Detection of *Legionella* Species and the Influence of Precipitation on the Amount of *Legionella* DNA Present in Aerosols from Outdoor Sites Near Asphalt Roads in Toyama Prefecture, Japan, with Microbiome Analysis

Jun-ichi Kanatani (junichi.kanatani@pref.toyama.lg.jp)
Toyama Institute of Health
Masanori Watahiki
Toyama Institute of Health
Keiko Kimata
Toyama Institute of Health
Tomoko Kato
Toyama Institute of Health
Kaoru Uchida
Toyama Institute of Health
Fumiaki Kura
National Institute of Infectious Diseases
Junko Amemura-Maekawa
National Institute of Infectious Diseases
Junko Isobe
Toyama Institute of Health

Research article

Keywords: Legionella species, Legionella pneumophila, legionellosis, aerosols, asphalt roads, microbiome

Posted Date: August 18th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-58152/v1

Version of Record: A version of this preprint was published at BMC Microbiology on July 17th, 2021. See the published version at https://doi.org/10.1186/s12866-021-02275-2.

Abstract

Background: Legionellosis can be caused by the inhalation of aerosolized water contaminated with *Legionella*. In this study, we investigated the prevalence of *Legionella* species in aerosols collected from outdoor sites near asphalt roads, bathrooms in public bath facilities, and other indoor sites such as buildings and private homes using culture methods, quantitative PCR with ethidium monoazide treatment (EMA-qPCR), and 16S rRNA gene amplicon sequencing.

Results: *Legionella* species were not detected in culture. However, *Legionella* DNA was detected in 114/151 (75.5%) air samples collected near roads (geometric mean ± standard deviation was 1.80 ± 0.52 $\log_{10}$ copies/m^3); these numbers were comparable to those obtained from bathrooms [15/21 (71.4%), 1.82 ± 0.50] and higher than those obtained from other indoor sites [11/30 (36.7%), 0.88 ± 0.56] ($P <$ 0.05). By EMA-qPCR, *Legionella* DNA was detected in 20/30 (66.7%) samples collected near roads, indicating the presence of membrane-intact *Legionella* cells in the air. The amount of *Legionella* DNA correlated with the monthly total precipitation ($r = 0.25$, $P < 0.01$). It was also directly and inversely correlated with the daily total precipitation for seven days ($r = 0.21$, $P = 0.01$) and one day ($r = -0.29$, $P <$ 0.01) before the sampling day, respectively. In addition, 16S rRNA gene amplicon sequencing revealed that the three most abundant bacterial genera in the samples collected near roads were *Sphingomonas* (21.1%), *Streptococcus* (14.6%), and *Methylobacterium* (1.6%); *Legionella* species were detected in 9/30 samples (30%) collected near roads (mean proportion of reads, 0.11%). At the species level, *L. pneumophila* was detected in 2/30 samples collected near roads (mean proportion of reads, 0.11% and 0.09% in the detected samples).

Conclusions: DNA from *Legionella* species, including *Legionella pneumophila*, were widely detected in aerosols collected from outdoor sites near asphalt roads, especially during the rainy season. Our findings suggest that there may be a risk of exposure to *Legionella* species in the areas surrounding asphalt roads.

Background

Legionella is the causative agent of Legionnaires' disease, a severe form of legionellosis and a potentially fatal pneumonia [1]. Although 61 species and over 70 serogroups of *Legionella* have been identified [2], more than 90% of legionellosis cases are caused by *Legionella pneumophila* [3, 4]. *Legionella* species are ubiquitously found in natural environments. They have also been found in artificial environments, such as cooling towers, baths, showers, and decorative fountains [5–8].

Legionellosis can be acquired through the inhalation of aerosolized water contaminated with *Legionella* [9]. Therefore, aquatic facilities are potential sources of sporadic cases and outbreaks of this disease. According to the National Epidemiological Surveillance of Infectious Diseases, public bath facilities are a major source of *Legionella* infections in Japan [10]. For the remaining cases, however, the sources of infection remain unknown. The results of our previous study suggest that puddles on asphalt roads can

serve as potential environmental reservoirs of *L. pneumophila* [11]. *Legionella* present in puddles on roads could be aerosolized by splashing or by wind. Recently, several studies have attempted to detect *Legionella* species in aerosols released from hot tap water in bathrooms, shower water, and compost [12–14]. These studies revealed that *Legionella* species were present in the aerosols derived from these environments. However, to date, the prevalence of *Legionella* species in aerosols from outdoor sites near asphalt roads has not been analyzed.

Typically, conventional plate culture methods have been used to detect *Legionella* in clinical and environmental samples. In some cases, amoebic co-culture has been used because of its high sensitivity [15]. In addition, some rapid detection methods, such as PCR/real-time quantitative PCR (qPCR), have been applied in the field [16, 17]. However, these methods lack the ability to differentiate between viable and dead cells. Ethidium monoazide (EMA) is a dye that allows the differentiation of viable and dead cells [18]. EMA can penetrate compromised cell walls and membranes, form covalent links with DNA, and cleave it into small fragments after photoactivation. Thus, viable *Legionella* cells can be selectively quantified through the combined use of photoactivated EMA and qPCR (EMA-qPCR) [19]. Furthermore, metagenomic analysis is a powerful tool for analyzing microbial communities and 16S rRNA gene amplicon sequencing has been widely used to detect bacterial pathogens in environmental samples [20].

The main objective of this study was to determine whether *Legionella* species present in aerosols derived from outdoor sites near asphalt roads could be a source of *Legionella* infection. We investigated the prevalence of *Legionella* species in aerosols from outdoor sites near asphalt roads, bathrooms next to bathtubs in public bath facilities, and other indoor sites such as buildings and private homes using culture methods, EMA-qPCR, and 16S rRNA gene amplicon sequencing.

Results

Prevalence of Legionella species in air samples

Legionella species were not isolated by direct culture or amoebic co-culture methods from any of the 202 air samples collected from roads or bathrooms and other indoor sites (Table 1). Although 49 *Legionella*-suspected colonies grew on glycine-vancomycin-polymyxin B-cycloheximide (GVPC) agar plates, they also grew on blood agar plates, indicating that they do not belong to the genus *Legionella*. There was low background growth on the GVPC agar plates.

Table 1
Prevalence of Legionella species in air samples

		No. (%) of positive samples by:			Geometric mean ± SD (log_{10} copies/m^3) in *Legionella* DNA-positive samplesd
Sampling site	No. of samples	Culture	Amoebic co-culture	qPCRc	
Road	151	0	0a	114 (75.5) A	1.80 ± 0.52 C
Bathroom	21	0	0	15 (71.4) A	1.82 ± 0.50 CD
Indoor site	30	0	NTb	11 (36.7) B	0.88 ± 0.56 D

a22 out of 151 samples were not tested.

bNot tested.

cValues with different letters are significantly different ($P < 0.05$). Data were analyzed by Fisher's exact test followed by *post hoc* Holm test.

dValues with different letters are significantly different ($P < 0.05$). Data were analyzed by the one-way ANOVA test followed by Tukey–Kramer multiple comparisons.

However, *Legionella* DNA was detected in 114/151 (75.5%) air samples collected near roads at all 12 sampling sites (locations A–L). *Legionella* DNA was also detected in 15/21 (71.4%) air samples collected from 14/17 bath facilities and 11/30 (36.7%) samples collected from 4/4 other indoor sites. The rates of positivity for the samples collected near roads and from bathrooms were significantly higher than those for samples collected from indoor sites other than bathrooms ($P < 0.05$; Fisher's exact test followed by *post hoc* Holm test). The geometric means ± standard deviation (SD) (log_{10} copies/m^3) of *Legionella*-specific 16S rRNA genes in the *Legionella* DNA-positive samples were 1.80 ± 0.52, 1.82 ± 0.50, and 0.88 ± 0.56 for roads, bathrooms, and other indoor sites, respectively. The values for these three sampling source types were determined to be significantly different by one-way analysis of variance (one-way ANOVA) test ($P < 0.05$). Moreover, the Tukey-Kramer method revealed that the amounts of *Legionella* DNA were significantly different among the samples from roads and indoor sites other than bathrooms ($P < 0.05$).

