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Vertical Stratification in Urban Green Space Aerobiomes

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Background: Exposure to a diverse environmental microbiome is thought to play an important role in "educating" the immune system and facilitating competitive exclusion of pathogens to maintain human health. Vegetation and soil are key sources of airborne microbiota—the aerobiome. A limited number of studies have attempted to characterize the dynamics of near surface green space aerobiomes, and no studies to date have investigated these dynamics from a vertical perspective. Vertical stratification in the aerobiome could have important implications for public health and for the design, engineering, and management of urban green spaces.

Objectives: The primary objectives of this study were to: *a*) assess whether significant vertical stratification in bacterial species richness and evenness (alpha diversity) of the aerobiome occurred in a parkland habitat in Adelaide, South Australia; *b*) assess whether significant compositional differences (beta diversity) between sampling heights occurred; and *c*) to preliminarily assess whether there were significant altitudinal differences in potentially pathogenic and beneficial bacterial taxa.

Methods: We combined an innovative columnar sampling method at soil level, 0.0, 0.5, 1.0, and 2.0 m, using passive petri dish sampling to collect airborne bacteria. We used a geographic information system (GIS) to select study sites, and we used high-throughput sequencing of the bacterial 16S rRNA gene to assess whether significant vertical stratification of the aerobiome occurred.

Results: Our results provide evidence of vertical stratification in both alpha and beta (compositional) diversity of airborne bacterial communities, with diversity decreasing roughly with height. We also found significant vertical stratification in potentially pathogenic and beneficial bacterial taxa.

Discussion: Although additional research is needed, our preliminary findings point to potentially different exposure attributes that may be contingent on human height and activity type. Our results lay the foundations for further research into the vertical characteristics of urban green space aerobiomes and their implications for public health and urban planning. https://doi.org/10.1289/EHP7807

Introduction

Vegetation and soil are known to be key sources of airborne microbiota—i.e., the *aerobiome* (Joung et al. 2017; Liu et al. 2018). Exposure to a diverse suite of microbes from the environment (including the aerobiome) is thought to be important for the development and regulation of the human immune system (Rook et al. 2003, 2013; Tasnim et al. 2017). Furthermore, studies now link the microbiome to a plethora of maladies from Alzheimer's disease (Kowalski and Mulak 2019) and myalgic encephalomyelitis (Hanson and Giloteaux 2017), through inflammatory bowel (Aschard et al. 2019) and skin diseases (Prescott et al. 2017), to respiratory health (Sokolowska et al. 2018). Environmental factors are thought to be more important than genetic factors in shaping the composition of the gut microbiome (Rothschild et al. 2018). Indeed, Browne et al. (2016) showed that spore-forming bacteria (which survive in aerobic conditions) dominated the human gut, comprising 50%–60% of bacterial genera, and displayed greater change in abundance and species over time in comparison with nonspore formers, suggesting that many gut bacteria may come and go from the environment.

Gut colonization aside, exposure to airborne microbiota has implications for the human skin and airways. For example, several studies (particularly in agricultural settings) have demonstrated that the composition of the human nasal microbiome is significantly influenced by airborne microbial communities from the surrounding environment (Shukla et al. 2017; Kraemer et al. 2018). A recent study also showed that the diversity of skin and nasal microbiota increased after exposure to urban green spaces (Selway et al. 2020). Furthermore, a recent systematic review highlights that despite the relative infancy of aerobiome–human health research, two studies have shown that rural aerobiomes shifted immune function away from allergic (Th2-type) responses (Flies et al. 2020). In the indoor environment, studies have also drawn the link between microbial composition and endotoxin levels in dust and immuno-protection (e.g., against asthma) (Gehring et al. 2008; Stein et al. 2016). Other indoor-based studies show airborne microbes contribute to nasal, oral, and skin microbiomes (Lai et al. 2017; Chen et al. 2019). Studies have also shown that up to 10^6 microbial cells can be found in a cubic meter of air (Šantl-Temkiv et al. 2018; Tignat-Perrier et al. 2019). Therefore, there is considerable potential for aerobiome–respiratory system interactions.

A limited number of studies have attempted to characterize the community structure and spatiotemporal dynamics of near-surface green space aerobiomes. For example, Mhuireach et al. (2016) compared bioaerosol samples in green spaces and parking lots and found compositional distinctions in bacterial communities between the two land cover types. Furthermore, Mhuireach et al. (2019) explored spatiotemporal controls on the aerobiome and suggested that localized site factors were likely to be important in driving bacterial

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All data and code used in this study are available on the *UK Data Service ReShare* at https://reshare.ukdataservice.ac.uk; Data Collection #854411. All 16S rRNA gene sequences have been deposited in the European Nucleotide Archive (accession no. ERC000025).
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community structure. However, no known studies have investigated the spatial and compositional factors from a vertical perspective. Support for the existence of aerobiome vertical stratification can be drawn from studies of pollution, allergenic pollen, and fluid dynamics of particulates, where stratification has been shown to occur at various scales. For example, in an indoor agricultural environment and under ventilated conditions, Miles (2008) showed that NH_3 molecule concentrations decreased vertically with increasing distance from source (i.e., the ground). Gao and Nui (2007) found that vertical concentration stratification of particles up to (10.0 μm) occurred under different ventilation conditions. Particles smaller than 2.5 μm were less affected by gravitational factors, and submicron particles with small relaxation times (i.e., the time required for particles to adjust their velocity to new conditions of forces) behaved more like trace gases following main airstreams. Alcázar et al. (1998) found higher concentration of *Urtica membranacea* pollen at the upper region of their sampling height range of 1.5 m–15 m and higher concentrations of *U. urens-Parietaria sp.* at lower heights, possibly due to pollen mass and different fluid dynamics. The size range of bacterial cells can vary by eight orders of magnitude (0.013 μm to 750 μm) (Levin and Angert 2015) and can clump together and adhere to larger suspended particles (Tham and Zuraimi 2005; Haas et al. 2013; Gong et al. 2020). These factors, along with turbulent mixed flow could conceivably influence aerobiome stratification.

The existence of aerobiome vertical stratification could have important implications for the design, engineering and management of urban green spaces, particularly those aimed at promoting public health via microbial exposure (Watkins et al. 2020). For example, do children receive the same exposure to airborne microbiota as taller adults? Do people who lie down or work close to the ground (e.g., gardeners bending over to dig) have different exposure levels to those who remain upright, and what are the downstream implications for health? Developing a refined understanding of this aerobiome–human interface could also have implications for the design and monitoring of nature-based health interventions, for example via green/nature prescribing (Robinson and Breed 2019; Shanahan et al. 2019; Robinson et al. 2020). Furthermore, protocols for sampling the aerobiome to date have often included a reasonable yet arbitrary sampling height of 2 m (Airaudi and Marchisio et al. 1996; Cordeiro 2010; Mhuireach et al. 2016; Domingue 2017). Therefore, investigating aerobiome composition at various heights could also provide important methodological insights to fine-tune future study protocols and public health recommendations.

In this proof of concept study, we combine innovative columnar aerobiome sampling methods along with remote sensing techniques and high-throughput sequencing of the bacterial 16S rRNA gene. The primary objectives of this study were to: *a*) assess whether significant vertical stratification in bacterial species richness and evenness (alpha diversity) of the aerobiome occurred; *b*) assess whether significant compositional differences (beta diversity) between sampling heights occurred; and *c*) to preliminarily assess whether there were significant altitudinal differences in putative pathogenic and beneficial bacterial taxa.

Materials and Methods

Site Selection

Our study site comprised three vegetated plots totaling 7 hectares (ha) of the southern section of the Adelaide Parklands (Kaurna Warra Pintyanthi), South Australia. The justification for the selected study site was as follows:

1. Its broadly consistent soil geochemistry, because the southern Parklands generally fall within the Upper Outwash Plain soil boundary (coalescing alluvial soil, draining the Eden Fault Block).
2. This area is managed by a single division of the City of Adelaide, minimizing variation in site management and allowing for simpler study logistics.
3. A single study site (i.e., the southern section) in the Parklands provided a degree of control over potential variation in landscape effects on the aerobiome (e.g., dominant vegetation type, distance to coast, elevation, orientation, aspect).
4. Urban Parkland is representative of conditions that both child and adult residents might be exposed to.

