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Effect of traffic congestion and vegetation on airborne bacteria in a city of a developing country

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Abstract

The study was designed to determine the variations in the diversity and the total abundance of airborne bacteria in the atmosphere of nine locations in Kandy City, the cultural capital of Sri Lanka. Culturable microorganisms were identified using 16S rDNA sequencing. Quantification of total bacterial abundance was calculated using real-time PCR. Twenty-eight bacterial species were identified by 16S rDNA sequencing. *Bacillus cereus, Bacillus pumilus, Pseudomonas aeruginosa, Pseudomonas stutzeri*, and *Brevundimonas vesicularis* were present in all the sampling sites. Most of the recorded species were opportunistic human pathogens of the respiratory tract (*Pseudomonas* spp., *B. cereus, B. vesicularis, Klebsiella pneumoniae*), gastro intestine (*B. cereus, K. pneumoniae*), and skin (*B. cereus*). The highest total bacterial load (1.42×10^{10} cells/m^2) was at the railway station where traffic congestion was the highest while significantly high mean culturable bacterial concentration (5.35×10^6 CFU/m^2) ($p < 0.05$) was recorded from the site close to a tea plantation with heavy vegetation cover. This study shows the impact of vegetation and traffic congestion on airborne microorganisms. The presence of opportunistic pathogens highlights the need for risk assessment and management of air quality in congested urban areas.

Keywords Opportunistic pathogens · Diversity · Total abundance · Real-time PCR · 16S rDNA sequencing

Highlights
- Total abundance of airborne bacteria bound to atmospheric dust samples is reported.
- Study location is Kandy City, the cultural capital of Sri Lanka.
- Twenty-eight bacterial species were identified by 16S rDNA sequencing.
- Most of the recorded species were opportunistic human pathogens.

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Introduction

Inhalation of air includes various gases, hydrocarbons, aerosols, and particulate matter. Bacteria, fungi, viruses, pollens, and byproducts of microorganisms such as endotoxins and mycotoxins are significant components of bioaerosols and particulate matter in the atmosphere (Després et al. 2012). Outdoor atmospheric microorganisms are considered a critical source of indoor atmospheric microbial communities because they enter indoor through ventilation systems (Onat et al. 2016). The impact of the airborne microorganisms on human, animal, and plant health can be significant. A study undertaken in the Virgin Islands in the United States has shown that the atmosphere constitutes 25% plant pathogens (*Bacillus megaterium, Curtobacterium citreum, Gibberella pulicaris, Sphingomonas pruni*, *Cochliobolus sativus*), 10% human opportunistic pathogens (*Cladosporium cladosporioides*, *Arthrobacter* sp.), and others are non-pathogens (*Pleospora rudis, Curtobacterium luteum*) (Griffin et al. 2002; Kellogg et al. 2004). Therefore, investigating the atmospheric microbial life can reveal solutions for current and future scenarios such as zoonotic diseases, climate change (through ice

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nucleation), bioweapon threats, and ecological impacts (nutrient cycles, pollution).

The atmosphere is considered the medium for dispersion of microorganisms, rather than a habitat (Smith et al. 2012). Similar to other environments, the atmosphere is an assorted congregation of microorganisms and the diversity can fluctuate spatially and temporally (Zhai et al. 2018). The heterogeneity, distribution, and sources of microorganisms in the atmosphere have not received adequate attention, particularly in developing countries. Knowledge of the diversity and distribution patterns of the airborne microorganisms is vital to understand the risk posed by them as it is commonly accepted that exposure to these microorganisms is one of the causes for many respiratory diseases such as pneumonia, aspergillosis, chronic pulmonary disease, tuberculosis, and allergic problems such as allergic alveolitis, allergic bronchopulmonary penicilliosis (Griffin 2010; Kim et al. 2018). Understanding the sources of microorganisms can contribute to the formulation of effective airborne infection control strategies for efficient reduction of microbial air pollutants.

Spatial and temporal diversity of airborne microorganisms is governed by a range of factors such as pH, temperature, UV intensity, humidity, and precipitation occurrence (Griffin 2010; Jang et al. 2018; Smets et al. 2016). The most common types of airborne bacteria that have been observed in atmospheric sampling belong to the genera *Arthrobacter, Bacillus*, *Pseudomonas*, *Staphylococcus*, *Streptomyces,* and *Microbacterium* (Griffin 2010). *Alternaria*, *Aspergillus*, *Penicillium*, *Phoma*, *Cladosporium*, and *Fusarium* are the most common fungal genera that have been discovered from atmospheric sampling (Griffin 2010). It has been estimated that total number of microorganisms in 0.7 m^3 is in the range of ~4×10^6 where the samples were collected 10 m above the ground level (Kakikawa et al. 2008). The topsoil of the land, aquatic ecosystems, traffic aerosols, and plants are considered major sources of airborne microorganisms (Griffin et al. 2017; Kakikawa et al. 2008; Mescioglu et al. 2019). In addition to that, volcanic eruptions, burning of biomass, and agricultural activities such as tilling and livestock grazing introduce microorganisms to the atmosphere (Griffin et al. 2017). These airborne microorganisms are transported via attaching to dust particles (Mazar et al. 2016), introducing microorganisms not only to terrestrial ecosystems but also to aquatic ecosystems (Mescioglu et al. 2019; Rahav et al. 2016). Therefore, it is essential to understand the quantity and types of bacteria in the atmosphere.

Although studies on airborne microorganisms have been undertaken in many developed countries (Serrano-Silva and Calderon-Ezquerro 2018; Genitsaris et al. 2017), developing countries such as Sri Lanka have not paid much attention to the issue of airborne microorganisms (Weerasundara et al. 2017). Even though some studies pay attention to both microbial and heavy metals in the atmosphere of Sri Lanka, only few types of bacterial species have been identified (Weerasundara et al. 2017). Indoor study conducted at the Kandy hospital in Sri Lanka by Sivagnanasundaram et al. (2019) showed the presence of pathogenic bacteria including *Micrococcus* sp., *Pseudomonas* sp. But, the information on microorganisms in breathing level in outdoor air is important to understand their effects on human health. Therefore, the current study was conducted to investigate the bacterial pollutants in the outdoor atmosphere. Accordingly, this study aimed to determine the site-specific variations in airborne bacterial communities within the heavily traffic-congested city environment of Kandy, Sri Lanka by using culturable methods and molecular techniques.

Materials and methods

Sample collection

The study was undertaken in Kandy, the second largest city of Sri Lanka. It is a major cultural, educational, commercial, administrative, and transport center. This is one of the most revered cities in the world, included in the UNESCO World Heritage List due to the presence of the Sacred Temple of the Tooth Relic. The City lies amid hills on a plateau (altitude 473 m) in the Central Province of Sri Lanka and its condition is similar to a bottom of a basin (Seneviratne et al. 2017). Due to its geographical placement, Kandy City is considered to have a highly polluted atmosphere due to vehicle emissions (Wickramasinghe et al. 2011).