Of the 202 air samples, 150 (129 from roads and 21 from bathrooms) were tested by amoebic co-culture. For one sample collected from a bathroom (*Legionella*-specific 16S rRNA genes, 1.45 log_{10} copies/m^3), the amount of *Legionella* DNA was found to be increased by 1.1×10^5-fold after amoebic co-culture. BLAST search showed that the macrophage infectivity potentiator (*mip*) gene sequence in the sample had 92% identity with the *mip* gene sequence of *L. nautarum*.

[Insert Table 1 here]

EMA-qPCR for the detection of viable Legionella cells in air samples

To detect DNA from viable but non-culturable (VBNC) *Legionella* cells, we collected another set of 30 air samples near roads and carried out EMA-qPCR. Considering the low airborne concentration of viable *Legionella* cells, the sampling time was extended to 30 min as it increased the volume of air sampled and thus, improved the limit of detection. Furthermore, Chang et al. reported that collection with deionized water yielded greater recovery of viable *L. pneumophila* than with tween mixture (containing 0.01% Tween 80, 1% peptone, and 0.005% Antifoam Y-30) by EMA-qPCR [19]. Thus, for EMA-qPCR, sterile deionized water was used as the sampling solution instead of 0.005% Tween-80 solution. Among the 30 air samples collected near asphalt roads, *Legionella* DNA was detected in 20 samples (66.7%) by EMA-qPCR and in 26 samples (86.7%) by qPCR (no EMA treatment, as a control). The geometric means ± SD (log_{10} copies/m^3) of *Legionella*-specific 16S rRNA genes in the *Legionella* DNA-positive samples were 0.97 ± 0.88 by EMA-qPCR and 1.21 ± 0.70 by qPCR.

Geographic And Meteorological Characterizations

The numbers of air samples collected near roads at the 12 sampling sites were as follows: A, N = 15; B, N = 12; C, N = 12; D, N = 11; E, N = 10; F, N = 13; G, N = 11; H, N = 11; I, N = 17; J, N = 13; K, N = 11; and L, N = 15. At these sampling sites, the detection rates of *Legionella* DNA ranged from 60.0–93.3%. The geometric means ± SD (log_{10} copies/m^3) of *Legionella*-specific 16S rRNA genes in the *Legionella* DNA-positive samples ranged from 1.54 ± 0.66 to 2.03 ± 0.42. The differences in the detection rates according to sampling sites and the amount of *Legionella*-specific 16S rRNA genes according to sampling sites were not significant by Fisher's exact test followed by *post hoc* Holm test and the one-way ANOVA test, respectively.

We assessed the correlation between the climatic conditions (air temperature, relative humidity, total precipitation, and wind speed) and the amount of *Legionella*-specific 16S rRNA genes (log_{10} copies/m^3) in the *Legionella* DNA-positive samples (Table 2). The amount of *Legionella* DNA correlated with the monthly total precipitation ($r = 0.25$, $P < 0.01$). It was also directly and inversely correlated with the daily total precipitation for seven days ($r = 0.21$, $P = 0.01$) and one day ($r = -0.29$, $P < 0.01$) before the sampling day, respectively. The scatter plots of total precipitation and the amount of *Legionella* DNA are shown in Fig. 1. The geometric mean ± SD of the *Legionella* DNA-positive samples at a monthly total precipitation of > 200 mm (2.00 ± 0.40 log_{10} copies/m^3) was higher than that at a monthly total precipitation of ≤ 200 mm (1.73 ± 0.54 log_{10} copies/m^3; $P < 0.05$, Student's *t-test*). However, the detection rates of *Legionella* DNA at monthly total precipitation of > 200 mm (87.9%, 29/33 samples) and ≤ 200 mm (72.0%, 85/118 samples) were not significantly different ($P \geq 0.05$, Fisher's exact test). The detection rate and the geometric mean of *Legionella* DNA seven days before the sampling day at a daily total precipitation of > 10 mm (100%, 16/16 samples; 2.07 ± 0.32 log_{10} copies/m^3) were also higher than those at a daily total

precipitation of ≤ 10 mm (72.6%, 98/135 samples; 1.75 ± 0.53 $\log_{10}$ copies/m^3) ($P < 0.05$; Fisher's exact test and Student's *t* test, respectively). However, the geometric mean of *Legionella* DNA one day before the sampling day at a daily total precipitation of ≤ 10 mm (1.83 ± 0.49 $\log_{10}$ copies/m^3) was higher than that at a daily total precipitation of > 10 mm (1.55 ± 0.64 $\log_{10}$ copies/m^3; $P < 0.05$, Student's *t-test*); the detection rates of *Legionella* DNA one day before the sampling day at daily total precipitation of ≤ 10 mm (75.0%, 99/132 samples) and > 10 mm (78.9%, 15/19 samples) were not significantly different ($P \geq 0.05$, Fisher's exact test).

Table 2
Correlation between the climatic conditions and the amount of Legionella DNAa

	Air temperature (mean, °C)		Relative humidity (mean, %)		Total precipitation (mm)		Wind speed (mean, m/s)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Daily value:								
on the sampling day	−0.10	0.22	0.13	0.11	0.17	0.04[c]	−0.10	0.22
one day before the sampling day	0.01	0.90	−0.19	0.02[c]	−0.29[b]	< 0.01[c]	0.16	0.049[c]
two days before the sampling day	−0.07	0.39	−0.15	0.06	0.12	0.16	0.08	0.34
three days before the sampling day	−0.10	0.24	−0.01	0.90	−0.11	0.17	−0.15	0.06
four days before the sampling day	−0.06	0.48	0.03	0.74	0.02	0.85	−0.19	0.02[c]
five days before the sampling day	−0.09	0.29	−0.05	0.53	−0.06	0.45	−0.08	0.35
six days before the sampling day	−0.11	0.17	0.04	0.65	−0.09	0.28	0.07	0.39
seven days before the sampling day	−0.13	0.12	0.14	0.09	0.21[b]	0.01[c]	−0.07	0.37
Monthly value	−0.10	0.24	−0.02	0.78	0.25[b]	< 0.01[c]	0.01	0.88

[a]Geometric mean (log_{10} copies/m^3) of *Legionella*-specific 16S rRNA genes in the *Legionella* DNA-positive samples.

[b]An absolute value of Pearson's $r \geq 0.20$ was considered to be correlated.

[c]$P < 0.05$ was considered significant.

[Insert Table 2 here]

16s Rrna Gene Amplicon Sequencing

16S rRNA gene amplicon sequencing was performed on randomly selected samples collected from roads (N = 30), and all samples collected from bathrooms (N = 21) and other indoor sites (N = 30). The median

number of reads after quality filtering, denoising, merging, and removing chimeric sequences was 62,246 (range, 9,852–246,625) from roads, 112,471 (range, 16,534–341,587) from bathrooms and 111,835 (range, 16,009–250,862) from other indoor sites. A total of 8,174,054 reads (100,914 reads per sample) were assigned to 18,426 amplicon sequence variants (ASVs).