Following site selection, boundaries of three plots (as polygons) were defined in QGIS 3 (version 3.0.2). These polygons were subsequently converted to shapefiles (.shp), and a random point algorithm was generated. This process provided randomly selected sampling points within each vegetated plot to include in our study (Figure 1). The spatial coordinates for each sampling point were recorded and programmed into a handheld global positioning system (GPS) device. This device was operated on site to allow us to identify the relevant locations for setting up the sampling stations.

Sampling Equipment

The sampling stations (Figure 2) were constructed using timber (SpecRite 42 mm × 28 mm × 2.7 m screening Merbau). The sampling stations comprised a timber stand with 45° leg braces. Hooks and guy ropes were also installed, ensuring stability in the field. We used standard lab-grade, clear plastic petri dishes (Nest Cell) supported by steel brackets (and attached to the brackets with Velcro tabs) to passively sample the aerobiome per Mhuireach et al. (2016).

The level of stability was tested in two phases. Phase 1: during windy conditions (∼ Beaufort scale No. 5) in a yard environment, and Phase 2: in situ, prior to the sampling phase.

Data Loggers

We installed temperature and relative humidity data loggers (Elitech RC-4HC) at each sampling station. Each logger was programmed to record data at 8-s intervals for the entire sampling period. The dataloggers were calibrated at the start of each sampling day using a mercury thermometer (Gerotherm) and a sling psychrometer (Sper Scientific 736700) taking the range between the two bulbs to determine baseline humidity.

On-Site Setup Procedure

The sampling stations were placed into position between 0600 hours and 0800 hours on 4, 5, and 6 November 2019. This procedure ensured that sufficient time was allocated to travel between the sampling locations. From 0800 hours onward and prior to installing the petri dishes for passive sampling, the sampling stations were decontaminated using a 5% Decon 90 solution. The microclimate data loggers were then decontaminated and installed on the sampling stations. The nearest trees (all <10 m height and 20 cm–50 cm in diameter at breast height) were between 2 m and 5 m from the sampling stations.

Sampling Protocol

The sampling procedure involved collecting soil samples (actively) and airborne microbiota (passively). Environmental metadata were also collected (e.g., wind speed, temperature, and relative humidity). Soil pH at each site was measured using a digital pH meter (Alotpower). The probe of the pH meter was inserted into the soil and left for a period of 1 min prior to taking a reading, per manufacturer's instructions.

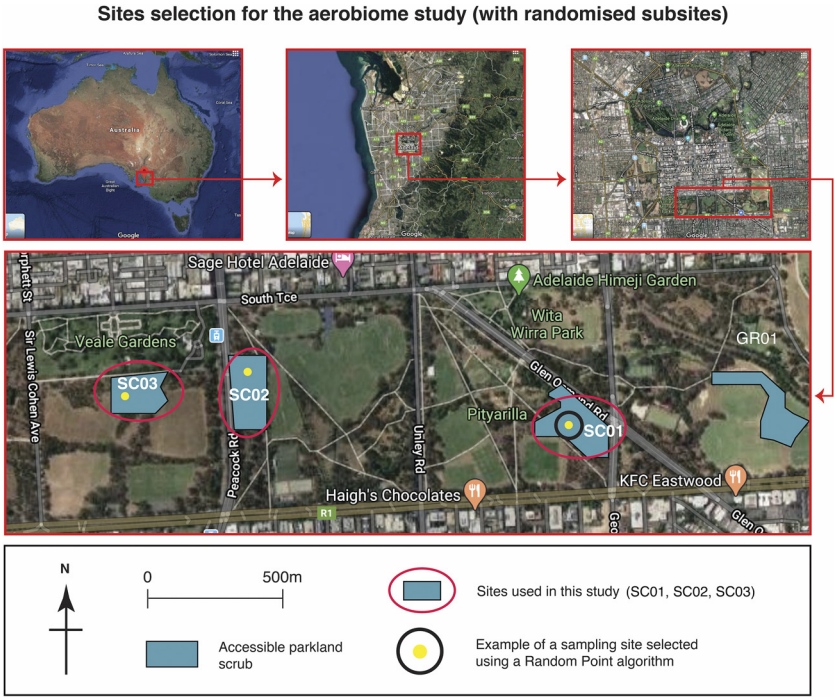

Figure 1. Location of study sites, showing the randomly selected sampling locations (indicated by yellow points). Accessible sites are indicated by the blue polygons, and the sites used in this study are surrounded by the red rings. SC01, SC02, and SC03 refer to the three scrub habitat study sites.

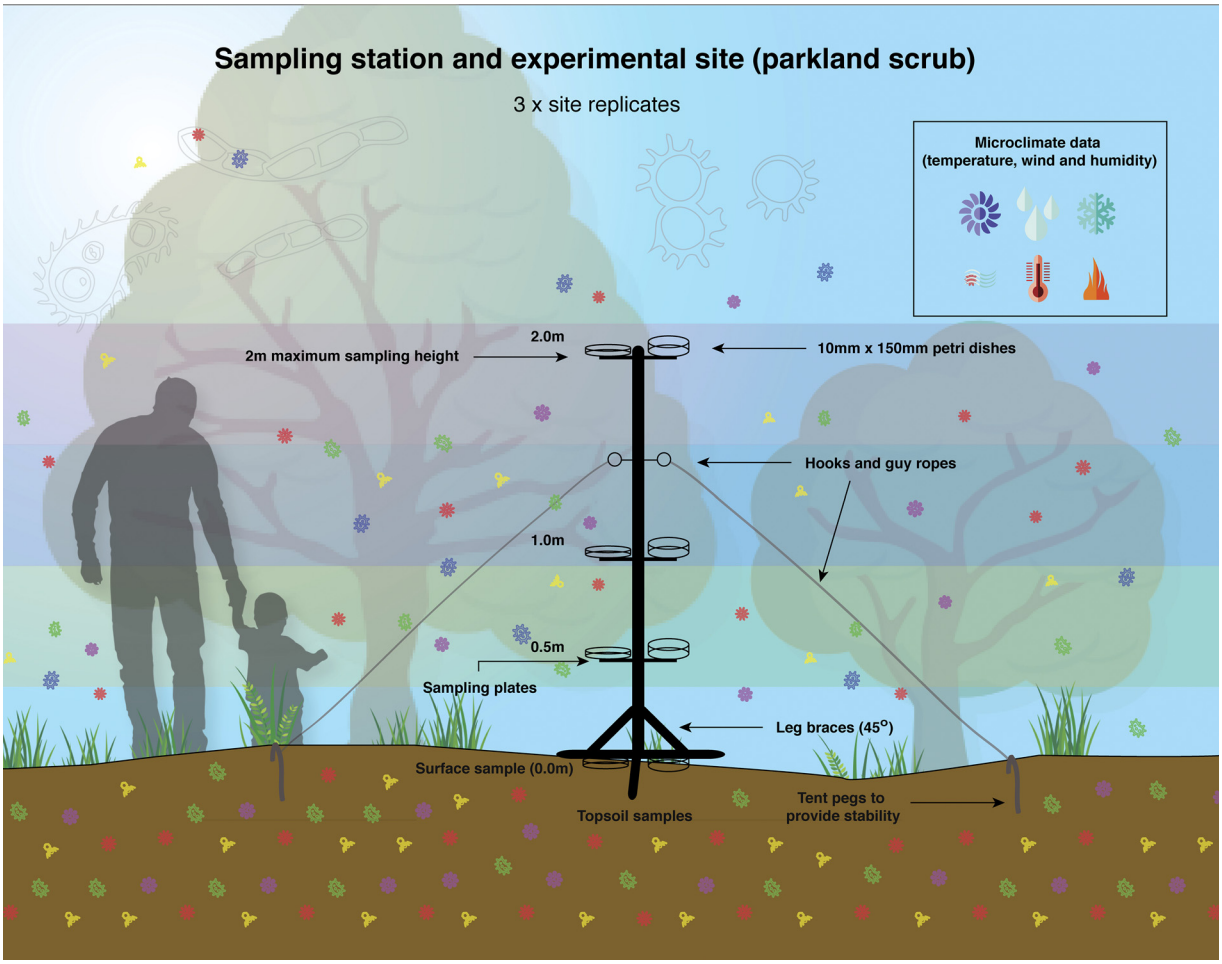

Figure 2. Design of the aerobiome vertical stratification sampling stations. These were deployed in scrub habitat in the Adelaide Parklands, South Australia. The figure also shows a silhouette of humans to provide perspective.