The area of the Kandy City region is approximately 26 km^2. Around 0.12 million population reside within the City. About 0.1 million people commute daily within the Kandy City limits and more than 100,000 vehicles move daily within the area (Premasiriet al. 2012). The average elevation varies between 1500 feet in the flat surface of the core area and about 792 m in the hilltops. Kandy urban area is demarcated by natural boundaries. The south boundary falls along the limits of the hills representing approximately 30% of the perimeter. The West, North, and East boundary is demarcated by the Mahaweli River and it is nearly 55% of the total urban boundary. The other 15% falls along the natural streams or over hilltops (Masakorala 2015). Day time ambient temperature in the city is between 27.6 and 31.8 °C, monthly rainfall is between 52 and 398 mm, and day time relative humidity is 63–83% (Wickramasinghe et al. 2011). As the City is surrounded by mountains, air circulation within the City is restricted. Nine sampling sites in the Kandy City limits were selected according to traffic and vegetation characteristics (Table 1). Five traffic-congested sites (UTS) were selected where the daily traffic volume was >6900. Except for site C (Children's Park), the rest had minimal vegetation cover. The other four sites were designated as F (Fire Brigade), P (Police

Table 1 Vegetation and traffic characteristics of the sampling sites

Site	GPS locations	Vegetation characteristics	Traffic characteristics	Other characteristics	Type
National Institute of Fundamental Studies (NIFS), Hantana road, Kandy (I)	Longitude: 80°37′56.9″E Latitude: 7°17′01.4″N	Surrounded by large number of large trees and small plants	Traffic congestion does not exist. However, vehicles are driven near the sampling site routinely.	Non-commercial, low residential activities, situated remote from the city	RNTS
Lewalla, Kandy (L)	Longitude: 80°39′02.6″E Latitude: 7°17′49.9″N	High vegetation cover with several large trees and small plants present.	None	Residential area with ten houses, situated in rural area	RNTS
Dodanwela , Kandy (D)	Longitude: 80°37′22.4″E Latitude: 7°18′08.1″N	High vegetation cover. Large trees, small plants present.	None	Residential area, number of houses are greater than at site L, situated in rural area	RNTS
Tea Research Institute, Hantana, Kandy (TRI)	Longitude: 80°38′05.7″E Latitude: 7°17′05.1″N	High vegetation cover, tea plantation	None	Non-residential area, on a hilltop, away from the City, situated in rural area	RNTS
Railway Station, Kandy (R)	Longitude: 80°37′55.9″E Latitude: 7°17′23.8″N	No vegetation cover	Highly traffic congested site near the major bus terminus in Kandy City, 24 h traffic volume >23,000	High density built-up area, minimal residential activities, situated in urban area	UTS
Police Station, Kandy (P)	Longitude: 80°38′01.9″E Latitude: 7°17′36.1″N	Low vegetation cover with 2 large trees and few small plants	Highly traffic congested site, with circular intersection of three merging main roads, 24 h traffic volume >30,200	High density built-up area, minimal residential activities, situated in urban area	UTS
Fire Brigade, Kandy (F)	Longitude: 80°38′07.2″E Latitude: 7°17′30.2″N	No vegetation cover	Highly traffic congested site, with intersection of three main roads, 24 h traffic volume >6900	High density build-up area, minimal residential activities, situated in urban area	UTS
Children's Park, Kandy (C)	Longitude: 80°38′52.0″E Latitude: 7°17′15.2″N	Situated in a grass field, having 2–3 large trees and few small plants	Highly traffic congested site, with intersection of three main roads, 24 h traffic volume >30,300	Density of buildings are low compared to sites P, F, and T, minimal residential activities, consisting of recreational activity facility, situated in urban area	UTS
Road side near Trinity College, Kandy (T)	Longitude: 80°38′04.6″E Latitude: 7°18′20.5″N	No vegetation cover	Near a school. Highly traffic congested site, 24 h traffic volume >18,000	High density build-up area, minimal residential activities, situated in urban area	UTS

RNTS, rural non traffic congested sites; *UTs*, urban traffic congested sites

Station), R (Railway Station), and T (Trinity College). Four sites with low traffic congestion (RNTS) were selected, wherein rich vegetation cover was present. These sites were designated as TRI (Tea Research Institute), I (National Institute of Fundamental Studies), L (Lewella), and D (Dodanwala) (Fig. 1).

The settle plate method was used for capturing depositing microorganisms by directly exposing the media plates to the atmosphere (Naruka and Gaur 2014). Deposition samples were collected with the use of direct impaction Luria–Bertani (LB) agar plates and Petri plates containing filter papers (diameter 8.5 cm) by using a sample collection system that was previously described by Weerasundara et al. (2017). The sampling system is illustrated in the Supplementary information (Fig. S1). The agar plates were mounted on the sampling system at each site where the mesh bases were connected to a star picket bar and fixed at the height of 1.5 m above ground in a relatively open area to determine the diversity of microorganisms at human respiration level and also to minimize contamination from re-suspended particles deposited on the ground (Gunawardena et al. 2013).

Duration of sampling was from May 2015 to April 2016 during weekdays (twice per week) and the deposition period was six-hourly (06.00–12.00 h and 12.00–18.00 h, day time). After sampling, LB agar plates were immediately transported to the laboratory and incubated for 1 to 2 days at 25 °C in order to obtain culturable bacteria. After transportation to the laboratory, filter papers were immediately cut into small pieces and shaken in 8 mL of sterile Mili-Q water at 200 rpm for 2 h. Two milliliters of the solution was used for DNA extraction for real-time PCR analysis.

Identification of bacteria

After incubation of LB agar plate for 24 to 48 h, bacterial colony morphologies were observed and Gram staining was performed for the morphologically distinct bacterial colonies. These bacterial colonies were subcultured. The pure isolates from the subcultures were used for DNA extraction. Bacterial DNA was extracted using the modified Cetyltrimethylammonium bromide (CTAB) method (Somerville et al. 2005), and extracted DNA was subjected to polymerase chain reaction (PCR) by using 16S rDNA primers, F: 5′ AGRGTTTGATCMTGGCTCAG 3′ and R: 5′ GGYTACCTTGTTACGACTT 3′. The reaction was carried out in a 25 μL mixture containing 375 ng of DNA, 0.1 mM of each dNTP, 0.4 μM of each forward and reverse primer, 1x Taq buffer (Promega), 1.5 mM $MgCl_2$ (Promega), and 1 unit of Taq DNA polymerase (Promega). PCR was conducted: 94 °C 1 min, 50 °C 1 min, and 70 °C 2 min (Chen et al. 2009).

