


Fig. 3. Change in a) explanatory power and b) statistical significance of the PERMANOVA model with varying buffer zone radii.

40% of the total bacterial load in the Willamette Valley airshed (Lighthart 1984).

At the same time, a relatively small component (13.6%) of the microbial communities clearly distinguished parks from parking lots. Related to this, each park tended to have its own unique bacterial signature of indicator taxa, whereas parking lots were more similar to each other (Fig. 4). These observations may be explained by the relatively homogeneous environmental conditions of parking lots – they are dry, covered in asphalt, exposed to high amounts of solar radiation, and receive continual inputs of heavy metals and fossil fuel products from automobiles. Thus, we might expect that parking lot microbial signatures are determined by the ability of certain taxa to persist in extreme conditions. Parks, on the other hand, vary widely in the plant species present, vegetation structure and layering, human management regimes, and landscape design. We conjecture that park microbial signatures may be governed by some, or all, of these factors, which would explain their wide variation. Of the five parks that were chosen for this exploration, one was mown and irrigated, two were undergoing prairie restoration, and one was sheltered from wind by a small urban forest. In particular, it seems likely that vegetation serves both as a source, emitting microbes, as well as a modifier of airflow, which could tend to retain locally emitted microbes in some situations.

As yet, we know little about the spatial scale of such influences, but it is notable that the proportion of vegetated area in the smallest buffer radius (50 m) provided the best separation of parks and parking lots, and successively larger buffer sizes produced poorer results. In terms of urban design, the distinction between the relatively strong association of vegetation and bacterial communities at smaller buffer sizes (50 m, 100 m, and 200 m) and the weak relationship at larger buffer sizes (400 m and 800 m) suggests that the “park-like microbiome” extends less than 400 m. However, we also noted that, in contrast to the distinct separation of parks and parking lots shown in Fig. 2, an unconstrained PCoA ordination showed two samples collected from parks (park.MAU and park.WEW) clustering near the parking lot samples (Fig. S3 in Supplementary material). These two parks had less vegetation in the larger surrounding area (Table S1 in Supplementary material, 800 m buffer) than any other site sampled, including the parking lots. Stated differently, in this study parking lot communities always resembled those from other parking lots, regardless of vegetation in the larger area; park communities were generally distinct from parking lots, but those that had less vegetation in their larger surrounding area were more similar to parking lots than those that had plentiful vegetation within the 800 m buffer zone. Additionally, the differentially abundant bacterial signatures in Fig. 5 show that both park.MAU and park.WEW have a relatively high proportion of one OTU (closest NCBI match: *Acidisoma sibiricum*, Accession #: NR\_042706.1, 95% match) that is also prevalent in all parking lots. Possibly this indicates built and paved environments as a distinct source of that OTU (and other OTUs identified by reds and browns in Fig. 5).