Wind speed and direction data for the entire study area were obtained from Adelaide's meteorological weather station at Ngayirdapira (West Terrace): Lat: −34.93, Lon: 138.58, Height: 29.32 m. Wind speed and direction was also recorded at each sampling site on an hourly basis (Mhuireach et al. 2016) using the handheld anemometer (Digitech QM-1644).

Soil samples. Topsoil samples were collected using a small shovel and stored in 50-mL sterile falcon tubes. The shovel was decontaminated using the 5% Decon 90 solution prior to use. Wearing gloves, we sampled five topsoil samples (depth: 5–7 cm) at equidistant sampling points, 20–30 cm from the central stem of each sampling station (Zarraonaindia et al. 2015). The soil samples were subsequently pooled and then homogenized, passed through a 1-mm pore sieve, and placed in new sterile 50-mL Falcon tubes. The sample tubes were labeled using a predefined labeling system. We included field controls of soil samples by opening 50-mL sterile falcon tubes for 60 s at each site (Mbareche et al. 2019). All soil and field control samples were immediately chilled by placing in an ice box in the field and then storing at −80°C in the lab prior to DNA extraction and sequencing (Zarraonaindia et al. 2015). In total, we collected 15 soil subsamples per sampling day across the three sampling stations for each of the three sampling days. Subsamples were pooled and homogenized by sampling station and day, which gave a total of nine homogenized samples (three per sampling station) plus three field controls.

Aerobiome samples. Passive sampling methods were used to collect low biomass aerobiome samples following established protocols (Mhuireach et al. 2016, 2019). Petri dishes (100 × 15 mm) were attached with decontaminated Velcro tabs on the sampling stations at four sampling heights: ground level, 0.5 m, 1 m, and 2 m. The total height of the sampling stations was 2 m from ground level (in a cohort study across Europe, North America, Australia, and East-Asia, 95% of adult human heights fell within 2 SD at 1.93 m for males and 1.78 m for females) (Jelenkovic et al. 2016). One meter is the average height of a 4-y-old child (RCPCH 2020)—typically the maximum weaning age (Mutch 2004; Clayton et al. 2006) and the time when the gut microbiome is thought to become less plastic (Milani et al. 2017)—and is the approximate height of a jogging stroller (Thule 2020). Fifty centimeters is the approximate height of an adult torso from the hip to the mouth (representing the height of an adult sitting on the floor) (Nikolova et al. 2017), although this will vary depending on size and age. The ground surface is also considered to be an important sampling level, for example, representing the point of contact for a crawling child or an adult lying on the floor. The petri dish sampling plates were also decontaminated using the 5% Decon 90 solution prior to use.

The petri dishes were secured to the sampling stations (Figure 2) and left open for 6–8 h (Mhuireach et al. 2016). At the end of the sampling period, we closed the petri dishes. A new set of gloves was worn for the handling of petri dishes at each vertical sampling point to reduce potential contamination. The petri dishes were then sealed using Parafilm, labeled, immediately placed on ice, and transported to the laboratory for storage at −80°C prior to DNA extraction (Mhuireach et al. 2019). Unused petri dishes were left open for 60 s in the equipment box carried on site and then sealed at each site as field controls. Dishes were later swabbed during the DNA extraction process using nylon flocked swabs (FLOQSwabs Cat. No. 501CS01, Copan Diagnostics, Inc.) (Mhuireach et al. 2019; Bae et al. 2019; Liddicoat et al. 2020).

DNA Extraction, Amplification, and Sequencing

We extracted DNA from samples at the Evolutionary Biology Unit (EBU), South Australian Museum. The order of processing samples was randomized using a digital number randomizer, including the soil samples (higher biomass), which were processed after the low biomass aerobiome samples to minimize cross-contamination.

The petri dishes for each sampling station were swabbed with FLOQSwabs for 30 s (with consistent back and forth strokes) in a laminar flow cabinet type 1 (License No. 926207). The base and lid samples for each height, station, and date were then pooled, prior to extraction. The swabs were cut with decontaminated scissors directly into labeled 2-mL Eppendorf tubes. Extraction blank controls were used to demonstrate the absence of sample contamination during extraction and were the last samples in the extraction. Sterile water and reagents were used instead of a sample, and all DNA extraction steps were performed as if they were normal samples. We used Qiagen QIAamp DNA Blood Mini Kits to extract DNA from the swabs together with extraction blank controls, and Qiagen DNAeasy PowerLyzer Soil Kits to extract DNA from the soil samples (and extraction blank controls). We followed the manufacturer's instructions throughout the extraction process.

Polymerase chain reaction (PCR) amplification was done in triplicate using the 341F/806R primer targeting the V3–V4 region of the 16S rRNA gene (5′-CCTAYGGGRBGCASCAG-3′/5′-GGACTACNNGGGTATCTAAT-3′). The 300-bp paired end run was sequenced on an Illumina MiSeq platform at the Australian Genome Research Facility Ltd (AGRF) using two flow cells (ID 000000000-CW9V6 and 000000000-CVPGT). Image analysis was done in real time by the MiSeq Control Software (MCS) (version 2.6.2.1) and Real Time Analysis (RTA) (version 1.18.54). Then the Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data. A minimum of 0.20 ng/uL of usable PCR product was required to generate sequencing output guarantee of 10,000 raw reads and to be included in the analysis.

Bioinformatics and Statistical Analysis

Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5). Primers were identified and trimmed. Trimmed reads were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8.4), USEARCH (version 8.0.1623), and UPARSE software. Using USEARCH tools, reads were quality filtered; full-length duplicate reads were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Reads were clustered and chimeric reads were filtered using the "rdp_gold" database as a reference. To obtain the number of reads in each operational taxonomic unit (OTU), reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned using QIIME.

We used the phyloseq (version 1.24.0) package (McMurdie and Holmes 2013) in R to import and analyze the sequencing data, and decontam (version 1.1.2) (Davis et al. 2018) to identify and exclude contaminants.

Lower biomass samples (i.e., air, field blanks, and extraction blank controls) were analyzed using the isNotContaminant() function, where contaminants were identified by increased prevalence in negative controls. Higher biomass samples (i.e., soil, and corresponding extraction blanks) were analyzed using the isContaminant() function. Using isContaminant(), contaminants were identified by the frequency that varies inversely with sample DNA concentration, or by increased prevalence in negative controls. All taxa identified as contaminants were pooled and removed from further analysis. To estimate OTU alpha diversity, we derived Shannon Index values based on rarefied abundances (Liddicoat et al. 2020) in phyloseq. The lowest number of reads in a sample was used to rarefy the data sets (Liddicoat et al. 2020). We generated box and violin plots with ggplot2 (version 3.0.0) to visualize the distribution of the alpha diversity scores for each sampling height. Microbial beta diversity was visualized using nonmetric multidimensional scaling (NMDS) ordination of Bray–Curtis distances based on rarefied OTU abundances.

The ordinations plots show low-dimensional ordination space in which similar samples are plotted close together, and dissimilar samples are plotted far apart.