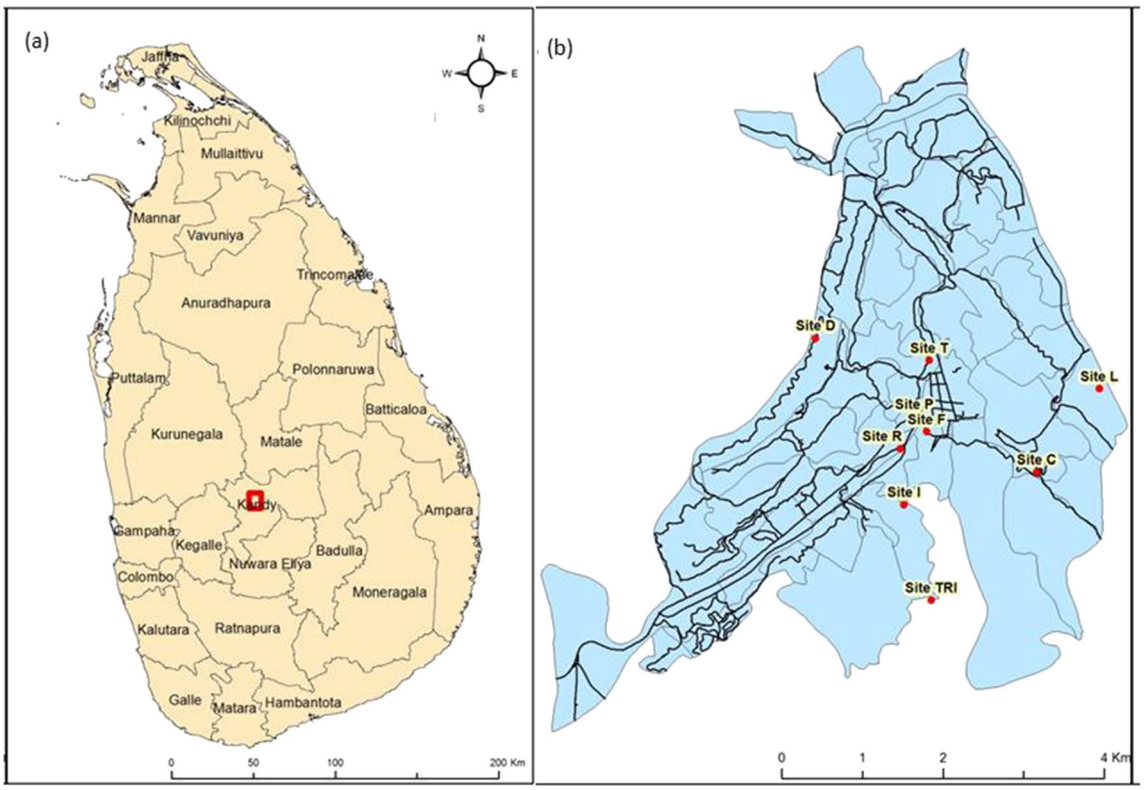


Fig. 1 (**a**) Location of Kandy City in the map of Sri Lanka; (**b**) Location of nine sample collection sites in the map of Kandy Municipal Council. I – National Institute of Fundamental Studies, R – Railway Station, P – Police Station, F – Fire Brigade, C – Children's Park, L – Lewalla, D – Dodanwela, T – Trinity College, TRI – Tea Research Institute

Amplified DNA fragments were purified using a gel extraction kit (Promega) and were commercially sequenced by Macrogen Inc., South Korea. All the sequences were compared with the National Center for Biotechnology Information (NCBI) database and identified. The sequences were submitted to NCBI GenBank (Accession numbers: KT985360–KT985382, KU510055–KU510075, KX641080–KX641083) (sequence identity > 97%).

Diversity indices

Shannon's diversity index (H) was determined using,

$$H = -\sum_{i=1} n(pi \ln pi)$$

where n is the total number of bacterial species, and pi is the number of CFU of each bacterial species divided by total bacteria (Fahlgren et al. 2010). H was calculated for each site and each month in which sampling was conducted.

Culturable bacterial concentration

A hundred microliters of the solution with the suspended particulate matter was spread on a LB agar plate and incubated at 25 °C for 1–2 days in order to obtain culturable bacteria as CFU/m^2 (Shaffer and Lighthart 1997). These bacterial colonies were tested for Gram staining characteristics.

Total bacterial concentration

Two milliliters of solution that was taken from the filter paper was subjected to Boom's DNA extraction (Boom et al. 1990). Total bacteria was measured using real-time PCR with the use of 5′ TCCTACGGGAGGCAGCAGT 3′ and 5′ GGACTACC AGGGTATCTAATCCTGTT 3′ primers (Nadkarni et al. 2002). The reactions were carried out in a 25 μL mixture containing 10 μL of environmental DNA sample, 0.1 mM of each dNTP, 0.4 μM of each forward and reverse primer, 0.1x SYBR Green I, 1x Taq buffer (Promega), 1.5 mM $MgCl_2$ (Promega), and 1 unit of Taq DNA polymerase (Promega).

The total number of cells was calculated using the equation given below (Oppliger et al. 2008).

$$\text{Genome weight in Dalton, W} = [\%\text{GC} \times \text{total length}] \times 618.4/100 + [(100-\%\text{GC}) \times \text{total length}] \times 617.4/100 + 36$$

$$\text{Genome copies per nanogram of DNA} = (\text{NL}/\text{W}) \times 10^{-9}$$

$$\text{NL is the Avogadro constant } \left(6.02 \times 10^{23} \text{ molecules per mol}\right)$$

The calibration curves were generated using Rotor-Gene Q 2.3 software version (Fig. S2). The calibration curve was used to obtain the initial numbers of cells in the environmental samples.

Results

Gram staining results

Gram-positive, rod-shaped bacteria were prominent in all sites (Table 2). The highest number was recorded at the site TRI as 4.64×10^6 CFU/m^2.

Culturable bacterial concentrations and total bacterial concentrations

Culturable bacterial concentration was significantly high at site TRI which is rich with vegetation while the highest total bacterial load was observed at site R, where congestion is high (≈23,000 vehicles per day) (Fig. 2).

The culturable bacteria that were collected from the atmospheric samples were taxonomically diverse. Twenty-eight bacterial species that were identified belonged to four major groups as Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, and Bacilli (Table 3). Fourteen bacterial species belonged to the Gammaproteobacteria group. Three species were from the Alphaproteobacteria group, and two and nine bacterial species were from the Actinobacteria and Bacilli groups, respectively. Identified *Kocuria* sp. and *Arthrobacter sanguinis* belong to the Actinobacteria group. Only these two species were Gram-positive cocci and culture isolates did not yield any Gram-negative cocci.