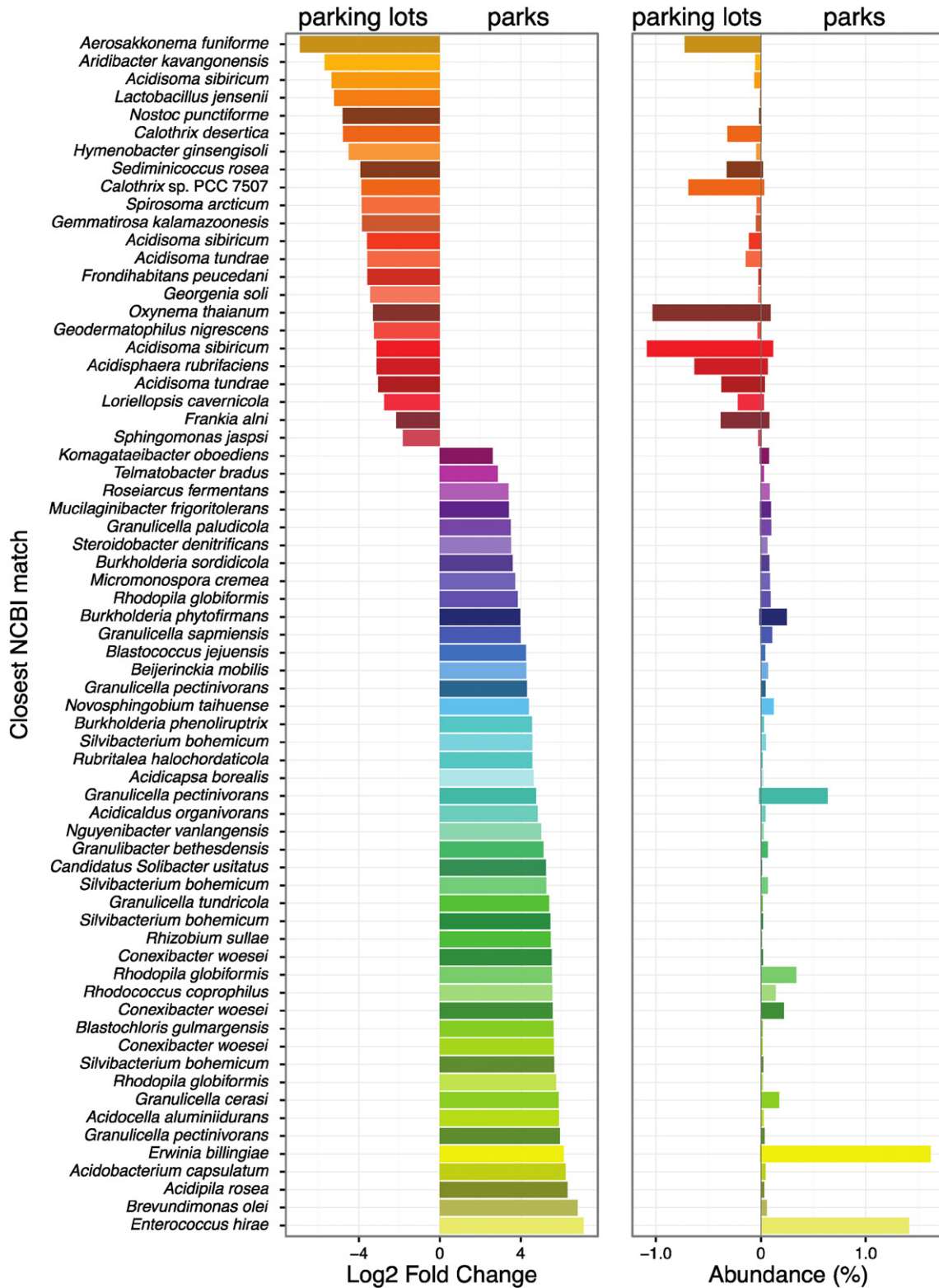
Currently the major evidence that suggests a potential linkage between human health and vegetation-associated microbes comes from studies at coarse spatial scales where differences in vegetation are confounded with differences in land use. For example, Hanski et al. (2012) found a relationship among diversity of skin Gammaproteobacteria, prevalence of atopic sensitization, and land use (forest, agriculture, built, water bodies, and wetlands), where the spatial scale of measure was a 3 km radius around the home. A larger follow-up study also documented associations among the relative abundances of several classes of Proteobacteria on the skin of healthy (versus atopic) individuals and land use within 2–5 km around the home (Ruokolainen et al., 2014). It remains to be seen whether such effects are related to specific vegetation factors, such as biomass, structural diversity, or species composition, and whether fine-scale differences such as those found in cities would play a role. Our results suggest that if plant-associated microorganisms are shown to be beneficial for human health, planners and designers might consider provisioning urban residents with green space within 400 m of their homes.

## 5. Conclusion

Although we know that urban green space has significant health benefits, we do not know the exact mechanism(s) through which those benefits arise. By 2050, the world population is expected to reach 9.3 billion and all population growth in the next 35 years is projected to occur in urban areas, bringing the percentage of people living in cities to about 66% (U.N., 2012). At the same time, cities are being built more densely so as to reduce their impact on surrounding landscapes by creating a smaller spatial footprint. This may have the side effect of reducing large green open spaces as well as the amount of vegetation in residential neighborhoods. We cannot predict how further loss of urban vegetation would affect human health, nor do we know enough about the mechanisms through which vegetation influences human health and well-being to design urban green space to maximize health benefits. Here, we provide the first evidence of fine-scale variability in outdoor urban microbiomes due to difference in vegetation. Future research may be able to elucidate how urban vegetation composition and structure, and open space distribution, influence urban airborne microbial communities, and in turn the degree to which this may influence human health. The current study thus provides a foundation for understanding how urban green space design impacts microbial communities, which could in time provide landscape architects and other urban design professionals the ability to better design cities and neighborhoods to foster human health.

## Competing interests

The authors disclose the following potential competing interests: JFM works at Phylagen, Inc.; JLG is Chief Technology Officer at Phylagen, Inc.