We used permutational multivariate analysis of variance (PERMANOVA) to test for compositional differences between sampling heights. The Pearson's product-moment and Spearman's rank correlation tests were used to examine correlations between sampling height and alpha diversity scores. A Mann-Whitney-Wilcoxon test was used to examine differences in alpha diversity between merged air sampling heights (0.0–0.5 m and 1.0–2.0 m) and a Kruskal-Wallace chi-square test to explore differences in correlations between sites and dates. We also calculated OTU relative abundances using the phyloseq package in R to examine the distribution of taxa that have potential implications for public health. To compare presence and proportions of taxa, we used two-sample tests for equality of proportions with continuity corrections and created radial charts using pivot tables with comma separated value (csv) files. A data point was considered to be an outlier if it was more than 1.5 × above the third quartile or below the first quartile.

Results

We obtained 3,781,284 raw reads from air samples with an average length of 300 base pairs and 3,278,433 reads after quality control (QC). For soil, we obtained 1,830,395 raw reads and 1,287,303 reads after QC. The range of reads per samples after QC was 19,966–251,822. Reads were clustered into 10,563 OTUs. Overall, bacterial communities were diverse at each sampling height and bacterial phyla were dominated by:

- Proteobacteria (at 2.0 m: 49.5%; 1.0 m: 43.8%; 0.5 m: 28.1%; 0.0 m: 27.1%; and soil level: 23.12%); and,
- Actinobacteria (at 2.0 m: 19.7%; 1.0 m: 17.5%; 0.5 m: 26.6%; 0.0 m: 43.5%; and soil level: 47.2%).

Ten bacterial phyla represented 100% of OTUs over 1% relative abundance, including: Proteobacteria, Actinobacteria, Bacteriodetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimondates, GN02, OD1, and TM7. Proteobacteria were dominant at upper sampling heights, and Actinobacteria were dominant at lower sampling heights.

We observed a significant negative correlation between alpha diversity (air and soil for all sites/dates) and sampling height [$r = -0.58$, degrees of freedom (df) = 38, $p = < 0.01$; Figure 3A; Table 1].

Alpha diversity ranged from 1 to 6 and was highest at soil level followed by the lower air sampling levels (0.0 m–0.5 m) and the upper sampling levels (1.0 m–2.0 m), respectively.

When the lower sampling heights and the upper sampling heights were merged (0.0 with 0.5 m; 1.0 m with 2.0 m), we observed a significant negative correlation between alpha diversity and sampling height ($r = -0.68$, df = 38, $p = < 0.01$) (Figure 3B). Following an examination of alpha diversity scores for individual sites and dates, all variants showed negative correlations between alpha diversity and sampling height. Four out of six indicated strong and significant relationships (Day 1: $r = -0.76$, $p = 0.00$; Day 3: $r = -0.64$, $p = 0.01$; SC01: $r = -0.68$, $p = < 0.01$; and, SC03: $r = -0.73$, $p = 0.01$; Table 2). It is important to note that we omitted six samples from the lower heights due to failure to reach minimum DNA concentrations (as denoted by "—" in Table 1).

With the merged sampling heights, all correlations increased in strength and were all statistically significant (Table 2). A Mann-Whitney-Wilcoxson test for differences in alpha diversity between the merged air sampling heights (0.0 m–0.5 m and 1.0 m–2.0 m) showed a statistically significant difference ($W = 188$, $p = < 0.01$). A Kruskal-Wallace chi-squared test indicated no significant difference in correlations between sites or dates ($p = 0.44$).

Using these same merged sampling heights, a two-sample test for equality of proportions with continuity correction showed a significant difference in proportions of taxa that occurred in lower air sampling heights (compared to upper sampling heights) that also occurred in the soil samples. The positive relationship

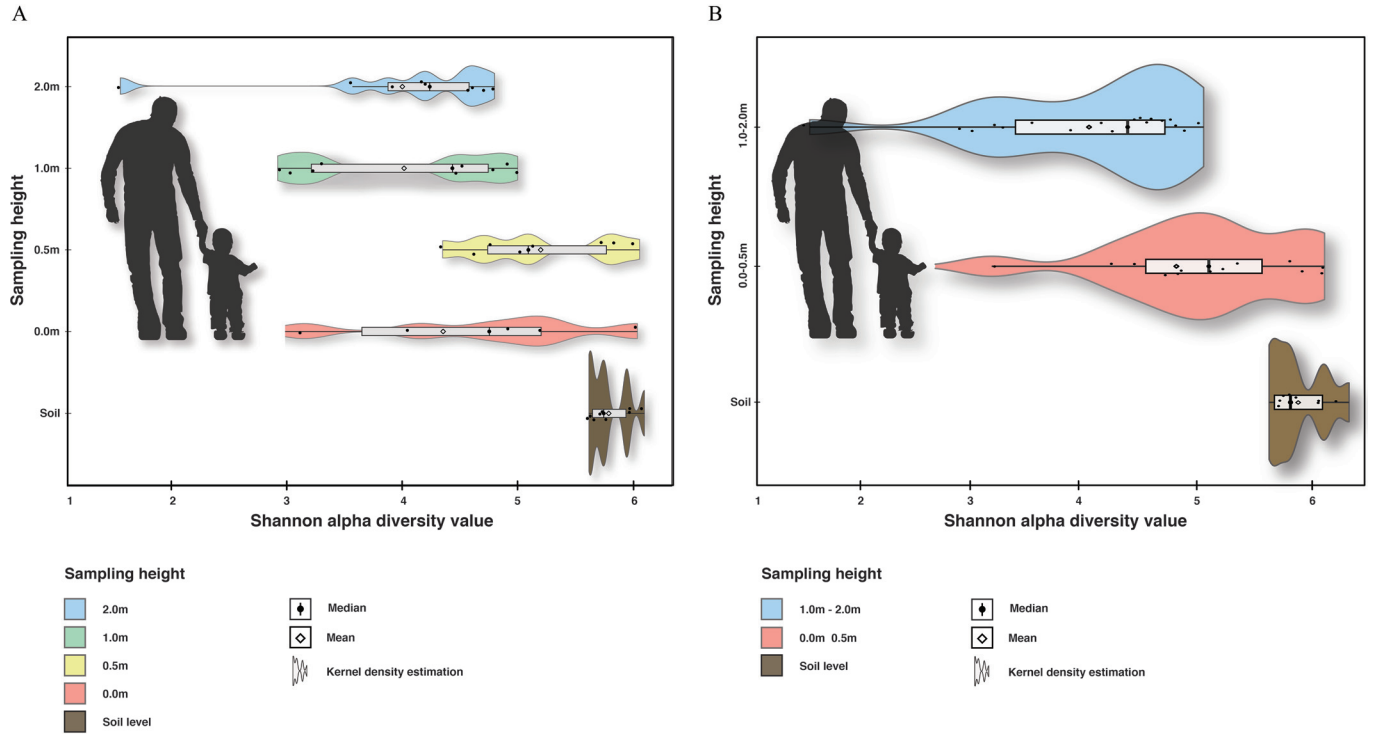

Figure 3. Box/violin plots of Shannon alpha diversity scores for each sampling height including soil (A) and for merged lower heights 0.0–0.5 m and upper heights 1.0–2.0 m, with soil (B). Plots also display mean values, interquartile range, and kernel density estimation. Box plots indicate a vertical stratification trend of airborne bacterial alpha diversity decreasing with increased sampling height.

Table 1. Shannon alpha diversity scores for each spatial and temporal replicate, along with means and standard deviations.