The phylogenetic relationship between the identified bacterial species is shown in Fig. 2. S2FP27-ia strain was a Gram-negative rod-shaped bacterium. The similarity of *Bacillus* sp. S2FP27-ia (KX641081) sequence could not be determined with a high degree of certainty to species level since the closest matches were *B. amyloliquefaciens* and *B. subtilis* having 98% similarity when compared with the data bank sequences. According to the phylogenetic tree, the sequence is mostly matching with *B. subtilis*. The strain E1 was also a Gram-positive, rod-shaped bacterium. *Exiguobacterium* sp. E1 (KT985362.1) showed 96% similarity to both *E. acetylicum* and *E. indicum*, but *Exiguobacterium* sp. E1 (KT985362.1) is most closely related to *E. indicum* according to the phylogenetic tree. *Sphingomonas* sp. S1 (KT985361.1) was sharing sequence similarity of 98% with *S. pseudosanguinis, S. yabuuchiae, S. parapaucimobilis*, and *S. sanguinis*. Therefore, this isolate could not be identified to species level with high degree of certainty (Fig. 3).

Most of the bacteria such as *Serratia marcescens*, *E. acetylicum, E. indicum,* and *Sphingomonas* sp. were pigmented. Spore forming genus *Bacillus* was abundant among the sequenced culturable bacteria.

Potential pathogens

Most of the bacterial species were potential pathogens (Table 4). Twenty-two out of the identified twenty-eight

Table 2 Concentrations of culturable bacteria in the atmospheric deposition samples according to Gram staining characteristics

Site	Mean culturable Gram positive rod-shaped bacteria (CFU/m^2)	Mean culturable Gram negative rod-shaped bacteria (CFU/m^2)	Mean culturable Gram positive cocci-shaped bacteria (CFU/m^2)	Mean culturable Gram negative cocci-shaped bacteria (CFU/m^2)	Total culturable bacteria (CFU/m^2)
C	5.23×10^5	8.92×10^4	2.34×10^3	0.00	6.14×10^5
F	1.36×10^6	1.43×10^5	2.63×10^3	0.00	1.50×10^6
P	1.57×10^6	2.89×10^5	1.99×10^4	0.00	1.75×10^6
R	9.11×10^5	5.73×10^5	7.25×10^4	0.00	1.56×10^6
I	6.56×10^5	4.17×10^4	2.60×10^4	0.00	7.24×10^5
L	1.45×10^6	7.93×10^4	0.00	0.00	1.53×10^6
D	1.28×10^6	3.09×10^5	0.00	0.00	1.59×10^6
T	1.84×10^6	5.23×10^5	0.00	0.00	2.36×10^6
TRI	4.64×10^6	1.88×10^4	6.87×10^5	0.00	5.35×10^6

C – Children's Park, F – Fire Brigade, P – Police Station, R – Railway Station, I – National Institute of Fundamental Studies, L – Lewella, D – Dodanwala, T – Trinity College, TRI – Tea Research Institute

bacterial types could be recognized as potential pathogens. Fifteen types were common to both UTS and RNTS.

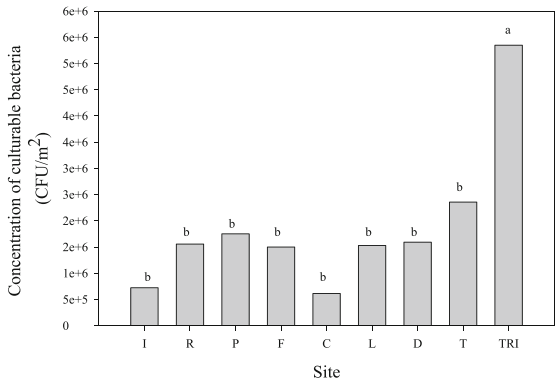
Spatial variation of bacterial diversity

The highest number of bacterial species was observed at site C with 25 species (Table 4), but the diversity index was 1.66 (Table 5). Eleven bacterial species were evident at the TRI site, where the diversity index was 0.31, which was the lowest diversity index when compared with all the sampling sites. The diversity indices ranged from 0.31 to 2.21. The highest Shannon's index could be observed at station P as 2.21. Bacterial species, *B. cereus, B. pumilus, Pseudomonas aeruginosa, P. stutzeri,* and *Brevundimonas vesicularis* were present in all the sampling sites. Some bacteria were observed only in a limited number of sites such as *B. megaterium* at sites C, *B. aryabhattai* at site C, *Enterobacter ludwigii* at the site I, and *Escherichia hermanni* at site P.

Temporal variation of bacterial diversity

In the present study, the samples were collected during weekdays throughout a year from May 2015 to April 2016. There was considerable variability in bacteria in the collected samples across the year. The lowest number of bacterial species was observed in April, and Shannon's diversity index for this month was 0.34, which was the lowest among the months (Table 6). The highest number of bacterial species (n=25) was recorded in September (Table 7), where the diversity index was 1.57. Shannon's diversity indices of the bacteria of the sampling months ranged from 0.34 to 2.00.

B. pumilus was the most dominant bacterial species in June, July, and December, while *B. cereus* was the most dominant bacterial species in November, January, February, and April. However, in August and September, both *B. pumilus* and *B. cereus* were equally dominant. *P. stutzeri* showed the highest percentage of bacteria at 73.85% in March. In October, *Exiguobacterium* sp. and *B. cereus* were dominant



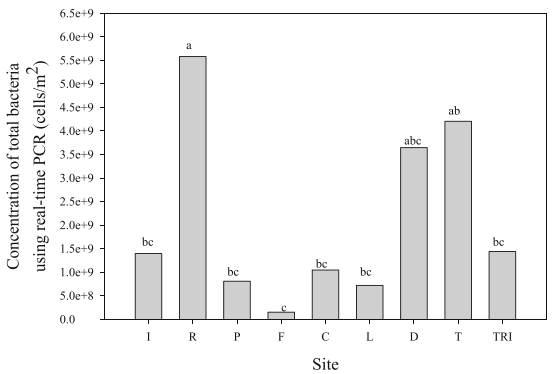


Fig. 2 Variation of concentration of culturable bacteria and total bacteria in nine sites within Kandy City. I – National Institute of Fundamental Studies, R – Railway Station, P – Police Station, F – Fire Brigade, C – Children's Park, L – Lewalla, D – Dodanwela, T – Trinity College, TRI – Tea Research Institute. The means denoted by the same letters on the bars in the graph are not significant different at $p \leq 0.05$