**Fig. 4.** Differentially abundant bacterial OTUs in parks (purples, blues, greens, yellows) and parking lots (reds, oranges), labeled by closest match in NCBI database. Left panel indicates the degree to which each OTU is differentially abundant, right panel shows the actual relative abundance of each OTU.

**Author's contributions**

GAM conceived and implemented the study, analyzed the data, and wrote the manuscript. BRJ helped conceive the study, helped analyze the data, and edited the manuscript. AEA helped implement the study, helped

analyze the data, and edited the manuscript. JL helped conceive and implement the study and edited the manuscript. JFM helped conceive and implement the study, helped analyze the data, and edited the manuscript. KSP helped interpret the data and edited the manuscript. JLG helped conceive the study, helped analyze the data, and edited the manuscript.

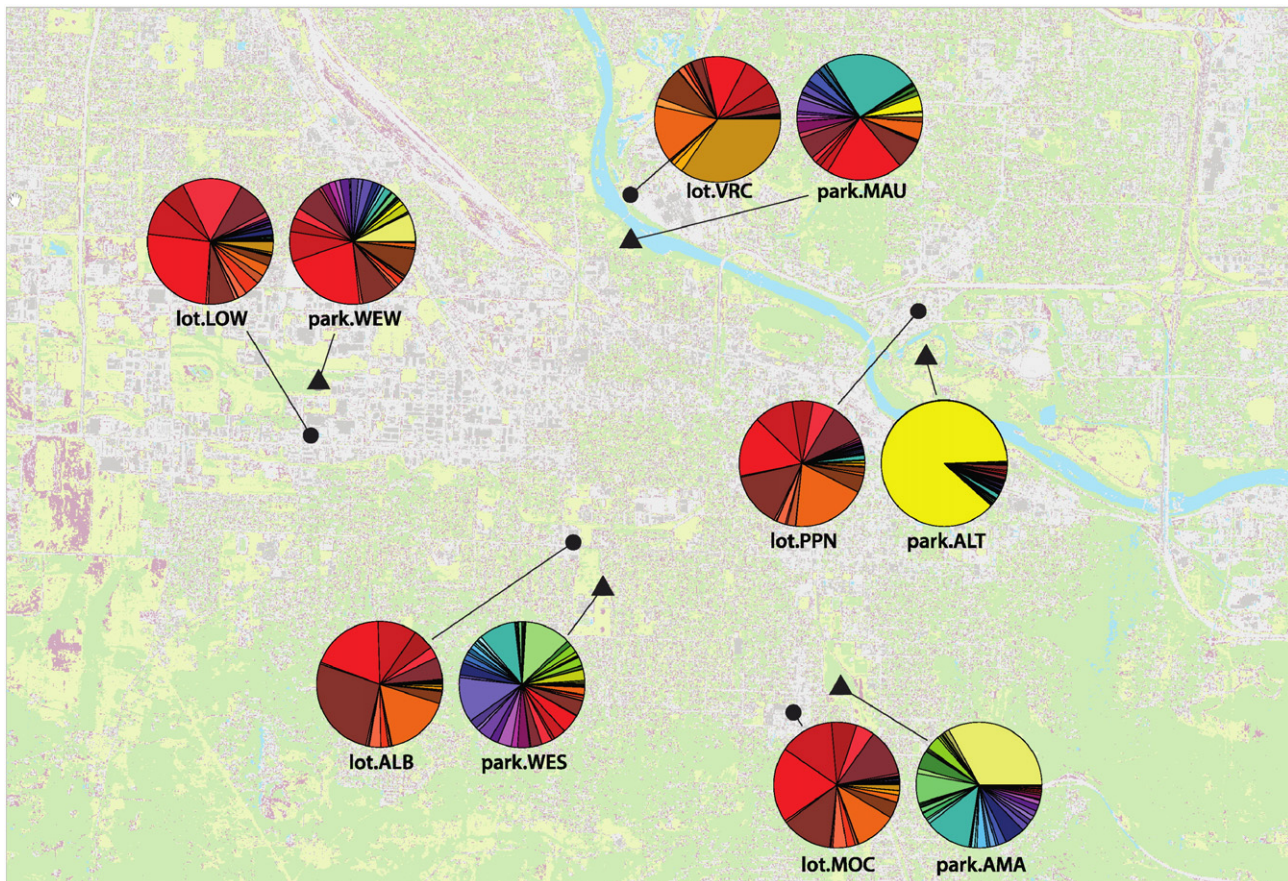


Fig. 5. Composition of differentially abundant taxa for each site, color-coded same as Fig. 4.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2016.07.037>.

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