Days/sampling height	Scrub 01 (SC01) Shannon α diversity score	Scrub 02 (SC02) Shannon α diversity score	Scrub 03 (SC03) Shannon α diversity score	Mean (±SD)
Day 1				
Soil	5.73	5.60	5.93	5.75 ± 0.16
0.0 m	5.26	6.01	4.74	5.34 ± 0.63
0.5 m	4.63	5.82	5.72	5.39 ± 0.66
1.0 m	4.43	3.21	4.48	4.04 ± 0.71
2.0 m	1.54*	3.87	4.53	3.31 ± 1.57
Day 2				
Soil	5.63	5.60	5.93	5.72 ± 0.18
0.0 m	—	3.15	4.15	3.65 ± 0.70
0.5 m	4.35	6.01	5.14	5.16 ± 0.83
1.0 m	3.01	4.86	2.90	3.59 ± 1.10
2.0 m	4.67	4.79	4.14	4.53 ± 0.34
Day 3				
Soil	5.68	5.74	6.00	5.81 ± 0.17
0.0 m	—	—	—	—
0.5 m	4.77	5.02	—	4.89 ± 0.17
1.0 m	3.28	4.98	4.74	4.33 ± 0.92
2.0 m	4.57	3.53	4.23	4.11 ± 0.53

Note: —, missing data (failed to reach minimum DNA concentrations: 0.20 ng/uL of usable PCR product was required to generate sequencing output of 10,000 raw reads); *A data point was considered to be an outlier if it was more than 1.5× above the third quartile or below the first quartile. Scrub 1, 2, and 3 refer to samples collected from the scrub habitat study sites. SD, standard deviation.

between the proportion of taxa occurring in the air that also occurred in the soil decreased as vertical distance from the soil increased. For example, at the genus level, 84.4% of taxa in the lower air samples also occurred in the soil samples, whereas only 76.1% of the taxa in the upper air samples occurred in the soil. This difference was statistically significant ($\chi^2 = 9.5376$, df = 1, $p = < 0.01$; Figure 4 shows taxonomic breakdown).

Sampling heights displayed distinct bacterial signatures (Figure 5A). Sampling height explained 22% of the variation in environmental microbiota when all air sampling heights and the soil level were included, and this was statistically significant (PERMANOVA df = 4, $F = 2.50$, $R^2 = 0.22$, $p = < 0.01$, permutations = 999).

When analyzing air samples in isolation, sampling height explained 11% of the variation in environmental microbiota, however, this was not significant (df = 3, $F = 1.18$, $R^2 = 0.11$, $p = 0.15$, permutations = 999). When we merged within lower and upper sampling heights, sampling heights explained 6% of the variation and this was statistically significant (df = 1, $F = 1.98$, $R^2 = 0.06$, $p = 0.01$, permutations = 999) (Figure 5B).

Table 2. Correlation scores of alpha diversity and sampling height based on all air and soil samples, followed by merged air sampling heights (0.0 m–0.5 m and 1.0 m–2.0 m) and soil samples.

Days/sites	*r* score	df	*p*-Value
Day 1 (04-11-19)	−0.76	11	<0.01***
Day 2 (05-11-19)	−0.31	12	0.17
Day 3 (06-11-19)	−0.64	11	0.01**
Scrub 01 (SC01)	−0.68	13	<0.01***
Scrub 02 (SC02)	−0.41	12	0.14
Scrub 03 (SC03)	−0.73	9	0.01**
Merged air sampling heights (0.0 m–0.5 m and 1.0 m–2.0 m):			
Day 1 (04 November 2019)	−0.76	11	<0.01***
Day 2 (05 November 2019)	−0.59	12	0.02*
Day 3 (06 November 2019)	−0.72	11	<0.01***
Scrub 01 (SC01)	−0.72	13	<0.01***
Scrub 02 (SC02)	−0.54	12	0.04*
Scrub 03 (SC03)	−0.86	9	<0.01***

Note: The Pearson's product moment correlation test was used. Correlation scores for each sampling date and site are included. Scrub 1, 2, and 3 refer to samples collected from the scrub habitat study sites. df, degrees of freedom. *0.05. **0.01. ***<0.01.

The dominant taxa in the soil and lower sampling heights were Actinobacteria (based on mean relative abundance >1%), and the dominant taxa in the upper sampling heights were Proteobacteria (Figure 6; segments 1 and 9). A significantly greater proportion of Actinobacteria were present in lower air sampling heights (merged 0.0 m–0.5 m; 43.52% and 26.61%, respectively; $\bar{x} = 35.07\%$) compared to upper air sampling heights (merged 1.0 m–2.0 m; 17.52% and 19.67%, respectively; $\bar{x} = 18.59\%$) ($\chi^2 = 6.1032$, df = 1, $p = 0.01$).

A significantly greater proportion of Proteobacteria was present in the upper air sampling heights (merged 1.0 m–2.0 m; 43.78% and 49.50% respectively; $\bar{x} = 46.64\%$) compared to the lower air sampling heights (merged 0.0 m–0.5 m; 27.11% and 28.14%, respectively; $\bar{x} = 27.63\%$) ($\chi^2 = 6.9471$, df = 1, $p = < 0.01$).

A number of relatively abundant and notable taxa (contingent primarily on their implications for public health) were identified in the samples (Figure 7). The relative abundance of these taxa differed across sampling heights, and all significantly correlated with sampling height, ranging from moderate to strong relationships (Table 3). The relative abundance of these taxa are as follows: *Streptomyces* (3.63% and 3.7% in soil and 0.0 m, respectively), *Kingella* (2% and 4.1% in 1.0 m and 2.0 m, respectively), *Lactobacillus* (5.9% and 3.8% in 1.0 m and 2.0 m, respectively), *Flavobacterium* (4.3% in 0.0 m, 7.5% in 0.5 m, 7.9% in 1.0 m, and 4.8% in 2.0 m), and *Sphingomonas* (4.3% in 0.0 m, 4.8% in 0.5 m, 6.5% 1.0 m, and 6.8% in 2.0 m). The potential implications of these taxa for public health are highlighted further in Table 4 in the Discussion.

Discussion

Vertical Stratification of Aerobiome Alpha Diversity

Here we show that vertical stratification of aerobiome alpha diversity occurred in an urban green space scrub habitat in Adelaide Parklands, South Australia. This transpired as a significant association in the reduction of bacterial alpha diversity as height increased (i.e., between the ground surface level and two vertical meters of the air column). When considering all sampling heights, alpha diversity reduced with greater height. This vertical stratification in alpha diversity was neither spatially (i.e., site specific) or temporally dependent. The strength of the negative relationship between alpha diversity and height increased when we merged lower sampling heights (0.0 m with 0.5 m) and the upper sampling heights (1.0 m with 2.0 m). This finding implies that the required spatial frequency to elucidate vertical stratification in alpha diversity—specifically, five sampling heights across a 2-m vertical transect—may have been overestimated. However, several omissions in the lower sampling heights due to failure to reach minimum DNA concentrations could have affected the strength of this association.

The decay in observed alpha diversity as height increased could be the result of increasing distance from the primary source, i.e., potentially the soil. It is widely accepted that soil represents one of the most microbially diverse terrestrial habitats (Briones 2014; Bender et al. 2016; Dumbrell 2019; Zhu et al. 2019). Therefore, it seems reasonable to suggest that lower sampling heights may possess a higher level of microbial diversity because they are closer to a potentially greater concentration of microbiota. We observed that a greater proportion of bacteria taxa found in the lower sampling heights (in comparison with the upper sampling heights) were also present in the soil samples,