Table 3 Major taxonomic groups of bacteria that were identified

Class	Family	Bacterial species	Accession numbers
Gammaproteobacteria	Enterobacteriaceae	*Serratia marcescens* SM1	KT985379
		Serratia marcescens SM2	KT985380
		Providencia rettgeri PR1	KT985381
		Enterobacter ludwigii EN1	KU510068
		Escherichia hermanni ES1	KU510070
		Escherichia hermanni ES2	KU510074
		Klebsiella pneumonia KP1	KT985366
		Leclercia adecarboxylata LA1	KT985369
	Pseudomonadaceae	*Pseudomonas aeruginosa* PSA1	KT985363
		Pseudomonas monteilii PSM1	KT985367
		Exiguobacterium acetylicum PS3	KU510064
		Pseudomonas stutzeri PS4	KU510066
		Pseudomonas brenneri PS5	KU510067
	Xanthomonadaceae	*Stenotrophomonas maltophilia* ST1	KU510056
		Stenotrophomonas maltophilia ST2	KU510059
	Moraxellaceae	*Acinetobacter soli* AC1	KU510058
		Acinetobacter baumannii AC2	KU510069
Alphaproteobacteria	Caulobacteraceae	*Brevundimonas vesicularis* BR1	KU510063
	Brucellaceae	*Ochrobactrum intermedium* OI1	KT985368
	Sphingomonadaceae	*Sphingomonas* sp. S1	KT985361
Bacilli	Bacillaceae	*Exiguobacterium acetylicum* EA1	KT985374
		Exiguobacterium indicum EI1	KT985378
		Bacillus pumilus BA1	KT985365
		Bacillus pumilus S5FP12b	KX641083
		B. subtilis BA12	KU510071
		B. subtilis BA14	KU510073
		Bacillus amyloliquefaciens BA3	KT985372
		Bacillus amyloliquefaciens BA5	KT985375
		Bacillus amyloliquefaciens BA13	KU510072
		Bacillus aryabhattai BA6	KT985376
		Bacillus aryabhattai BAB2	KT985382
		Bacillus megaterium BA7	KT985377
		Bacillus cereus BA8	KU510055
		Bacillus cereus BA9	KU510057
		Bacillus cereus BA11	KU510062
		Bacillus thuringiensis BA10	KU510061
Actinobacteria	Micrococcaceae	*Arthrobacter sanguinis* AR1	KU510065
		Kocuria kristinae KK1	KT985360

with percentages of 48.16% and 38.48%, respectively (Table 7).

B. megaterium, which could be observed at site C was detected in the months of June and August. *B. aryabhattai* could be observed at site C in August and September. *E. ludwigii* was detected in three months (July, August, and September) at site I, and *E. hermanni* in the months of May, June, August, and September at P (Tables 7 and 8).

Discussion

Although the atmosphere can appear to be clear, it is rich with the organisms which are unseen through the naked eye. Thousands of microorganisms travel searching for their habitat. The study provides evidence for this phenomenon through the number of culturable and total bacteria.

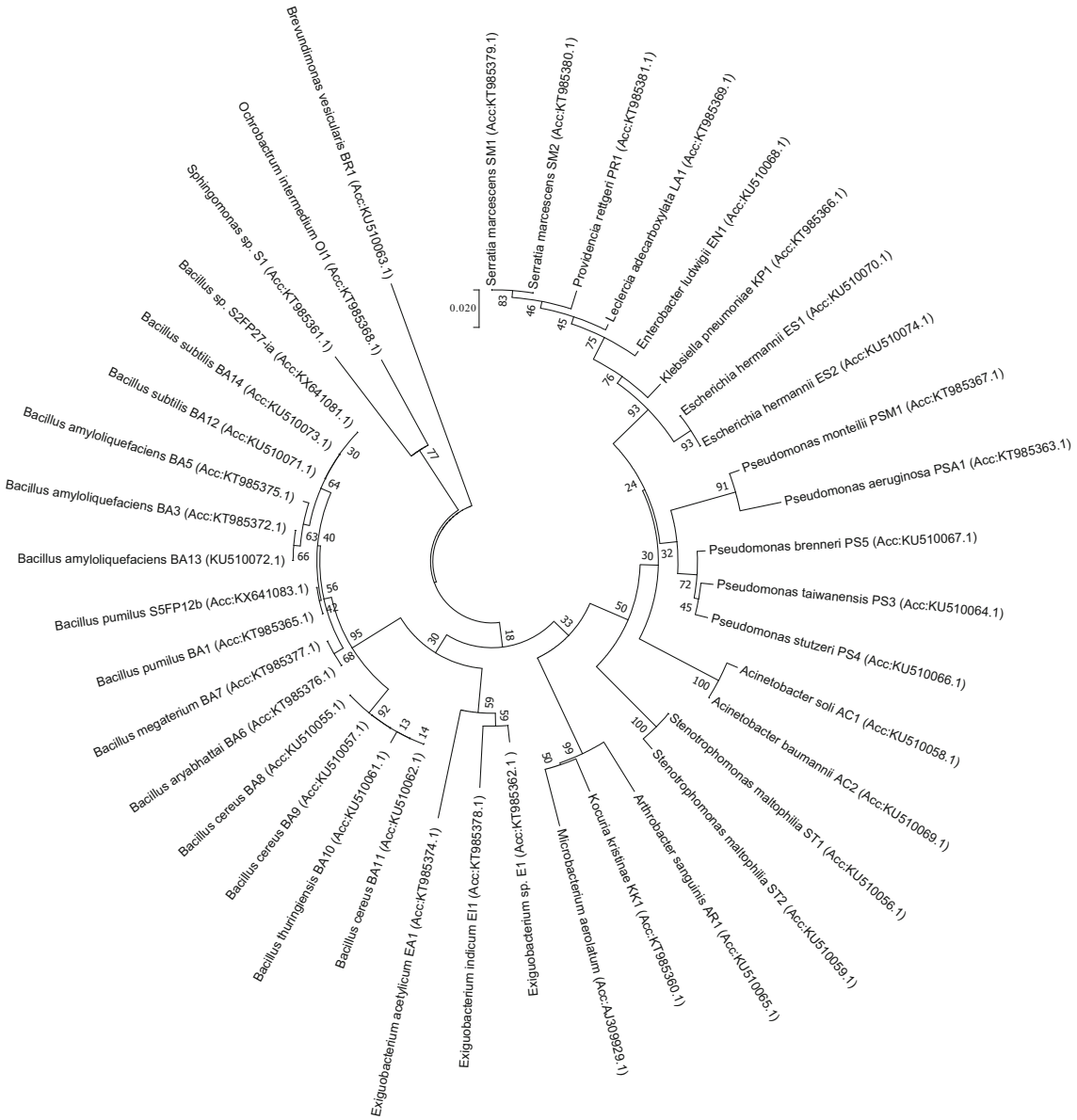


Fig. 3 Phylogenetic tree showing the relationships of sequenced culturable bacterial species

The significantly highest culturable microbial concentration ($5.35\times10^6 \pm 1.23\times10^5$ CFU/m^2) was detected at the site of the Tea Research Institute, which is a RNTS site (p = 0.0040). This result suggests that the high number of culturable bacteria is introduced to the air by vegetation. Moisture level at this site is high compared to the other sites (personal observations) as the vegetation introduces water vapor by transpiration. Culturability and viability of bacteria increases with the moisture level of the environment (Bragoszewska et al. 2017).

