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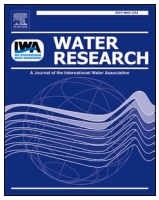

Particle-associated and free-living *Legionella* spp. in drinking water

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ABSTRACT

The *Legionella* genus houses several opportunistic pathogens that cause legionellosis, which ranges from mild (Pontiac Fever) to severe (Legionnaires' disease). *Legionella pneumophila* (*Lp*), the leading observed cause of legionellosis, persists in drinking water distribution systems (DWDSs) and building plumbing systems due to factors such as intrinsic disinfectant resistance and shielding by biofilms and free-living amoebae. Remediation efforts, therefore, might be hindered if *Lp* is primarily associated with biofilms, free-living amoebae, or other particles. However, the extent to which *Lp* and other *Legionella* spp. in drinking water occur as free-living organisms versus associated with free-living amoebae or other particles is unclear. In this study, we quantified free-living and particle-associated *Lp* and *Legionella* spp. in first-draw and flushed drinking water samples. Citizen scientists collected 1-L cold tap samples (first-draw and flushed) at 19 sites across the U.S. and across three seasons. Free-living and particle-associated microorganisms were size fractionated via membrane filtration, and *Legionella* spp. and *Lp* were quantified by digital polymerase chain reaction. Further, amplicon sequencing targeting the *Legionella* genus was used to explore the diversity of *Legionella* spp. at each site. The concentration of free-living *Legionella* spp. was significantly higher than that of particle-associated *Legionella* spp. ($p=2.86\times10^{-9}$) in paired samples at each site, and significantly higher concentrations of total *Legionella* spp. were found in surface water than in groundwater systems ($p=0.020$). *Lp* detection was geographically and temporally sporadic, with detections occurring at seven sites. Sequencing results showed that the site strongly influenced the specific *Legionella* spp. present. These results emphasize the influence of source water type and site on *Legionella* spp. in DWDSs and building plumbing. Further, this study suggests that consumers are more frequently exposed to free-living than to particle-associated *Legionella* spp. at the tap.

1. Introduction

Certain locations in drinking water distribution systems (DWDSs) and building plumbing systems provide favorable conditions for microbial persistence and growth, including warm temperatures, high surface-to-volume ratios, and disinfectant residuals that might be insufficient for microbial inactivation (Lin et al., 1998). Some *Legionella* spp. are opportunistic human pathogens and cause legionellosis, which includes Legionnaires' disease and Pontiac Fever; cases of legionellosis have risen in the U.S. from approximately 1,000 in the year 2000 to a peak of 10,000 in 2018 (U.S. CDC 2022). Humans become infected with *Legionella* spp. primarily through inhalation of contaminated droplets and aerosols from drinking water fixtures (e.g., showers, sinks), cooling towers, and misters (Holsinger et al., 2022; Medicine, 2020). Of the *Legionella* spp. that cause human infections, approximately 90 % of cases are attributed to *Legionella pneumophila* (*Lp*) (U.S. CDC 2022). However,

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eighteen other species of *Legionella* have been documented as causing human infection (Muder and Yu, 2002).

Many studies investigating *Legionella* spp. and *Lp* concentrations in DWDSs have reported that *Legionella* spp. are frequently detected when using DNA-based methods but that recovery of culturable *Lp* is less common (Aw et al., 2022; Dowdell et al., 2023; Hozalski et al., 2020; Ley et al., 2020; Liu et al., 2019a; Omoregie et al., 2022; Wang et al., 2012). Detection of *Legionella* spp. in drinking water has been reported to vary seasonally, with increased concentrations in summer months when water temperatures are typically higher (Huo et al., 2021; Liu et al., 2019a). Further, *Legionella* spp. concentrations can increase in building plumbing systems after periods of low water use, such as during building shutdowns (Dowdell et al., 2023; Hozalski et al., 2020) or in green water-efficient buildings (Ley et al., 2020; Rhoads et al., 2016).