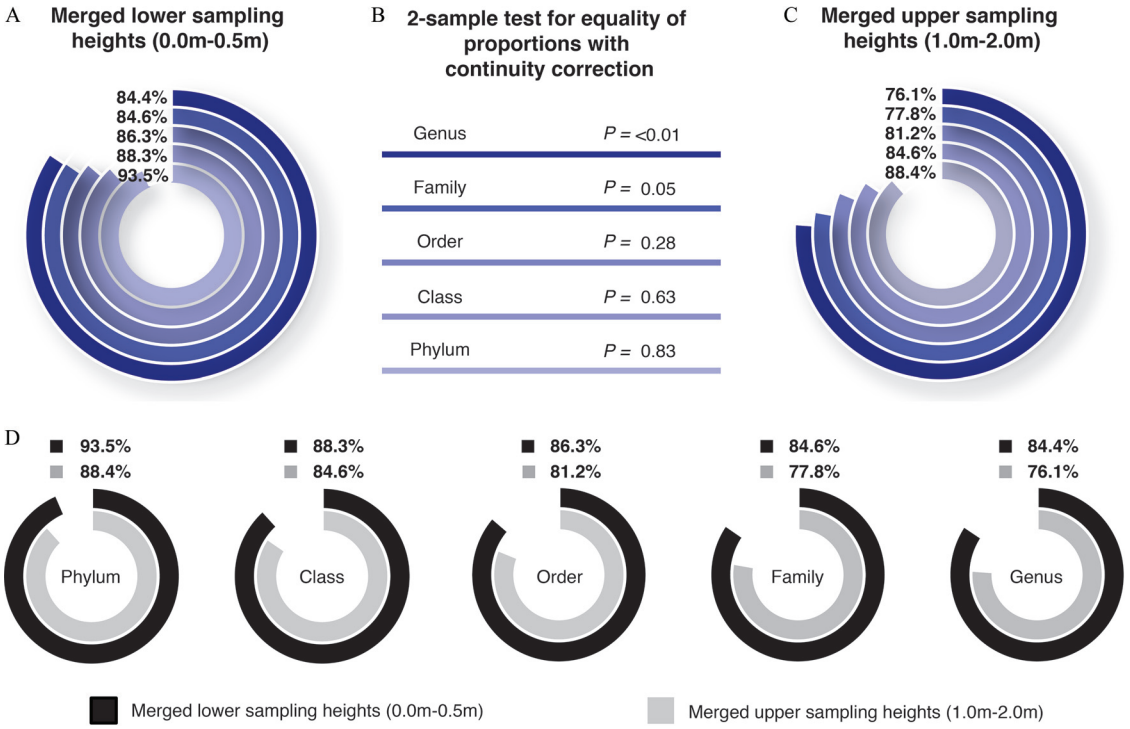

Figure 4. Radial charts showing proportions (as percent) of taxa from the air samples that also occurred in the soil samples for each sampling height and across all available taxonomic levels. A two-sample test for equality of proportions shows significant differences between lower and upper sampling heights for both genus and family taxonomic levels. Merged lower sampling heights are shown on the left (A), with the radial bar colors corresponding to the taxonomic level shown in (B), and merged upper heights are shown on the right (C). Proportional differences for individual taxonomic levels are compared in (D), with black radial bars indicating lower sampling heights, and gray indicating upper heights.

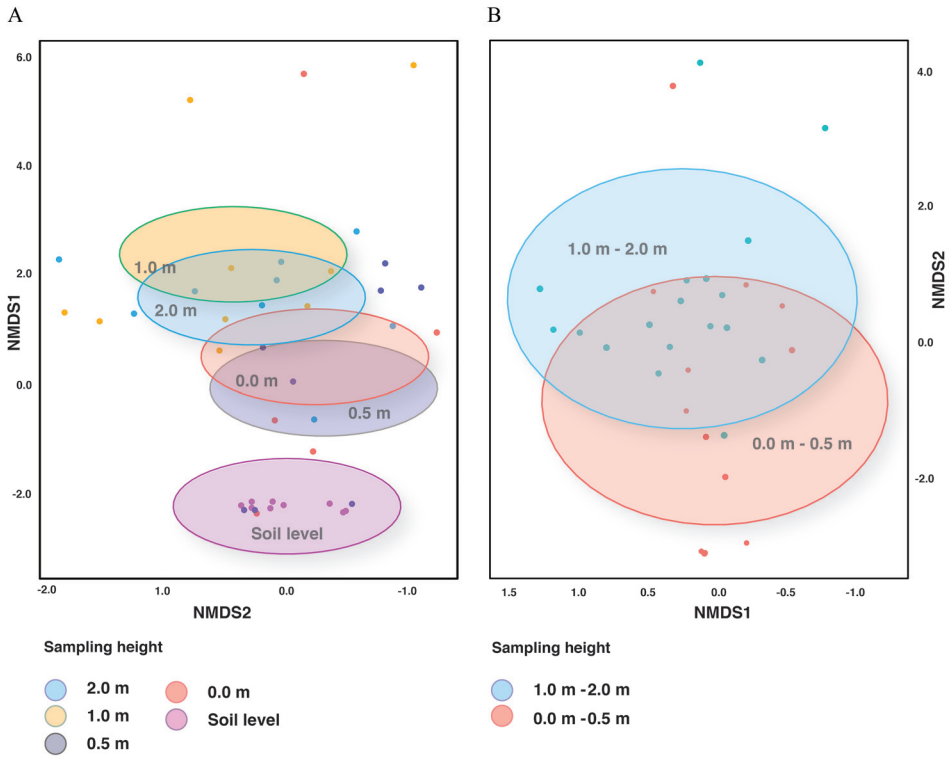

Figure 5. Nonmetric multidimensional scaling (NMDS) ordination plots for visualizing bacterial beta diversity (community composition) for all sampling heights, including soil (A) (Stress: 0.09, $R^2 = 0.22$) and for all sampling heights, excluding soil and merging within lower and upper samples (B) (Stress: 0.10, $R^2 = 0.06$). Ellipses represent Euclidian distance from the center, with the radius equal to the confidence level (0.95). Clusters suggest clear differences between communities at different sampling heights (indicated by the colors).

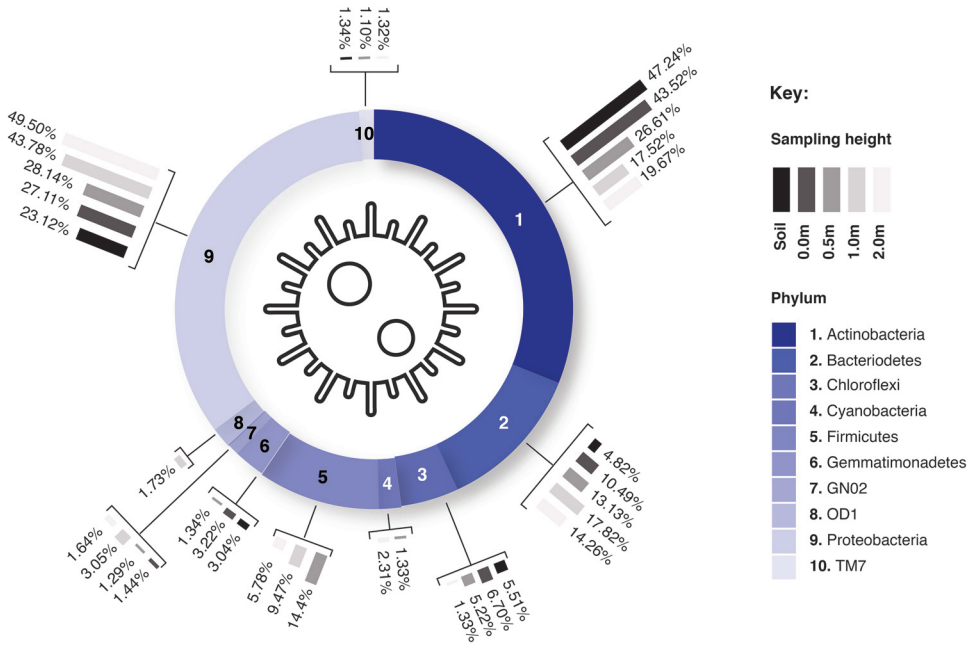

Figure 6. Relative abundance of bacterial operational taxonomic units (OTUs) at the phylum taxonomic level (based on mean relative abundance >1% for each sampling height). Ring segments relate to phyla via the number key on the right; segment size corresponds to mean relative abundance across all heights; mini bar charts relate to relative abundance of taxa for individual sampling heights where applicable. Actinobacteria (1) dominate lower sampling heights (indicated by the darker colored bars), Proteobacteria (9) dominate upper sampling heights (indicated by the lighter colored bars).