At the genus level, 485, 421, and 368 bacterial genera were detected in samples collected from roads and from bathrooms and other indoor sites, respectively (Fig. 2). The top three most abundant bacterial genera in the samples collected near roads were *Sphingomonas* (21.1%), *Streptococcus* (14.6%), and *Methylobacterium* (1.6%); those in the samples collected in bathrooms were *Sphingomonas* (17.6%), *Pseudomonas* (5.4%), and *Methylococcus* (4.3%); and those in the samples collected from indoor sites other than bathrooms were *Sphingomonas* (19.2%), *Achromobacter* (5.0%), and *Arthrobacter* (3.8%). Reads from *Legionella* species were detected in 9/30 samples (30%) collected near roads (mean proportion of reads, 0.11%), 5/21 samples (24%) in bathrooms (mean, 0.04%), and 1/30 samples (3%) from indoor sites other than bathrooms (mean, 0.03%). The rate of positivity for the samples collected near roads was significantly higher than the positivity rate for samples collected from indoor sites other than bathrooms ($P < 0.05$; Fisher's exact test followed by *post hoc* Holm test).

At the species level, *L. pneumophila* was detected in 2/30 samples collected near roads (mean proportion of reads, 0.11% and 0.09% in the detected samples) and in 1/21 samples collected in bathrooms (mean, 0.15% in the detected sample). Furthermore, *L. birminghamensis* and *L. geestiana* were detected in 1/30 samples collected from roads (mean, 0.81% and 0.02% in the detected samples, respectively), and *L. nautarum* was detected in 1/30 samples collected from indoor sites other than bathrooms (mean, 0.79% in the detected sample).

Alpha diversity indices in air samples are shown in Fig. 3. Shannon diversity indexes (a quantitative measure of bacterial community richness) and Pielou's evenness (a measure of community evenness) were not significantly different between the three sampling source types ($P \geq 0.05$, Kruskal-Wallis test). However, Faith's phylogenetic diversity indices, a qualitative measure of bacterial community richness that incorporates phylogenetic relationships between the features associated with samples from road exhibited a much higher value than those from other indoor sites (FDR-adjusted $P < 0.05$; Kruskal-Wallis test, Benjamini-Hochberg correction). The principal coordinates plot showed that a portion of the air samples collected from roads and indoor sites other than bathrooms plotted separately from those from bathrooms (Fig. 4). Linear discriminant analysis (LDA) effect size (LEfSe) analysis revealed 15 genera with an LDA score of at least 3.0, which were significantly more abundant in the three sampling source types (Fig. 5; $P < 0.05$, pairwise Wilcoxon test). Specifically, we found the following four genera to be more abundant in the samples (LDA score > 4.0): *Pseudomonas*, *Vibrio*, and *Staphylococcus* from bathrooms (red color), and *Achromobacter* from other indoor sites (green color).

Discussion

We demonstrate that DNA from *Legionella* species is widely present in aerosols derived from outdoor sites near asphalt roads, especially during the rainy season, regardless of the sampling site. Aerosols collected near roads contain viable *Legionella* cells, as shown by EMA-qPCR. In addition, 16S rRNA gene amplicon sequencing revealed that DNA from several *Legionella* species, including *L. pneumophila*, which is a major causative agent of Legionnaires' disease, were detected in aerosols collected near roads.

Legionella DNA was detected in more than 70% of the air samples collected near roads. The positivity rate for samples collected near roads was almost the same as that for samples collected in bathrooms in public bath facilities, which are a major source of legionellosis in Japan, and the rate was significantly higher than that for samples collected from other indoor sites ($P < 0.05$). Montagna et al. reported that *Legionella* DNA was detected in 72.7% (8/11) of air samples from bathrooms in healthcare facilities by qPCR using a Coriolis μ air sampler [12]. In this study, although the sampling height was not at 150–180 cm above ground level, which is the average height of an adult, owing to the stability of the air sampler, our results showed the existence of aerosols, including *Legionella* species near asphalt roads. Further investigation is required concerning the relationship between the sampling height and the prevalence of *Legionella* species in aerosols to reveal the risk of inhalation of aerosols, including *Legionella* species.

However, no *Legionella* species were isolated from any of the collected air samples (roads, bathrooms, and other indoor sites) by culture or amoebic co-culture methods. This may be due to the relationship previously reported, which indicated that a high concentration of *Legionella* species (> 300,000 CFU/l) was required to be present in a water sample near an air sampling site to isolate colonies from aerosols by culture [14]. In this study, the highest concentration of *Legionella* species in bath water near air sampling sites was not high (8,100 CFU/l in 16/21 tested samples; Kanatani, unpublished observations). In puddle water, the concentration of *Legionella* species was not high; the highest concentration of *Legionella* species in puddle water was 75,200 CFU/l based on the results of our previous study [11]. The reason *Legionella* species could not be isolated may be related to the stress encountered during aerosolization and the air sampling process, which may have led to a loss of culturability [21]. Montagna et al. reported that viable *Legionella* cells could not be isolated by the culture method from air samples collected using a Coriolis μ air sampler [<link rid="bib12">12</link>]. Sampling solution may be another factor that influenced the viability of the *Legionella* cells collected. Chang et al. reported that the samples collected with deionized water yielded greater recovery of viable *L. pneumophila* by EMA-qPCR than those collected with the tween mixture [19]. However, bacteria cells may become damaged in deionized waster due to differences in osmotic pressure; this influences the survival activity of *Legionella* cells as stated in the manufacturer's instruction of Coriolis μ air sampler. Additional improvements in culture methods, such as strains of amoeba used for co-culture and incubation periods with the amoeba, may also allow the isolation of *Legionella* from aerosols. Therefore, new sampling and/or culture methods to isolate *Legionella* species from aerosols need to be established.

It is also necessary to consider the possibility of VBNC *Legionella* cells. Although various stress factors may induce *Legionella* cells to enter a VBNC state, these cells can still directly infect human macrophages

and amoebae, indicating that VBNC *Legionella* cells are able to cause disease in humans [22, 23]. Several studies have shown that VBNC *Legionella* in water samples regain culturability in amoebic co-culture [24, 25]. Our study also showed that amoebic co-culture increased the amount of *Legionella* DNA in one air sample collected from a bathroom, but not in any sample collected near roads. To eliminate free DNA released from dead or membrane-impaired cells [21], EMA-qPCR was performed on air samples collected near roads, and *Legionella* DNA was detected in about 70% of the samples. This indicates that aerosols from roads contain viable *Legionella* cells. Although *Legionella* is sensitive to environmental stresses such as desiccation and UV irradiation, amoeba cysts, which are resistant amoebal forms that can survive under stress conditions [26], may play a role in airborne survival and transmission. Further investigations are needed to obtain a detailed understanding of the state of *Legionella* in aerosols.

It seems plausible that precipitation is positively associated with the occurrence of legionellosis [27]. Our study also showed a weak but positive correlation between the monthly total precipitation and the amount of *Legionella* DNA present in aerosols collected near roads. According to previous studies, the number of legionellosis cases peaked in July, the second half of the rainy season, in Japan [28, 29]. Thus, there is a risk of contracting legionellosis near asphalt roads, especially during the rainy season. The amount of *Legionella* DNA was also directly and inversely correlated with the daily total precipitation of seven days and one day before sampling, respectively. Our results suggest that *Legionella* may have multiplied within amoeba present in the environment after precipitation at the seven-day-before sampling time point, whereas the dry condition during the day before the sampling time point may have favored the release of aerosols of small particle sizes from the ground into the atmosphere because of splashing or the effect of wind. Alternatively, the survival rate of *Legionella* in aerosols may be different due to climatic conditions.