Free-living amoebae (FLA), single-celled eukaryotes that are ubiquitous in soil and aquatic ecosystems, also are present in drinking water treatment trains and DWDSs (Delafont et al., 2013, 2018; Liu et al., 2019a; Stockman et al., 2011). FLA graze on smaller microorganisms (e.g., fungal cells, protozoa, and bacteria) and organic particles through phagocytosis (Khan, 2006). However, many bacterial opportunistic pathogens, including *Lp*, have evolved to resist uptake or digestion by phagocytes (McDougald and Longford, 2020). Instead, *Lp* can infect FLA, utilizing them as a replication niche (Barbaree et al., 1986). Inside FLA, *Lp* are shielded from unfavorable environments, including disinfectants and heat (Cervero-Aragó et al., 2015; Dupuy et al., 2011; Dupuy et al., 2014). Furthermore, *Lp* that pass through FLA become "protozoan primed," exhibiting increased infectivity and combined chlorine resistance as compared to *Lp* grown in typical laboratory media (Cambronne et al., 2023; Cirillo et al., 1994).

Previous studies have quantified *Legionella* spp. and *Lp* in drinking water (Angert et al., 2023; Aw et al., 2022; Hozalski et al., 2020; Liu et al., 2019a; Wang et al., 2012). However, key knowledge gaps remain, which are enumerated as follows. First, very few studies have surveyed *Legionella* spp. and *Lp* in geographically diverse drinking water systems (Donohue et al., 2019a, 2023; Dowdell et al., 2023). Second, the impact of combined factors among disinfectant type, source water type, and drinking water sample type (first-draw and flushed), when studied simultaneously, is not well understood. Physicochemical characteristics in drinking water have been shown to be building-specific and strongly influenced by source water quality (Gora et al., 2020), stressing the importance of investigating the fundamental relationships between sample characteristics/physicochemical parameters and *Lp* in both the free-living and particle-associated forms. Previous research suggests that when the effects of these characteristics/parameters are studied individually, *Lp* are detected more frequently in first-draw than in flushed samples (Angert et al., 2023; Cristina et al., 2014; Grimard-Conea et al., 2022; Ra et al., 2023), in DWDSs supplied by surface water than by groundwater (Boer et al., 2008; Kim et al., 2023; Zacheus and Martikainen, 2011), and in systems with free chlorine as the secondary disinfectant than with combined chlorine (Donohue, 2021; Donohue et al., 2019b; LeChevallier, 2019; Lytle et al., 2021). Third, particle-associated *Legionella* spp. and free-living *Legionella* spp. usually are not distinguished from each other in the drinking water literature. Understanding the proportion of *Legionella* spp. associated with particles in drinking water is crucial for remediation efforts and treatment decisions due to the increased disinfectant resistance conferred by the association of *Legionella* spp. with biofilms and particles, as well as increased infectivity and disinfectant resistance when *Legionella* spp. infect FLA.

In this study, we address the three aforementioned knowledge gaps. The occurrence of particle-associated and free-living *Legionella* spp. and *Lp* across 19 geographically diverse DWDSs in the U.S. was assessed; further, the effects of drinking water characteristics, including source water type, disinfectant type and residual, temperature, pH, and stagnation, were investigated. To the authors' knowledge, this is the first study to quantify particle-associated and free-living *Legionella* spp. and *Lp* and to report trends in their occurrence by type of sample (first-draw or flushed), source water, and secondary disinfectant.