In contrast to the site at the Tea Research Institute, total bacterial count ($5.58 \times 10^9 \pm 1.65\times 10^8$ cells/m^2) was more significant at the site of Railway Station, which is a UTS site. Total bacterial count depicts all viable, nonviable, culturable, and also unculturable bacteria. Therefore, this result highlights the influence of traffic congestion, because the particular site has limited vegetation cover. Traffic congestion introduces particulate matter to the atmosphere. These particulates provide surfaces for the airborne bacteria to adhere resulting in the highest number of bacterial loads at that site. Further, the site is a highly populated area which includes elderly people and also children who could be immune-compromised. There is a teaching hospital near to railway station (collecting site at Railway Station), where there are around 224,917 inpatient admissions daily (https://www.nhk.health.gov.lk/, accessed date: 2020.05.06). Most of these people could be immune-compromised, who would be at risk from

Table 4 Potential pathogenic bacteria in urban traffic congested sites, remote areas covered with vegetation and common to both

Urban traffic congested areas	Remote areas covered with vegetation	Common to both
Bacillus megaterium	*Enterobacter ludwigii*	*Bacillus cereus*
Exiguobacterium acetylicum		*Bacillus pumilus*
Acinetobacter baumannii		*Stenotrophomonas maltophilia*
Escherichia hermanni		*Acinetobacter soli*
Leclercia adecarboxylata		*Pseudomonas monteilii*
Serratia marcescens		*Pseudomonas aeruginosa*
		Pseudomonas stutzeri
		Pseudomonas brenneri
		Providencia rettgeri
		Kocuria sp.
		K. pneumoniae
		Arthrobacter sanguinis
		Sphingomonas sp.
		Ochrobactrum intermedium
		Brevundimonas vesicularis

pulmonary diseases via inhalation of the polluted air comprising potential pathogens.

Eighty-eight percent of the culturable bacteria were Gram-positive. At all sites, Gram-positive bacteria were the most abundant. The unique adaptations of these Gram-positive bacteria increase their survival ability in dry, nutrient-deficient environments, including the atmosphere. Gram-positive bacteria have a thick cell wall (≈ 50 nm), which is a three-dimensional multilayered net-like structure. This cell wall provides them protection from mechanical and osmotic lysis, and proteins in it serve as an attachment to interact with the environment (Mai-Prochnow et al. 2016). Some Gram-positive bacteria (e.g., *B. subtilis*) produce spores that are heat and drought-resistant, dormant under unfavorable conditions and regenerate in favorable conditions (Kellogg and Griffin 2006). Therefore, they are able to colonize and multiply. They are capable of tolerating acids and organic solvents (Cotter and Colin 2003), which are constituents of the atmosphere. These adaptations have made Gram-positive bacteria to be abundant in the atmosphere. The peptidoglycan in the cell wall of some Gram-positive bacteria have inflammatory characteristics of endotoxins and induce respiratory symptoms (Oppliger et al. 2008). Pathogenic Gram-positive bacteria can cause diseases ranging from dental caries to fetal gastrointestinal infections (Cotter and Colin 2003; Woodford and Livermore 2009). Therefore, the high concentrations of Gram-positive bacteria could be a risk to human health.

A considerable amount of Gram-negative bacteria (12.1%) were also observed and certain Gram-negative bacteria produce endotoxins that can cause acute and chronic respiratory effects (Oppliger et al. 2008). Inhalation of these Gram-negative bacteria causes mucous membrane irritations, extrinsic allergic alveolitis, organic dust toxic syndrome, bronchitis, asthma, and many other infections (Naruka and Gaur 2014). Dead Gram-negative bacteria have the potential to influence lung function and also cause inflammatory reactions (Seedorf et al. 1998). In comparison to Gram positives, Gram-negative bacteria are less likely to survive in the atmosphere due to their fragile cell wall (Morris et al. 2011).

Certain airborne bacteria and fungi have been identified from most parts of the world as a potential health risk to humans, animals, and plants (Makino and Cheun 2003; Oppliger et al. 2008; Polymenakou 2012; Williams et al. 2001). Although the atmosphere is considered an inhospitable environment for microbial life due to low moisture and nutrient levels, and high levels of ultraviolet (UV) radiation (Lai et al. 2009), twenty-eight bacterial species belonging to four classes; namely, Gammaproteobacteria, Alphaproteobacteria, Actinobacteria, and Bacilli were identified by culture in this study. Most of the sequenced taxonomic groups belonged to the class Gammaproteobacteria. Similar results have also been reported in previous studies from other countries (Mescioglu et al. 2019; Bowers et al. 2009). The number of bacterial species that were identified from this study was more significant compared to the similar studies where culture-based techniques were used (Naruka and Gaur 2014).

Gram-positive cocci, Actinobacteria were also identified in the study. They are capable of tolerating extreme conditions, such as high UV radiation (Qin et al. 2016). *Kocuria* spp., one of the Actinobacteria identified in this study, can survive even under strong oxidants such as H_2O_2, which is commonly used as an antimicrobial agent that can destroy bacterial membranes. *Kocuria* genus is capable of multiplying under stress conditions. It has been found that they contain genes that

Table 5 The bacterial species that were observed at each sampling site

Identified bacterial species	Observed sites								
	I	R	P	F	C	T	L	D	TRI
Bacillus cereus	√	√	√	√	√	√	√	√	√
Bacillus amyloliquefaciens	√	√	√	√	√	√		√	√
Bacillus subtilis	√	√	√	√	√	√	√	√	
Bacillus pumilus	√	√	√	√	√	√	√	√	√
Bacillus aryabhattai					√				
Bacillus megaterium					√				
Bacillus thuringiensis		√	√	√	√				√
Exiguobacterium acetylicum				√	√				
Exiguobacterium indicum			√		√	√			
Stenotrophomonas maltophilia	√	√	√	√	√				
Acinetobacter soli		√	√	√	√		√	√	√
Acinetobacter baumannii	√	√	√	√	√	√	√		
Pseudomonas monteilii	√	√	√	√	√	√			
Pseudomonas aeruginosa	√	√	√	√	√	√	√	√	√
Pseudomonas taiwanensis	√		√	√	√				
Pseudomonas stutzeri	√	√	√	√	√	√	√	√	√
Pseudomonas brenneri		√			√		√		
Escherichia hermanni			√						
Klebsiella pneumoniae	√	√	√	√	√		√	√	√
Leclercia adecarboxylata		√	√		√				
Enterobacter ludwigii	√								
Providencia rettgeri	√	√	√	√	√				
Serratia marcescens			√	√	√				
Kocuria sp.	√	√	√	√	√				
Arthrobacter sanguinis	√	√	√	√	√				√
Sphingomonas sp.	√	√	√	√	√		√		√
Ochrobactrum intermedium	√	√		√	√				
Brevundimonas vesicularis	√	√	√	√	√	√	√	√	√

C – Children's Park, F – Fire Brigade, P – Police Station, R – Railway Station, I – National Institute of Fundamental Studies, L – Lewella, D – Dodanwala, T – Trinity College, TRI – Tea Research Institute

Table 6 Shannon's diversity index for each sampling site

Sample collection site	Shannon's diversity index (H)
I	1.64
R	1.53
P	2.21
F	2.02
C	1.66
T	1.19
L	0.68
D	1.48
TRI	0.31

Table 7 Shannon's diversity index for each sampling month

Month of sample collection	Shannon's diversity index
May, 2015	2.00
June, 2015	1.56
July, 2015	1.48
August, 2015	1.56
September, 2015	1.57
October, 2015	1.41
November, 2015	0.76
December, 2015	1.63
January, 2016	1.71
February, 2016	0.99
March, 2016	0.85
April, 2016	0.34

encode proteins that are responsible for DNA repair system, oxidative stress response, and biosynthetic pathways of sugars such as trehalose and mannosyglycerate, which are responsible for mitigating damage caused by UV radiation (Qin et al. 2016).