16S rRNA gene amplicon sequencing revealed that *Sphingomonas* was the most frequently detected genus in the air samples. This genus has been found in various environments such as soil, water, clinical specimens, air, and other locations [30–32], indicating the opportunity for the bacteria to be released into the air. *Pseudomonas* and *Achromobacter* were significantly more abundant in air samples from bathrooms and other indoor sites, respectively. They have frequently been detected in bathwater and moist indoor environments [33, 34]. Thus, our results were congruent with those reported in other studies. In this study, reads from *Legionella* species were detected in 30% of the air samples collected near roads, which was equivalent to those in bathrooms and significantly higher than those from other indoor sites. The detection rate of *Legionella* reads by 16S rRNA gene amplicon sequencing was lower than that by qPCR (75.5%). This may be due to PCR amplification bias of the 16S rRNA gene. However, the detection rate and the geometric mean of *Legionella* DNA by qPCR in the samples in which *Legionella* reads were detected by 16S rRNA gene amplicon sequencing (93.3%, 14/15 samples; 2.04 ± 0.56 $\log_{10}$ copies/m^3) were significantly higher than those in the samples in which *Legionella* reads were not detected by 16S rRNA gene amplicon sequencing (57.6%, 38/66 samples; 1.49 ± 0.56 $\log_{10}$ copies/m^3) ($P < 0.05$; Fisher's exact test and Student's *t* test, respectively). Thus, the qPCR results of *Legionella* DNA were correlated with those obtained by 16S rRNA gene amplicon sequencing. Furthermore, *L. pneumophila*, which is a

major causative agent of Legionnaires' disease, was also detected in aerosols collected near roads and in bathrooms. These results suggest that legionellosis may be acquired by inhaling aerosols from areas near asphalt roads and in bathrooms. Thus, the possibility of contracting legionellosis in daily life should be considered. Further studies are needed to evaluate the risk of legionellosis and to develop prevention strategies.

Conclusions

Here, we demonstrated that DNA from *Legionella* species, including *L. pneumophila*, were widely present in aerosols collected from outdoor sites near asphalt roads, especially during the rainy season, regardless of the sampling site. The detection rate and amount of *Legionella* DNA in the aerosols collected near roads were similar to those in aerosols collected from bathrooms and were significantly higher than those collected from other indoor sites. Our findings suggest that there may be a risk of exposure to *Legionella* species in the areas surrounding asphalt roads, especially during the rainy season.

Methods

Sample collection

We investigated outdoor sites near asphalt roads, bathrooms next to bathtubs in public bath facilities, and other indoor sites during the period of 2016–2018 in Toyama Prefecture, Japan. A total of 202 air samples were collected. Among these, 151 samples were collected from 12 outdoor sites (locations A–L) near asphalt roads, on any given days such as sunny, cloudy, and rainy, 1–4 times per month from June 2016 to April 2017; samples from locations A, I, and L were collected in all months except in November 2016 and March 2017. Samples from locations F, H, and K were collected in all months except in September and November 2016 and March 2017. Samples from locations B–E, G, and J were collected in all months except in September and November 2016 and February 2017. The average traffic volume per minute during the sampling period was approximately 10–50 vehicles (cars and trucks), indicating that aerosolization of water would occur especially on rainy days. Twenty-one samples were collected from bathrooms next to bathtubs in 17 public bath facilities from June to October 2016 (16 were collected while operating aerosol-generating devices in the bathtub, such as jet circulation with or without air induction bubbles). The remaining 30 samples were collected in rooms and corridors from two buildings and two private houses from March 2017 to February 2018 (indoor sites other than bathrooms) where aerosolization of water could not occur. For each sampling, a portable cyclone-based air sampler (Coriolis μ; Bertin Technologies, France) was placed approximately 70 cm from the ground. The air samples were collected in 15 mL of a solution containing 0.005% Tween 80 at a flow rate of 300 l/min for 10 min. Under these conditions, spiked *L. pneumophila* was recovered by approximately 60% recovered by culture (Kanatani, unpublished observations). The volume remaining after each sampling was 6–14 mL, and each sample solution was vortexed for 1 min.

Isolation of Legionella **spp.**

We attempted to detect *Legionella* species through culture and amoebic co-culture methods. For the culture investigations, 0.1 mL of the sample was spread onto a GVPC agar plate (Nissui Pharmaceutical Co., Tokyo, Japan). The agar plate was incubated at 35 °C for seven days in a humidified chamber. Any candidate colonies that were smooth gray with characteristic outward structures that were cut-glass-like or mosaic-like in appearance were viewed under a stereomicroscope with oblique illumination [35] and were sub-cultured on a buffered charcoal-yeast extract (BCYE) agar plate with L-cysteine (bioMérieux, Lyon, France) and a blood agar plate (Eiken Chemical, Tokyo, Japan). Colonies growing only on the BCYE agar plate and not the blood agar were presumed to belong to the genus *Legionella*.

Amoebic co-culture was carried out as described previously [36]. *Acanthamoeba* species isolated from cooling tower water were incubated in Proteose peptone-Yeast extract-Glucose-Cysteine medium at 30 °C for 5–7 days. Cells were washed and resuspended in phosphate-buffered saline, and then 0.5 ml of the suspension (approximately 1.0×10^5 cells) was added to the remaining amount of the sampling solution (4–12 mL) after culture (0.1 mL) and qPCR (2 mL). To prevent evaporation, the sample was incubated at 35 °C in 50 ml screw-cap tubes. After four weeks, the sample was mixed with equal volumes of 0.2 mol/L KCl-HCl buffer (pH 2.2) for 15 min at room temperature, and 0.2 mL was spread onto a GVPC agar plate. Then, the candidate *Legionella* colonies were analyzed as described above.

Qpcr

For qPCR, DNA was extracted from 2 mL of sample solution. The suspension was centrifuged at 20,000 × *g* at room temperature for 5 min and then resuspended in 100 µl of 5% (w/v) Chelex-100 solution (37; Bio-Rad Laboratories, CA, USA). The suspension was boiled at 100 °C for 10 min and then centrifuged at 20,000 × *g* at room temperature for 5 min. The supernatant was used as the DNA template, and qPCR was carried out using the CycleavePCR *Legionella* (16S rRNA) Detection Kit (Takara Bio, Shiga, Japan) and a Thermal Cycler Dice Real Time System II (Takara Bio) according to the manufacturer's instructions.

Ema-qpcr

To detect DNA derived from viable *Legionella* cells, air samples were subjected to EMA-qPCR. For this investigation, another set of 30 samples was collected from eight outdoor sites near asphalt roads. These sampling sites were different from those described in the sample collection subsection (locations A–L), although the traffic volume at the sites was approximately the same as locations A–L. Air samples were collected in 15 mL of sterile deionized water at a flow rate of 300 l/min for 30 min, and distilled water was added every 10 min to bring the sampling solution volume up to 15 mL. Sample solution (2 mL) was centrifuged at 20,000 × g at room temperature for 5 min and then resuspended in 40 µL of sterile deionized water. After treatment with EMA using the Viable *Legionella* Selection Kit for PCR Ver. 2.0 (Takara Bio) and LED CrossLinker 12 (Takara Bio), the DNA was extracted with Lysis Buffer for *Legionella* (Takara Bio) [38]. All protocols were carried out according to the manufacturer's instructions. qPCR was carried out as described above in the qPCR subsection.

Sequencing of the *mip* gene

For samples in which the amounts of *Legionella* DNA increased after amoebic co-culture, the species of *Legionella* was determined. DNA was extracted from a 2 ml sample of the amoebic co-culture with Chelex-100 solution as described above. The *mip* gene was directly amplified and sequenced as described previously [39].

Next-generation Sequencing

DNA samples extracted with Chelex-100 solution were used for next-generation sequencing. For each air sample from roads, bathrooms, and other indoor sites, the bacterial 16S V3–4 region was amplified using Tks Gflex DNA Polymerase (Takara Bio) with the primers 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'). A 16S rRNA gene amplicon library was prepared according to the 16S Metagenomic Sequencing Library Preparation protocol for Illumina MiSeq (Illumina, San Diego, CA, USA) and then sequenced using an Illumina MiSeq instrument with the MiSeq Reagent Kit v3 (600 cycles).