2. Materials and methods

2.1. Preliminary testing for culturable Lp

Preliminary testing was performed during Summer 2021 to collect data on culturable *Lp* concentrations at a subset of DWDSs used for the subsequent full study (Section 2.2). Each sampling site is named according to its source water (S=surface, G=groundwater, B=blend) followed by its secondary disinfectant (C=combined chlorine, F=free chlorine). For preliminary testing, citizen scientists collected cold-water tap samples from drinking water fixtures at 12 sites across the U.S.; one sampling site was utilized per DWDS, except that a single DWDS housed Sites SC_2 and SC_3 (Table S1). DWDSs were selected based on geographic diversity, source water type (groundwater, surface water, or a blend of surface water and groundwater), and secondary disinfectant type (free or combined chlorine). A training video was shared with participants to demonstrate the sampling methods. Sampling kits were shipped to the participants; the kits contained sterile 50-mL polyethylene centrifuge tubes for water collection and 5-in-1 water quality test strips (Hach, Loveland, CO) to measure free and total chlorine, total hardness, total alkalinity, and pH. Residual disinfectant was quenched using 0.705 μmol of sodium thiosulfate added to each 50-mL sampling tube. Samples were taken at the tap of kitchen and bathroom sinks, showers, and drinking water fountains (Table S1).

At each site, a 100-mL first-flush sample was collected from the cold-water tap after overnight stagnation to represent water from the building plumbing. The tap was then flushed for at least 15 min, and another 100-mL sample was collected to represent water from the DWDS. The samples were shipped overnight at ambient temperature to the laboratory. Samples were tested upon receipt for *Lp* using the Legiolert® kit (IDEXX Laboratories, Inc., Westbrook, ME) according to the manufacturer's 100-mL potable water protocol, which has a lower limit of detection (LLOD) of 1 most probable number (MPN) per 100 mL and upper limit of detection (ULOD) of 2272.6 MPN/100 mL.

2.2. Full-study sampling locations

At the completion of preliminary testing, samples for the full study were collected in 2022–2023 from the 12 previously sampled sites. The sampled sites were the same between the preliminary and full studies, with the exception that GC_1, SF_1, and SC_4 were collected from different buildings within the same DWDSs as the preliminary testing. Seven additional locations were selected for sampling in the full study, for a total of 19 geographically diverse sites across the U.S. (Table 1). Samples were collected from cold-water taps in homes and offices. Each sampling site is named according to its source water (S=surface, G=groundwater, B=blend) followed by its secondary disinfectant (C=combined chlorine, F=free chlorine). Sections 2.2–2.9 describe the full study.

2.3. Sample collection and on-site analysis

Similar to preliminary testing, sampling for the full study was performed by citizen scientists, who were provided sampling kits, an instructional video, and a written step-by-step protocol to ensure proper sample collection (Text S1). The sampling kits consisted of insulated coolers containing two sterile 1-L polypropylene bottles (containing 0.19 μmol sodium thiosulfate to quench the disinfectant residual) for sample collection, a thermometer, an ice pack, and two 5-in-1 water quality test strips (Hach). Sampling events occurred three times over the course of six months (August-September 2022, October-November 2022, and January 2023) and across three seasons (summer, fall, and winter), for a total of 110 samples. Four samples were either not

Table 1
Sampling site characteristics.

Site	Source Water	Secondary Chlorine Disinfectant	State	Tap Type	Building Type
SC_1	Surface	Combined	Indiana	Sink	House
SC_2^			Virginia	Sink	House
SC_3^			Virginia	Sink	House
SC_4*			Texas	Sink	House
SC_5			Texas	Sink	Apartment
GC_1*	Ground	Combined	California	Sink	House
GC_2			Florida	Sink	House
BC_1	Blend	Combined	California	Tub	Apartment
BC_2			Colorado	Shower	House
BC_3			Florida	Sink	Apartment
SF_1*	Surface	Free	Illinois	Sink	Condominium
SF_2			New Jersey	Sink	House
SF_3†			New York	Sink	Apartment
SF_4†			New York	Sink	Apartment
SF_5			New York	Sink	House
GF_1	Ground	Free	California	Sink	House
GF_2			Illinois	Shower	House
GF_3			Montana	Sink	House
GF_4			Texas	Sink	House

^ A single DWDS housed both Sites SC_2 and SC_3.
† A single DWDS housed both Sites SF_3 and SF_4.
* The sampling site for the full study was in a different building located in the same DWDS as the preliminary study.