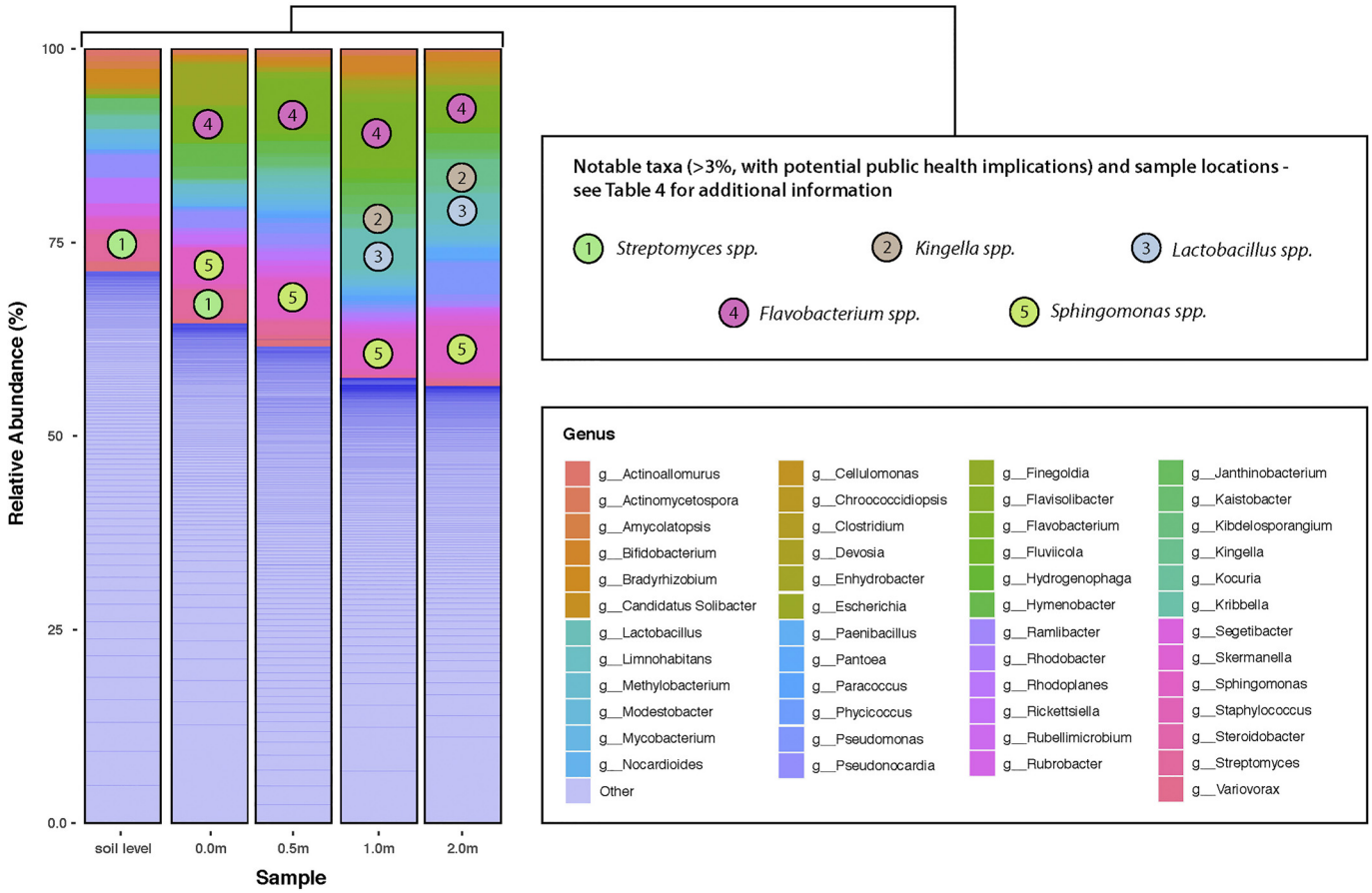

Figure 7. Relative abundance of bacterial operational taxonomic units (OTUs) at the genus taxonomic level and identification of notable taxa. Refer to Table 4 for potential public health implications of notable taxa. Reference numbers within the relative abundance bars correspond to the number key and notable taxa displayed in the upper-right pane.

Table 3. Correlations for notable taxa at the genus level across sampling heights, based on mean relative abundance (>1%) for each sampling height.

Reference number	Taxa (genus)	r_s score	S	p-Value
1	*Streptomyces*	−0.66	23,596	<0.01***
2	*Kingella*	+0.39	8,606	<0.01***
3	*Lactobacillus*	+0.54	6,470	<0.01***
4	*Flavobacterium*	+0.53	6,639	<0.01***
5	*Sphingomonas*	+0.39	8,577	<0.01***

Note: The Spearman's rank order correlation test was used. $S = (n3 - n) \times (1 - rs)/6$, where n is the number of bivariate observations and rs is Spearman's rank correlation coefficient. Reference number refers to the number key and corresponding notable taxa in Figure 7 and Table 4. *0.05. **0.01. ***<0.01.

both at genus and family levels. Together, these results suggest that soil does appear to play a key role in supplementing the local aerobiome, particularly at lower heights.

The presence of vertical stratification of bacterial diversity in the aerobiome could have important implications for human health. Indeed, exposure to environmental microbes is thought to prime and "educate" the immune system (Belkaid and Hand 2014; Hanski 2014; Mincham et al. 2020) particularly in early life, and a recent mouse study suggests that exposure to environmental microbes such as the butyrate-producer *Kineothrix alysoides* could also have anxiolytic (anxiety-reducing) effects (Liddicoat et al. 2019). The vertical stratification concept could also be important for exposome researchers, who investigate the types and methods of exposures to both endogenous and exogenous chemical composites (including microbes and their biological compounds across the life course) (Escher et al. 2017; Daiber et al. 2019; McCall et al. 2019). The presence of vertical stratification implies that the potential for exposure to environmental microbial diversity may differ throughout the human life course due to age and gender differences in height, activity types, and methods of motion. However, our static experimental conditions fail to capture the dynamics of human movement and activity within and between environments. Further research is required to understand how vertical stratification may affect human colonization, with particular focus on the dynamic nature of human movement through environments. Additional research into aerobiome stratification could lead to improved design and management of three-dimensional urban structures and vegetation assemblages, which may influence aerobiome dynamics. In the future, this research could lead to ways of optimizing human–environmental microbe interactions.

Humans are spending more time indoors (Ergan et al. 2019). Therefore, future aerobiome studies should also consider whether vertical stratification occurs indoors and consider the relative influence of the outdoor environment and the potential health implications of these dynamics. Understanding how patterns of human behavior influence exposure to airborne microbiota will also be important. For example, ongoing changes to commuting, recreation, and living environments may have important implications for aerobiome characteristics and exposure potential.

Table 4. Notable taxa (operational taxonomic units at the genus level) identified during the examination for bacterial relative abundance, based on mean relative abundance (>1%) for each sampling height.

Reference number	Notable taxa	Potential public health implication
1	*Streptomyces spp.*	These Actinobacteria are relatively more abundant at lower (vertically) sampling levels. They are soil-associated but also considered to be "old friends" with potential beneficial implications for human health (Bolourian and Mojtahedi 2018).
2	*Kingella spp.*	Higher relative abundance at upper (vertical) levels. The Gram-negative *K. kingae* is considered to be pathogenic to humans, causing osteomyelitis and septic arthritis, particularly in children (Kiang et al. 2005; Nguyen et al. 2018).
3	*Lactobacillus spp.*	Gram positive Firmicutes, relatively more abundant at upper levels. Some species are widely considered to be beneficial "old friends" and probiotics in humans and other ecosystems (Rook et al. 2014) (e.g., *L. acidophilus*; *L. plantarum*; *L. rhamnosus*).
4	*Flavobacterium spp.*	Soil and water-dwelling Bacteroidetes bacteria. These are present in all levels but with highest relative abundance at upper levels. Generally not considered to be pathogenic to humans. Spatial distribution suggests potential allochthonous deposition.
5	*Sphingomonas spp.*	These are Proteobacteria, found in a variety of environments. Relatively abundant in all sampling heights but less so in the soil level. These organisms are not considered to be pathogenic to humans and can in fact be highly beneficial via their ability to break down polycyclic aromatic hydrocarbons, which are deleterious to human health (Macchi et al. 2017; Asaf et al. 2020).