Microbiome bioinformatics was performed using QIIME 2 version 2019.7 [40]. Imported demultiplexed sequence data were denoised with DADA2 [41] (via q2-dada2). All ASVs were aligned with mafft [42] (via q2-alignment) and used to construct a phylogeny with fasttree2 [43] (via q2-phylogeny). Taxonomy from kingdom to species was assigned to ASVs using the q2-feature-classifier [44] based on the classify-sklearn naïve Bayes taxonomy classifier against the Greengenes 13_8 99% OTU reference sequences [45]. Alpha-diversity metrics (Shannon diversity [46], Pielou's evenness [47], and Faith's phylogenetic diversity [48]) and beta-diversity metrics (weighted UniFrac [49]) were estimated using q2-diversity after samples were rarefied (subsampled without replacement) to 9,852 sequences per sample. Alpha-diversity indexes were compared in QIIME2 using the Kruskal-Wallis test with the Benjamini-Hochberg false discovery rate (FDR) correction. The FDR cutoff was set at 0.05. Different taxa with significance between sampling source type were assessed using the LEfSe program with parameters (alpha value for the pairwise Wilcoxon test was set at 0.05, the logarithmic LDA score threshold was set at 3.0) through the Galaxy Hutlab online platform [50, https://huttenhower.sph.harvard.edu/galaxy/].

Meteorological Data Collection

During the study period (2016–2018), meteorological data were obtained from two main weather stations in Fushiki and Toyama, Toyama Prefecture, Japan [51]. The meteorological data used in this study were monthly or daily values of air temperature (mean, °C), relative humidity (mean, %), total precipitation (mm), and wind speed (mean, m/s).

Statistical analysis

To investigate the relationships between the rates of *Legionella* DNA detection and the sampling source type and between the detection rates and sampling site (locations A–L), Fisher's exact test followed by *post hoc* Holm test were performed. We also investigated the relationships between the amount of *Legionella* DNA and the sampling source type, and between the amount of *Legionella* DNA and sampling site (locations A–L) by using the one-way ANOVA test, followed by Tukey–Kramer multiple comparisons. The Student's *t-test* was performed to compare the amounts of *Legionella* DNA between samples associated with high and low total precipitation. Fisher's exact test was performed to compare the rates of *Legionella* DNA detection between samples associated with high and low total precipitation. These tests were performed using the R statistical software package (version 3.0.0). Pearson's correlation coefficients (*r*) between the climatic conditions (air temperature, relative humidity, total precipitation, and wind speed) and the amount of *Legionella* DNA were investigated using Microsoft Excel (Microsoft, Tokyo, Japan). An absolute value of Pearson's $r \geq 0.20$ was considered indicative of a correlation, and a *P* value < 0.05 was considered significant.

Abbreviations

qPCR
quantitative PCR; EMA:ethidium monoazide; GVPC:glycine-vancomycin-polymyxin B-cycloheximide; SD:standard deviation; one-way ANOVA:one-way analysis of variance; *mip*:macrophage infectivity potentiator; VBNC:viable, but non-culturable; ASV:amplicon sequence variants; LDA:linear discriminant analysis; LEfSe:linear discriminant analysis effect size; BCYE:buffered charcoal-yeast extract; FDR:false discovery rate.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the current study are available from the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) under the accession number LC472487 for the *mip* gene, and DRA008310 and DRA009422 for the 16S rRNA gene amplicons.

Competing interests

The authors declare no conflicts of interest regarding this study.

Funding

This work was supported by Health and Labor Sciences research grants (grant numbers H28-Shinko-Ippan-006 and 19LA0601 to J.A.-M).

Authors' contributions

JK carried out the experiments, performed the bioinformatics and statistical analysis, and was a major contributor in writing the manuscript. MW, KK, TK, and KU participated in the analysis. FK, JA-M, and JI participated in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Editage for the English language review. A part of the results of this study was presented as an oral communication at the 6th meeting of the ESGLI Study Group for *Legionella* Infections Conference, Athens, Greece, 10 to 12 September 2019.

References

1. Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, et al. Legionnaires' disease: Description of an epidemic of pneumonia. N Engl J Med. 1977;297:1189–97.
2. 10.1099/ijsem.0.002786
   Parte AC. LPSN—List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. Int J Syst Evol Microbiol. 2018;68:1825–9. doi: 10.1099/ijsem.0.002786.
   https://www.bacterio.net/genus/legionella. Accessed 20 July 2020.
3. Yu VL, Plouffe JF, Pastoris MC, Stout JE, Schousboe M, Widmer A, et al. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: An international collaborative survey. J Infect Dis. 2002;186:127–8.
4. Amemura-Maekawa J, Kura F, Chida K, Ohya H, Kanatani J, Isobe J, et al. *Legionella pneumophila* and other *Legionella* species isolated from legionellosis patients in Japan between 2008 and 2016. Appl Environ Microbiol. 2018;84:e00721-18. doi:10.1128/AEM.00721-18.
5. Cordes LG, Wiesenthal AM, Gorman GW, Phair JP, Sommers HM, Brown A, et al. Isolation of *Legionella pneumophila* from hospital shower heads. Ann Intern Med. 1981;94:195–7.
6. Hlady WG, Mullen RC, Mintz CS, Shelton BG, Hopkins RS, Daikos GL. Outbreak of Legionnaire's disease linked to a decorative fountain by molecular epidemiology. Am J Epidemiol. 1993;138:555–62.
7. Ito I, Naito J, Kadowaki S, Mishima M, Ishida T, Hongo T, et al. Hot spring bath and *Legionella pneumonia*: An association confirmed by genomic identification. Intern Med. 2002;41:859–63.
8. Keller DW, Hajjeh R, DeMaria A, Fields BS, Pruckler JM, Benson RS, et al. Community outbreak of Legionnaires' disease: An investigation confirming the potential for cooling towers to transmit *Legionella* species. Clin Infect Dis. 1996;22:257–61.

9. Arnow PM, Chou T, Weil D, Shapiro EN, Kretzschmar C. Nosocomial Legionnaires' disease caused by aerosolized tap water from respiratory devices. J Infect Dis. 1982;146:460–7.
10. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare
National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare. Legionellosis, April 1999-July 2000. Infect Agents Surveill Rep. 2000;21:186–7. http://idsc.nih.go.jp/iasr/21/247/tpc247.html. Accessed 20 July 2020.
11. Kanatani J, Isobe J, Kimata K, Shima T, Shimizu M, Kura F, et al. Close genetic relationship between *Legionella pneumophila* serogroup 1 isolates from sputum specimens and puddles on roads, as determined by sequence-based typing. Appl Environ Microbiol. 2013;79:3959–66.
12. Montagna MT, De Giglio O, Cristina ML, Napoli C, Pacifico C, Agodi A, et al. Evaluation of *Legionella* air contamination in healthcare facilities by different sampling methods: An Italian multicenter study. Int J Environ Res Public Health. 2017;14(7). pii: E670. doi:10.3390/ijerph14070670.
13. Conza L, Pagani SC, Gaia V. Presence of *Legionella* and free-living amoebae in composts and bioaerosols from composting facilities. PloS One. 2013;8(7):e68244. doi:10.1371/journal.pone.0068244.
14. Wiik R, Krøvel AV. Necessity and effect of combating *Legionella pneumophila* in municipal shower systems. PloS One. 2014;9(12):e114331. doi:10.1371/journal.pone.0114331.
15. Conza L, Casati S, Gaia V. Detection limits of *Legionella pneumophila* in environmental samples after co-culture with *Acanthamoeba polyphaga*. BMC Microbiol. 2013;13:49. doi:10.1186/1471-2180-13-49.
16. Medema G, Wullings B, Roeleveld P, van der Kooij D. Risk assessment of *Legionella* and enteric pathogens in sewage treatment works. Water Sci Technol: Water Supply. 2004;4(2):125–32.
17. Wang H, Edwards M, Falkinham JO 3rd, Pruden A. Molecular survey of the occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa*, and amoeba hosts in two chloraminated drinking water distribution systems. Appl Environ Microbiol. 2012;78:6285–94.
18. Pan Y, Breidt F. Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl Environ Microbiol. 2007;73:8028–31.
19. Chang CW, Chou FC. Assessment of bioaerosol sampling techniques for viable *Legionella pneumophila* by ethidium monoazide quantitative PCR. Aerosol Sci Technol. 2011;45:343–51.
20. Borthong J, Omori R, Sugimoto C, Suthienkul O, Nakao R, Ito K. Comparison of database search methods for the detection of *Legionella pneumophila* in water samples using metagenomic analysis. Front Microbiol. 2018;9:1272. doi:10.3389/fmicb.2018.01272.
21. Zhen H, Han T, Fennell DE, Mainelis G. Release of free DNA by membrane-impaired bacterial aerosols due to aerosolization and air sampling. Appl Environ Microbiol. 2013;79:7780–9.
22. Ducret A, Chabalier M, Dukan S. Characterization and resuscitation of 'non-culturable' cells of *Legionella pneumophila*. BMC Microbiol. 2014;14:3. doi:10.1186/1471-2180-14-3.