Most of the bacteria in the collected sample sets were pigmented (e.g., *B. subtilis, Sphingomonas* sp., *S. maltophilia, S. marcescens, Exiguobacterium* sp.). Pigmentation protects these bacteria from UV radiation to which they are regularly exposed in the atmosphere (Kellogg et al. 2004). Pigmentation also could be a result of the nutrient composition of the medium used (Shaffer and Lighthart 1997). Some bacterial species, such as *Bacillus megaterium*, produce pigmented spores, which are highly resistant to ultraviolet radiation and exhibit ability to remain dormant for a long period. These carotenoid pigmented spores have the natural anti-oxidant capacity, thus giving the spores the ability to survive under harsh conditions (Hong et al. 2009).

Twenty-one bacterial species from the identified culturable bacterial species are opportunistic pathogens. Some are respiratory pathogens of humans and animals (e.g., *P. aeruginosa, P. stutzeri, P. brenneri, K. pneumoniae, P. monteilii, S. marscence, A. baumannii, S. maltophilia*). They enter directly via the inhalation pathway of humans and causing disease. The other pathogens cause enteric infections (e.g., *P. rettgeri, E. ludwigii, P. monteilii*) and skin infections in humans (*K. pneumoniae, B. cereus, P. stutzeri, Kocuria kristinae*). Enteric type of airborne pathogens can be deposited on water or edible leaves and fruits and washing out by rainfall. They can enter via the ingestion pathway and cause diseases.

In contrast to past studies, this study showed that the diversity of indices in urban areas (site at Children's Park, site of Police Station, and site at Fire Brigade) was higher than that of

Table 8 Proportional abundance of identified culturable bacteria in each month from May, 2015 to April, 2016

Identified bacterial species	May, 2015	June, 2015	July, 2015	August, 2015	September, 2015	October, 2015	November, 2015	December, 2015	January, 2016	February, 2016	March, 2016	April, 2016
B. cereus	15.73	5.84	7.07	40.22	38.61	38.48	78.65	17.85	37.66	62.75	13.85	89.44
B. amyloliquefaciens	9.86	1.69	3.68	0.23	0.59	0.02	0.22	3.03	14.29	0.00	0.00	0.00
B. subtilis	0.11	0.00	0.35	3.80	6.74	1.45	0.00	2.22	1.30	0.00	1.54	0.00
B. pumilus	0.33	52.86	62.21	40.24	38.04	1.04	1.69	46.31	9.09	11.90	9.23	10.54
B. aryabhattai	0.00	0.00	0.00	0.32	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B. megaterium	0.00	0.26	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B. thuringiensis	11.41	0.00	0.06	0.00	0.22	0.00	0.03	0.00	0.00	0.00	0.00	0.00
Exiguobacterium spp.	1.00	3.25	2.32	0.47	5.17	48.16	0.10	1.11	19.48	0.00	0.00	0.00
S. maltophilia	7.53	0.00	1.90	0.26	0.43	0.10	0.00	0.00	0.00	0.00	0.00	0.00
A. soli	1.55	0.00	0.00	1.02	0.30	3.57	11.34	4.15	11.69	0.00	0.00	0.00
A. baumannii	22.70	12.86	2.36	2.20	0.04	0.00	0.00	0.61	3.90	0.00	0.00	0.00
P. monteilii	24.81	0.00	0.00	0.12	0.42	0.00	0.00	0.00	0.00	2.04	0.00	0.00
P. aeruginosa	0.00	0.39	0.06	0.84	0.06	0.00	0.00	0.00	0.00	22.78	0.00	0.00
P. taiwanensis	0.44	0.13	0.06	0.14	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P. stutzeri	0.22	0.00	0.03	0.15	0.10	0.00	0.00	0.00	0.00	0.00	73.85	0.00
P. brenneri	0.00	0.00	0.06	0.01	0.16	0.00	0.05	0.00	0.00	0.00	0.00	0.00
E. hermanni	0.11	1.43	0.00	3.14	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
K. pneumoniae	2.99	0.52	2.84	1.40	3.40	0.00	0.03	4.20	0.00	0.00	0.00	0.02
L. adecarboxylata	0.11	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
E. ludwigii	0.00	0.00	0.03	1.00	0.92	0.00	0.00	0.00	0.00	0.00	0.00	0.00
P. rettgeri	0.00	0.13	4.26	0.65	0.10	0.02	0.00	0.00	0.00	0.00	0.00	0.00
S. marcescens	0.11	0.00	0.00	1.53	0.00	5.42	0.00	0.00	0.00	0.00	0.00	0.00
Kocuria sp.	0.00	0.00	0.26	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A. sanguinis	0.44	12.99	2.36	0.54	0.34	0.00	0.63	0.00	0.00	0.53	0.00	0.00
Sphingomonas sp.	0.55	7.40	9.84	0.48	3.19	0.17	0.17	16.23	0.00	0.00	0.00	0.00
O. intermedium	0.00	0.13	0.23	0.14	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B. vesicularis	0.00	0.13	0.00	1.03	0.55	1.55	7.07	4.30	2.60	0.00	1.54	0.00

the remote areas (sites at the Institute of Fundamental Studies, Dodanwala, Tea Research Institute, and Lewella) (Després et al. 2007; Liu et al. 2018). Some bacterial species such as *B. aryabhattai* and *B. megaterium* were found only from site in Children's Park. Air sampler at the site of Children's Park was placed in a grass field close to high traffic and congested road with an intersection which gave rise to three main roads. There is considerable vegetation cover and a bus stop near the site. Besides, a pre-school is also situated close to the site and children and parents can be seen in the vicinity enjoying the park facilities. The Police Station and Fire Brigade were situated in the middle of the City, where high traffic congestion could be observed. Therefore, these natural and anthropogenic factors could be influencing the high diversity in the indices which were observed at the sites.

Another different factor from the other studies was that, although the diversity was low (H=0.31), the culturable bacterial concentration was the highest at the rural site Tea Research Institute, compared to the urban sites (Després et al. 2012; Fahlgren et al. 2010). The reason could be the survival and culturable ability of bacteria at high moisture levels at the site due to vegetation (Bragoszewska et al. 2017). Due to the lack of other anthropogenic factors, the bacterial types which inhabit vegetation are abundant at the site, although the diversity was low.