Note: The taxa in this table may have important public health implications as highlighted in the third column. Reference number refers to the number key and corresponding notable taxa in Figure 7.

Vertical Stratification of Aerobiome Beta Diversity

We also showed vertical stratification of aerobiome beta diversity, where sampling height explained 22% of the variation in environmental microbiota when all sampling heights were included. This variation was corroborated by the analysis of equality of taxonomic proportions between the air and the soil samples. As mentioned, the proportion of bacterial taxa from the air samples that were also present in the soil decreased as altitude increased. This finding provides preliminary evidence that soil has a stronger influence on aerobiome composition at lower heights, and allochthonous sources make a key contribution to the aerobiome higher up.

It is likely that distance to source makes a key contribution to aerobiome vertical stratification. However, there may be other important biophysical driving factors. For example, the size range of bacterial cells can vary by eight orders of magnitude (from 0.013 μm to 750 μm) (Levin and Angert 2015). However, many bacteria are thought to occur in the 0.3–5 μm range (Schaechter 2016). Bacteria can also nucleate and exist as "clumps" or adhere to larger suspended particles (Tham and Zuraimi 2005; Haas et al. 2013; Gong et al. 2020), thus altering their net particle size that would influence their fluid dynamics. Airborne bacterial concentrations can be influenced by several factors, including ambient temperature, humidity, wind dynamics, and particulate matter concentrations (Gong et al. 2020), and these factors could also play important roles in vertical stratification and warrant further research. There also appeared to be some mixing of aerobiome signals within fine vertical resolution strata, whereas more sensible patterns emerged in larger vertical strata. These findings are consistent with the phenomenon of turbulent mixed (nonlaminar) flow, and we might expect some level of vertical mixing in the aerobiome where turbulent flow occurs over and around obstacles and over rough surfaces.

Vertical stratification in bacterial beta diversity could also have important implications for public health. For example, our results point to intriguing questions, such as: *a*) Are there

significant and consistent differences in potentially beneficial and pathogenic bacterial assemblages at different altitudes in the aerobiome? *b*) Does this affect exposure and colonization in humans across the life course? and *c*) What are the downstream health implications of this, if any? We provide a preliminary contribution towards answering the first question, as discussed in the section, "Relative Abundances and Notable Taxa."

Future research could also consider the potential influence of physicochemical (e.g., antimicrobials, pesticide use) and social (e.g., crowd gathering, isolation, or distancing) practices on microbial vertical stratification. For example, efforts to reduce infectious agents such as COVID-19 may disrupt out relationship with environmental microbiomes. Therefore, understanding whether and how these changes affect human–environmental microbial interactions will be essential in the future.

Relative Abundances and Notable Taxa

Following the analyses of relative abundances, the dominant taxa in the soil and lower sampling heights were found to be Actinobacteria, and the dominant taxa in the upper sampling heights were Proteobacteria. This finding is not surprising, given that a large proportion of terrestrial Actinobacteria are soil-dwelling organisms (Barka et al. 2016; Zhang et al. 2019), and both phyla are among the largest in the bacterial domain (Verma et al. 2013; Polkade et al. 2016; Rizzatti et al. 2017). Other studies have shown similar dominant roles for these phyla in the aerobiome (Arfken et al. 2015; Maki et al. 2017; Li et al. 2018), but vertical stratification has not, to our knowledge, been explored.

We identified a number of notable dominant taxa at the genus level, including *Streptomyces*, *Kingella*, *Lactobacillus*, *Flavobacterium*, and *Sphingomonas*. With the exception of *Flavobacterium*, species in these genera are considered to have potentially beneficial or pathogenic impacts on human health. For example, the Actinobacteria *Streptomyces* spp., is considered to be a microbial "old friend" and potentially beneficial to human health via production and regulation of antiproliferative, antiinflammatory and antibiotic compounds (Bolourian and Mojtahedi 2018; Nguyen et al. 2020). This genus had higher relative abundance at lower sampling heights. On the other hand, members of the *Kingella* genus such as *K. kingae* are considered to be pathogenic to humans, for example—causing debilitating conditions such as osteomyelitis and septic arthritis, particularly in children (Kiang et al. 2005; Nguyen et al. 2018; Ingersoll et al. 2019). These findings warrant further research because, if consistent across time and space, the spatial and compositional differences in microbiota have the potential to be important considerations for public health through the modulation of exposure.

Limitations

As a proof of concept study, we have demonstrated, for the first time, the presence of vertical stratification of microbial alpha and beta diversity at lower levels of the biosphere (ground level to 2.0 m high). However, data from a larger number of replicates from different environments and geographical areas will be required to establish the generalizability of our findings; i.e., will our results be consistent outside of the Adelaide Parklands environment? We also used OTU picking methods at the bioinformatics stage. We recognize that although this approach has value for short-read platforms and many studies still use this approach (Dei-Cas et al. 2020; Derilus et al. 2020; Sato et al. 2020), Amplicon Sequence Variant (ASV) analysis would have provided a more detailed taxonomic picture of vertical stratification. Further, following the DNA extraction process, three samples (each at SC03 0.0 m) failed to reach sufficient DNA concentrations to enable PCR and sequencing, which may have affected the vertical stratification relationship; we can only speculate that the relationship would have been stronger with their inclusion. There are many sensitive variables involved with processing low biomass samples (Eisenhofer et al. 2019; McArdle and Kaforou 2020), and perhaps even more stringent workflows are required for passive sampling.

Conclusions

We provide support for the presence of aerobiome vertical stratification in bacterial diversity (alpha and beta), and demonstrate that significant spatial differences in potentially pathogenic and beneficial bacterial taxa may occur. Although the need to promote healthy ecosystems and understand environmental microbial exposures has always been important, in light of the COVID-19 pandemic, it is now justifiably at the forefront of many public health agendas worldwide. As discussed, there is growing evidence to suggest that exposure to the microbiome in biodiverse green spaces contributes towards educating the immune system (Rook et al. 2003, 2013; Tasnim et al. 2017; Liddicoat et al. 2020). Furthermore, the microbiome is thought to support the immune system's defensive role against pathogens and prevent hyperinflammatory responses and metabolic dysregulation, which are risk factors for severe COVID-19 (Torres et al. 2019; Guo et al. 2020). Gaining a greater understanding of the transmission routes and physical factors (such as the vertical differential) affecting our exposure to environmental microbiomes—including potentially beneficial and pathogenic species—is likely to play an increasingly important role in the health sciences.

Strategies to explicitly consider the microbiome as part of health-promoting urban green spaces have recently been proposed, such as Microbiome-Inspired Green Infrastructure (MIGI) (Robinson et al. 2018; Watkins et al. 2020). Further exploration of aerobiome vertical stratification could make an important contribution to this approach. For example, there could be value in determining whether different habitats and vegetation management regimes affect vertical stratification in urban green spaces and in elucidating the downstream health effects on urban dwellers. Building on our findings—that vertical stratification did occur in an urban green space aerobiome—has the potential to inform future exposome research, urban biodiversity management, and disease prevention strategies.

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J.M.R. and M.F.B. contributed to the conception and design of the study; J.M.R. and C.C.D. conducted the field and lab work; J.M.R. and C.L. conducted the bioinformatics and data analysis; J.M.R. wrote the manuscript; J.M.R. produced the figures and data visualizations; J.M.R., C.C.D., C.L, P.W., R.C., and M.F.B. contributed to manuscript internal critical review process and revisions. All authors read and approved the submitted version.

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