23. Dietersdorfer E, Kirschner A, Schrammel B, Ohradanova-Repic A, Stockinger H, Sommer R, et al. Starved viable but non-culturable (VBNC) *Legionella* strains can infect and replicate in amoebae and human macrophages. Water Res. 2018;141:428–38.
24. García MT, Jones S, Pelaz C, Millar RD, Abu Kwaik Y. *Acanthamoeba polyphaga* resuscitates viable non-culturable *Legionella pneumophila* after disinfection. Environ Microbiol. 2007;9:1267–77.
25. Steinert M, Emödy L, Amann R, Hacker J. Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl Environ Microbiol. 1997;63:2047–53.
26. Greub G, Raoult D. Microorganisms resistant to free-living amoebae. Clin Microbiol Rev. 2004;17:413–33.
27. Sakamoto R. Legionnaire's disease, weather, and climate. Bull World Health Organ. 2015;93:435–6.
28. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare
National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare. Legionellosis, January 2003-September 2008, Japan. Infect Agents Surveill Rep. 2008;29:327–8. http://idsc.nih.go.jp/iasr/29/346/tpc346.html. Accessed 20 July 2020.
29. Ozeki Y, Yamada F, Saito A, Kishimoto T, Tanno S, Nakamura Y. Seasonal patterns of legionellosis in Saitama, 2005–2009. Jpn J Infect Dis. 2012;65:330–3.
30. Lee JS, Shin YK, Yoon JH, Takeuchi M, Pyun YR, Park YH. *Sphingomonas aquatilis* sp. nov., *Sphingomonas koreensis* sp. nov., and *Sphingomonas taejonensis* sp. nov., yellow-pigmented bacteria isolated from natural mineral water. Int J Syst Evol Microbiol. 2001;51:1491–8.
31. Kim SJ, Moon JY, Lim JM, Ahn JH, Weon HY, Ahn TY, et al. *Sphingomonas aerophila* sp. nov. and *Sphingomonas naasensis* sp. nov., isolated from air and soil, respectively. Int J Syst Evol Microbiol. 2014;64:926–32.
32. Zhu L, Si M, Li C, Xin K, Chen C, Shi X, et al. *Sphingomonas gei* sp. nov., isolated from roots of *Geum aleppicum*. Int J Syst Evol Microbiol. 2015;65:1160–6.
33. Barben J, Hafen G, Schmid J, Swiss Paediatric Respiratory Research Group. *Pseudomonas aeruginosa* in public swimming pools and bathroom water of patients with cystic fibrosis. J Cyst Fibros. 2005;4:227–31.
34. Nakamoto S, Sakamoto M, Sugimura K, Honmura Y, Yamamoto Y, Goda N, et al. Environmental distribution and drug susceptibility of *Achromobacter xylosoxidans* isolated from outdoor and indoor environments. Yonago Acta Med. 2017;60:67–70.
35. Morimoto Y. Usefulness of selection of *Legionella* by colony appearance. Jpn J Environ Infect. 2010;25:8–14. (In Japanese.).
36. Furuhata K, Okabe Y, Dogasaki C, Hara M, Fukuyama M. Isolation of *Legionella* spp. from soil in Japan. Bokin Bobai. 2002;30:555–61. (In Japanese.).

37. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 1991;10:506–13.
38. Inoue H, Takama T, Yoshizaki M, Agata K. Detection of *Legionella* species in environmental water by the quantitative PCR method in combination with ethidium monoazide treatment. Biocontrol Sci. 2015;20:71–4.
39. Ratcliff RM. Sequence-based identification of *Legionella*. Methods Mol Biol. 2013;954:57–72.
40. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–7.
41. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.
42. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30:3059–66.
43. Price MN, Dehal PS, Arkin AP. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLoS One. 2010;5:e9490. doi:10.1371/journal.pone.0009490.
44. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 2018;6:90. doi:10.1186/s40168-018-0470-z.
45. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 2012;6:610–8. doi:10.1038/ismej.2011.139.
46. Shannon CE. A mathematical theory of communication. Bell Syst Tech J. 1948;27:379–423.
47. Pielou E. The measurement of diversity in different types of biological collections. J Theor Biol. 1966;13:131–44.
48. Faith DP. Conservation evaluation and phylogenetic diversity. Biol Cons. 1992;61:1–10.
49. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol. 2007;73:1576–85.
50. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60. doi:10.1186/gb-2011-12-6-r60.
51. Japan Meteorological Agency. Past weather data search. 2019. http://www.data.jma.go.jp/obd/stats/etrn/index.php. Accessed 20 July 2020. (In Japanese.).

Figures

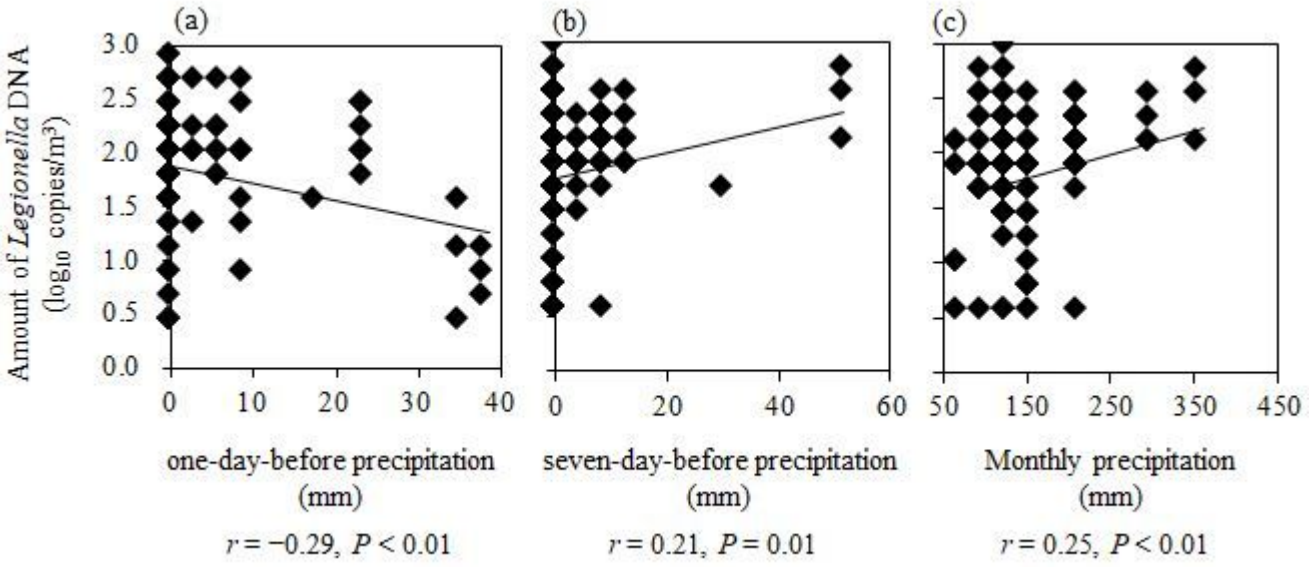

Figure 1

The scatter plots of total precipitation and the amount of Legionella DNA Total precipitation (mm) at each sampling timepoint and the amount of Legionella-specific 16S rRNA gene (log10 copies/m3) in the Legionella DNA-positive air samples collected near roads in Toyama Prefecture, Japan. (a) Daily precipitation one day before sampling. (b) Daily precipitation seven days before sampling. (c) Monthly precipitation