The identified bacteria are of soil, plants, or animal origin. The soil inhabitant bacteria, such as *Bacillus*, are capable of responding to stresses and develop counter-strategies such as efficient resistance mechanisms (Mai-Prochnow et al. 2016). Genus *Kocuria* has been found in Mali, West Africa, and the Virgin Islands, and are inhabitants of soil and human skin (Kellogg et al. 2004). The presence of bacteria such as *Kocuria* in the Kandy atmosphere as well shows that some of the bacterial assemblages discovered could be generally observed in the atmosphere regardless of the geographic location. Fierer et al. (2008) hypothesized that the reason for this is

that these common bacterial types share adaptations which are uncharacterized and these adaptations improve their ability for aerosolization and survival in harsh environments. The presence of bacteria such as *P. rettgeri, E. hermanni, L. adecarboxylata*, and *P. monteilii* which are associated with feces could be an indication of deposition of animal (e.g., dog) feces which could have been aerosolized to the atmosphere. *S. marscence* is a pigmented bacterium, colored in red which were found only from the Police Station, Fire Brigade, and Children's Park. It is suggested that pigmentation of bacteria is a mechanism to protect itself under unfavorable conditions when the growth of cells is delayed. Most importantly, *S. marscence* is responsible for respiratory diseases, pneumonia, and lung abscesses. They are capable of utilizing a wide range of nutrients, which is a mechanism for surviving in harsh environments such as the atmosphere (Hejazi and Falkiner 1997). *E. ludwigii* were collected from site National Institute of Fundamental Studies (site I). This is a plant-associated bacterium having the potential to degrade hydrocarbons (Yousaf et al. 2011). Therefore, the potential source of this bacterial type could be the vegetation in the vicinity of the site. Genus *Pseudomonas* is another crucial group of pathogenic bacteria identified and five species of this genus were recorded in the study. The cells of *Pseudomonas* and also their endotoxins are responsible for hypersensitive pneumonitis (Selman et al. 2012).

The bacterial diversity was dynamic over time. The number of bacterial species declined from September to October. October was the month where the highest average rainfall was observed (410 mm). During high rainfall, agar plates were damaged because of 6-h exposure, which was a limiting factor of the study. This could be a significant reason for the reduction in the number of species detected during this period relative to periods with light showers (September, August). However, during September, a high number of bacterial species could be seen. In this month, light showers occurred and as such, a higher number of bacterial species were able to survive. Precipitation moistens the environment and the survival and culturability of the bacteria are enhanced (Weerasundara et al. 2017). Bacterial aerosol in the soil and various surface upsurges due to the rain (Joung et al. 2017). Sample collection sites, namely, Trinity College, Lewella, Dodanwala, and Tea Research Institute, had a low bacterial diversity compared to the other sites. The differences in the diversity level depend on the location, as described by Liu et al. (2019). However, the number of culturable bacteria was high at these sites indicating that though the species variation is low, bacteria are present in large quantities. *B. cereus, B. amyloliquefaciens, B. pumilus, B. subtilis, B. thuringiensis, E. indicum, A. soli, A. baumannii, P. monteilii, P. aeruginosa, P. stutzeri, P. brenneri, K. pneumoniae, A. sanguinis, Sphingomonas* sp., and *B. vesicularis* were the species observed in these locations. All of these species are soil inhabitants or plant-associated microorganisms. Sites Lewella, Dodanwala, and Tea Research Institute are highly vegetated sites, which confirm that plants introduce large quantities of bacteria to the atmosphere (Lymperopoulou et al. 2016).

B. cereus and *B. pumilus* could be cultured from all the sites throughout the period of sampling. *Bacillus* genus is capable of forming endospores which give them an advantage in surviving under extreme environmental conditions (Hong et al. 2009). The reservoir of these *Bacillus* spore formers is mainly the soil (Be et al. 2015). Sri Lanka is a tropical country which receives solar radiation during most of the year. It has been hypothesized that under hot conditions, thermal convection and photophoresis affect the adhesive forces between soil and bacterial cells. Consequently, the soil-inhabiting bacteria are more attracted to mix in the air (Genitsaris et al. 2017). Tong and Lighthart (2000) also provide evidence that summer conditions improve the efflux of bacteria to the atmosphere due to dry soil conditions. Therefore, soil aerosolizes to the atmosphere contributing to bacterial constituents which are circulating within Kandy City.

Bubble bursting introduces the microorganisms in the water sources to the atmosphere (Morris et al. 2011). *P. brenneri* has been isolated previously from natural water sources (Leclerc and Moreau 2002). This species was recorded from the sites at Railway Station, Lewella, and Children's Park. The Children's Park is very close to the Kandy Lake and Lewella site is close to the Mahaweli River. Therefore, the sources of *P. brenneri* could be the lake and the river. However, Railway Station is in the middle of Kandy City and there are no close water sources except for the central storm water drainage canal. Although some studies have shown that traffic volume has a major influence on diversity as well as the quantity of culturable microorganisms (Naruka and Gaur 2014; Raj and Joshi 2016), in this study, vegetation was also found to have influenced the diversity and quantity, not only the traffic volume. The similarity in major bacterial types at different locations throughout the year suggests their pervasive transmission over a long distance and their circulation in the air within Kandy City due to the surrounding mountain ranges. The aerosols which are emitted to the atmosphere can be deposited in the area of origin or another area away from the source due to circulation (Weerasundara and Vithanage 2016). Therefore, the sources of airborne microorganisms could be local or distant.

In summary, the population living and traveling in the city of Kandy is being exposed to high bacterial loads consisting of opportunistic pathogens. Therefore, the elders and children who are immunocompromised could be at a risk of respiratory diseases. Further, disease vulnerability is increased by the geographical location of the city surrounded with mountains.

Conclusion

Traffic-congested Kandy City harbors a dynamic and diverse range of atmospheric bacteria. These bacteria are originated via vegetation (e.g., *Enterobacter ludwigii, Exiguobacterium acetylicum*), soil (*Bacillus cereus, Bacillus amyloliquefaciens, Bacillus subtilis, Pseudomonas monteilii*), aquatic ecosystems (*Exiguobacterium indicum, Pseudomonas brenneri*), and animals (*Escherichia hermanni, Leclercia adecarboxylata*). Among the identified 28 bacterial types, 22 species were recognized as opportunistic pathogens. High quantity of culturable bacteria and total bacteria enhances the health risk to immune-compromised people. Due to the mountains surrounding Kandy City, air circulation within the City reduces the mingling of fresh air. Therefore, thousands of people who commute to the City on a daily basis and the people who are residents are at risk of a range of illnesses. Results obtained indicate that vehicular congestion and the emissions contribute in large quantities to the total bacterial load.

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