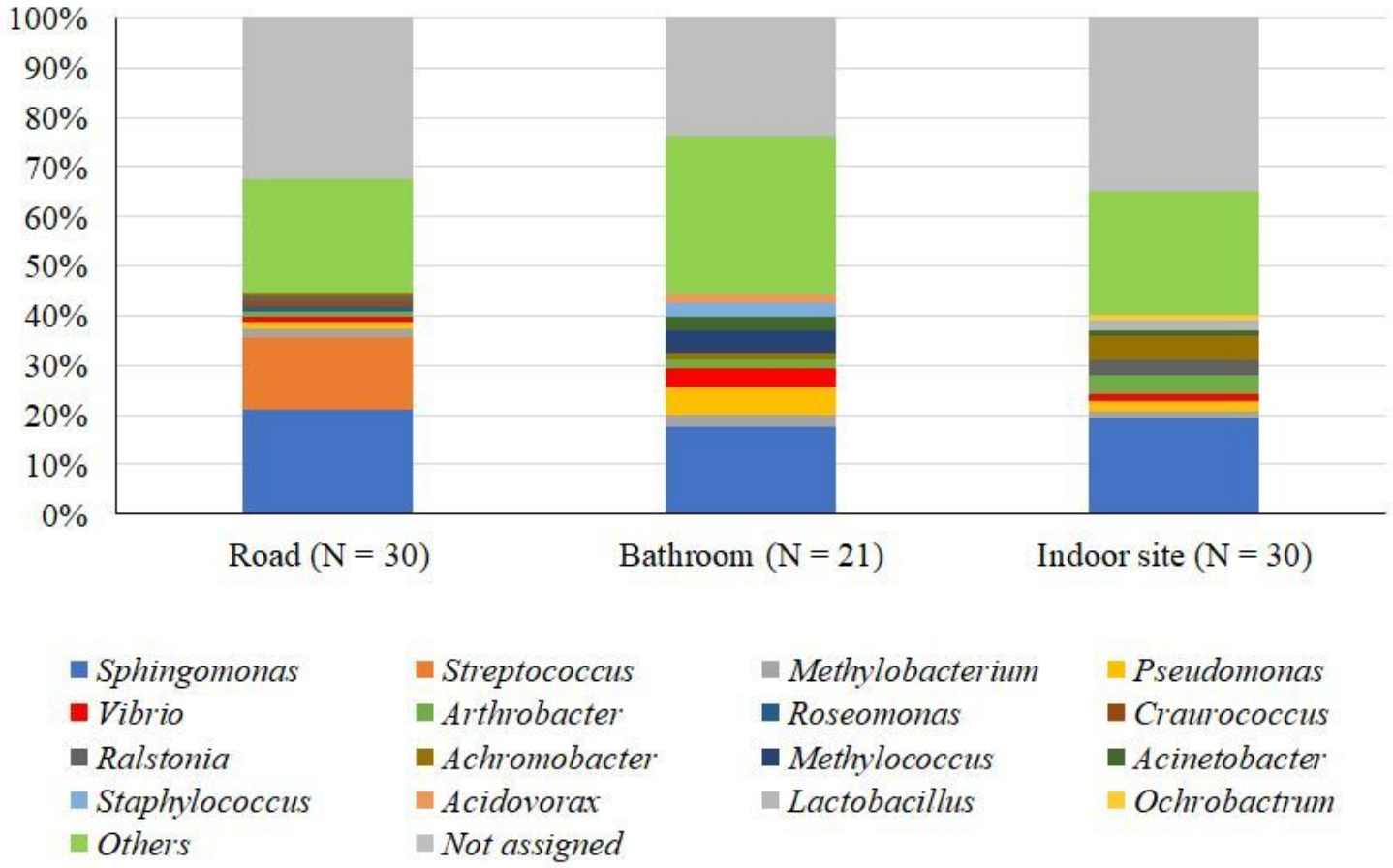

Figure 2

Taxonomic composition Taxonomic composition of bacterial reads at the genus level in air samples.

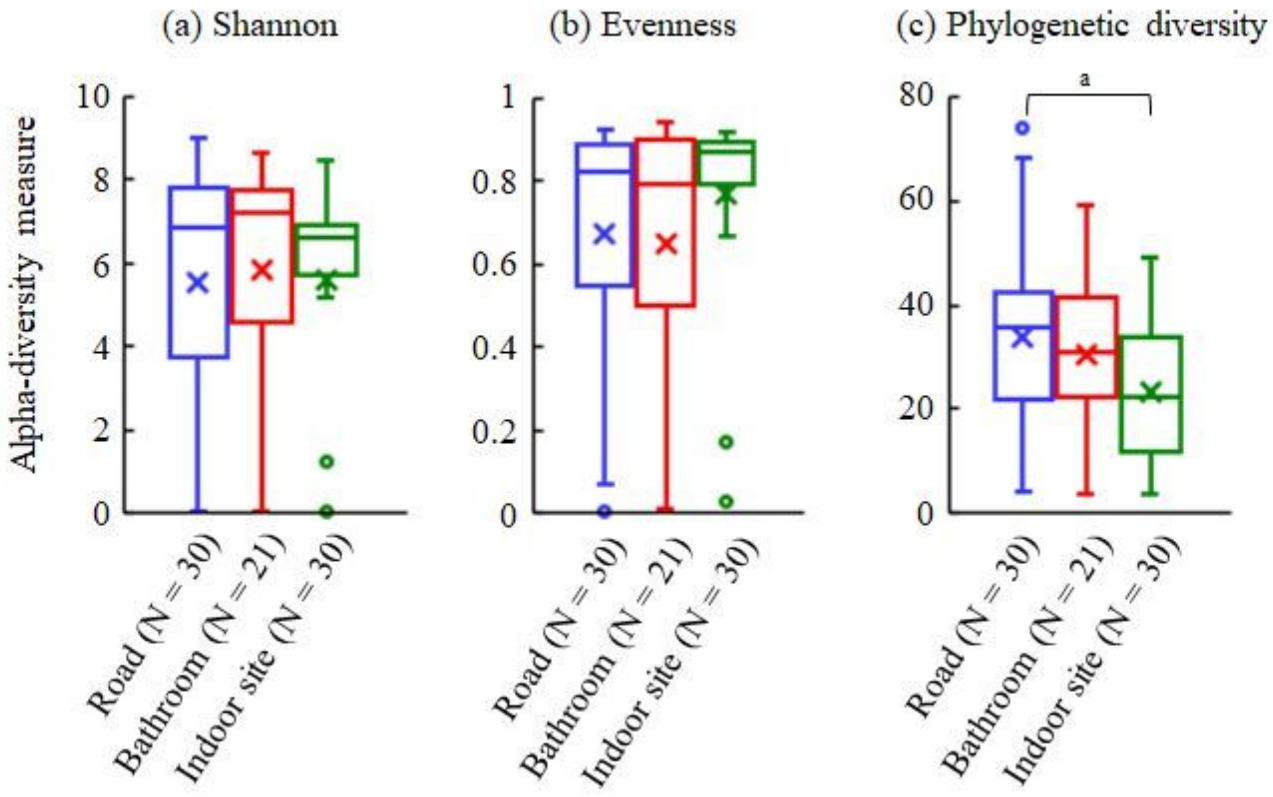

Figure 3

Alpha-diversity Comparison of alpha-diversity indices in air samples (a) Shannon diversity (b) Pielou's evenness (c) Faith's phylogenetic diversity aFDR-adjusted P < 0.05 (Kruskal-Wallis test, Benjamini-Hochberg correction).

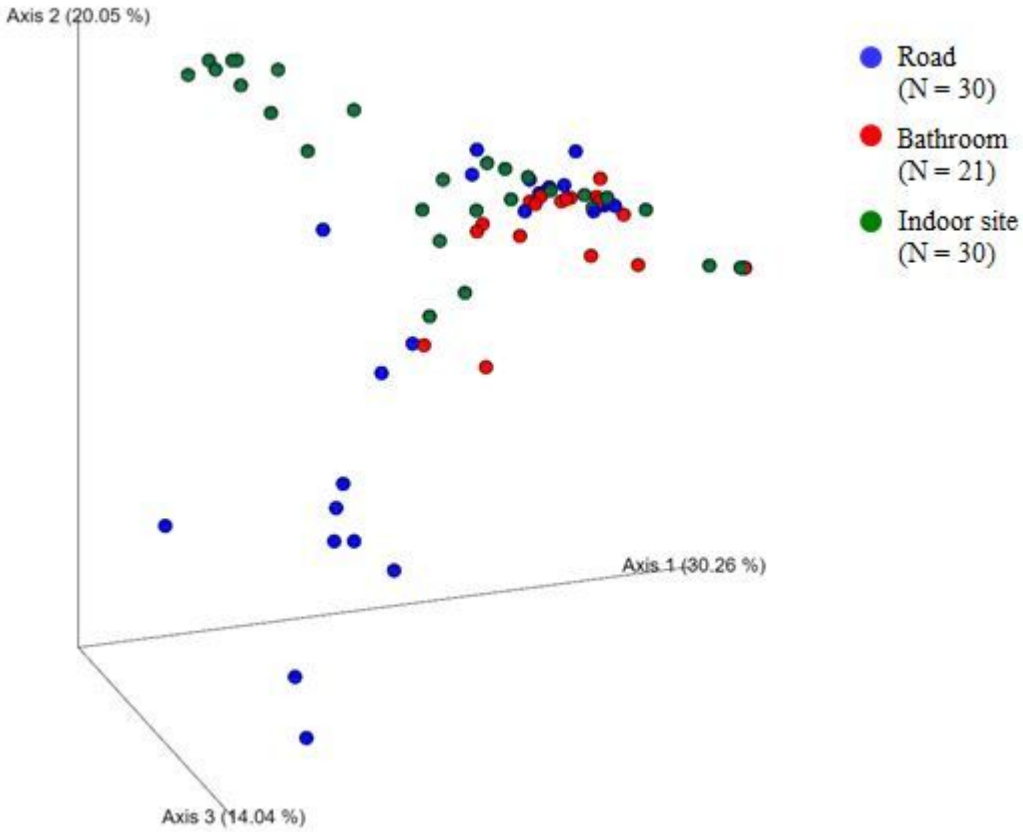

Figure 4

Principal coordinates plot Principal coordinates plot of beta-diversity based on weighted UniFrac distances shows overall variation in the bacterial community. A total of 81 air samples from roads (N = 30), bathrooms (N = 21), and other indoor sites (N = 30) were plotted with three dimensions.

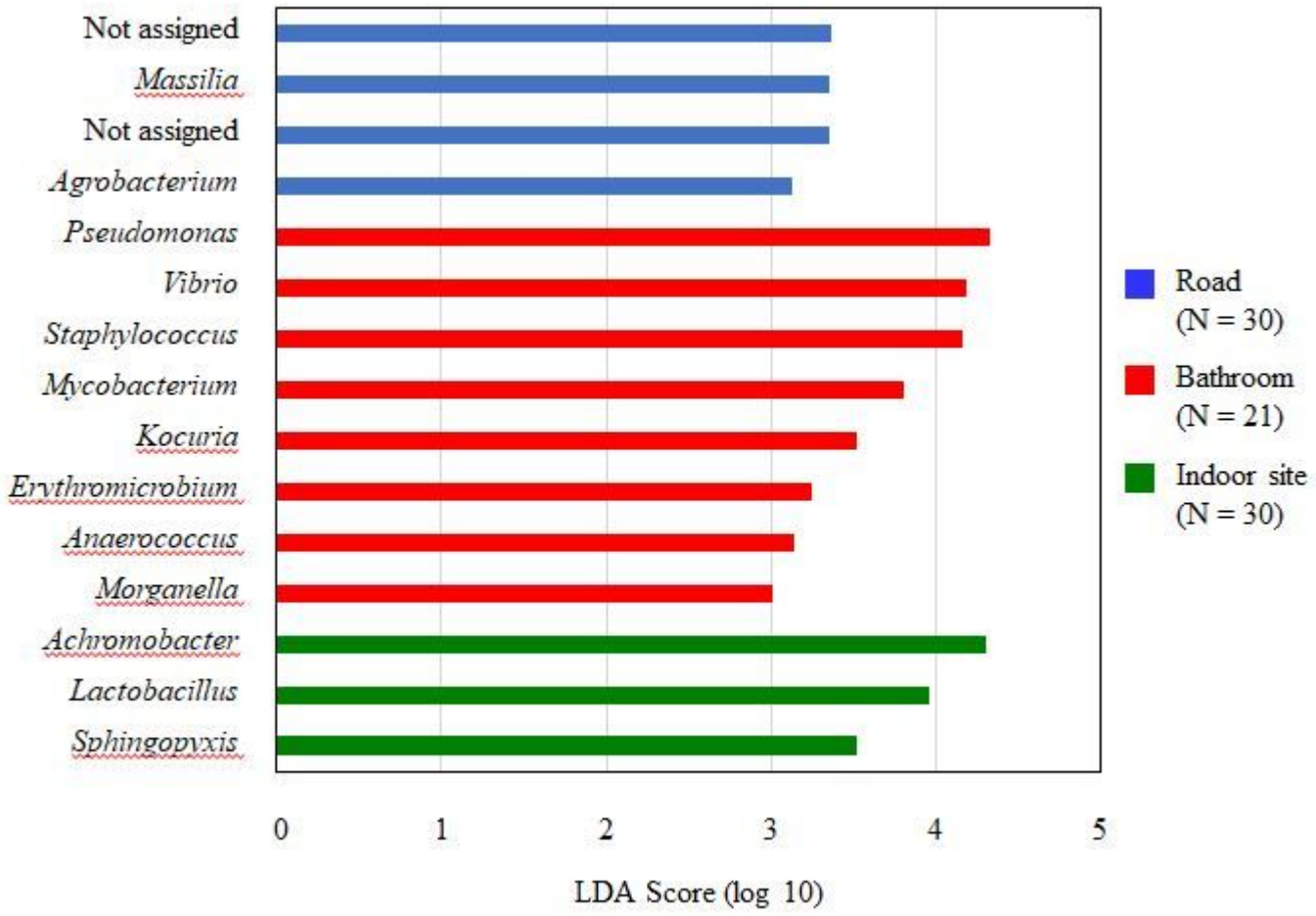

Figure 5

LEfSe analysis The bacterial genera with significant differences in relative abundance among sampling source type were identified by LDA effect size (LDA > 3.0; P < 0.05, pairwise Wilcoxon test). Blue bar: more abundant in samples from roads, red bar: more abundant in samples from bathrooms, green bar: more abundant in samples from other indoor sites.