collected or lost during sample processing. Two samples were collected at each site: a 1-L first-draw sample, collected after overnight stagnation to represent water from the building plumbing, and a 1-L flushed sample, collected after the tap was flushed to represent water from the DWDS. First-draw samples were collected by briefly turning on the cold-water tap at a low flow rate, during which time the samplers wetted the water quality test strip. The 1-L first-draw sample was then collected at the same low flow rate. Next, the flow rate was increased, and the taps were flushed for at least 15 min or until the water temperature stabilized; at this time, the 1-L flushed sample was collected, and the second water quality test strip was wetted. Citizen scientists shipped the samples overnight with the frozen ice pack to the laboratory for analysis and provided photos of the water quality test strips. For certain sampling events, one 1-L blank sample (sterile Milli-Q water) was analyzed as a negative control (n=6).

2.4. Physicochemical analyses

Physicochemical parameters, including free and total chlorine, total hardness, total alkalinity, and pH were measured on-site by citizen scientists for each sample using the water quality test strips. The strips contained either five or six bins for the results, listed as follows: total and free chlorine: 0, 0.5, 1.0, 2.0, 4.0, and 10 mg/L as Cl_2; total hardness: 0, 25, 50, 120, 250, and 425 mg/L as $CaCO_3$; total alkalinity: 0, 40, 80, 120, 180, and 240 mg/L as $CaCO_3$; pH: 6.2, 6.8, 7.2, 7.8, and 8.4. Results were assigned based on the value most similar in color to the test strip, and interpolation was not performed. Temperature was measured using the thermometer included in the kit.

2.5. Size fractionation of microbial community via membrane filtration

The microbial community of each sample was size fractionated by a double-filtration method. Within 24 hours of receipt, each sample was vacuum filtered through a sterile 3-μm polycarbonate filter (MilliporeSigma™, Burlington, MA, USA) using sterile glass funnels and filter flasks, where 3-μm filters have been used previously to capture FLA from drinking water (Delafont et al., 2013). The filtrate was collected and filtered again through a sterile 0.4-μm polycarbonate filter (MilliporeSigma™). Membrane filters were aseptically removed from the funnels and cut into pieces using sterile scalpels. The filter pieces were placed in Lysing Matrix E bead tubes (MP Biomedicals, Irvine, CA, USA), which had been previously emptied by transferring the beads to sterile microcentrifuge tubes. The samples were stored at -80°C until DNA extraction.

In the following sections, we use the operationally defined terms *particle-associated* to refer to *Legionella* spp. or *Lp* DNA retained by the 3-μm filter (e.g., DNA associated with biofilm, FLA, or other particles), and *free-living* to refer to *Legionella* spp. or *Lp* DNA retained by the subsequent 0.4-μm filter. Validations for the sequential filtration method and subsequent operational definitions are included in the Supplementary Materials (Text S2).

2.6. DNA extraction

DNA extraction was performed following a modified QIAGEN (Germantown, MD, USA) DNeasy PowerWater Kit® protocol (Vosloo et al., 2022). First, enzymatic lysis was performed by adding 294 μL of 10x Tris-EDTA (Sigma-Aldrich, Burlington, MA, USA) and 6 μL of 50 mg/mL lysozyme solution (ThermoFisher Scientific, Waltham, MA, USA) to each tube. The tubes were incubated for 60 min in a 37°C water bath. Next, a chemical and enzymatic lysis step was performed by adding 300 μL of PW1 from the QIAGEN DNeasy PowerWater Kit® and 30 μL of 20 mg/mL Proteinase K (ThermoFisher Scientific) to each tube. Samples were then incubated for 30 min in a 56°C water bath. The reserved beads from the Lysing Matrix E tubes and 630 μL of 24:1 chloroform/isoamyl alcohol (VWR, Radnor, PA, USA) were added prior to homogenization for 40 s using the FastPrep-24 Classic Instrument (MP Biomedicals). The tubes were centrifuged at 4°C for 10 min at 14,000 x g, and the upper aqueous phase was transferred to a sterile microcentrifuge tube.

At this point, the samples were processed starting at step 11 of the QIAGEN DNeasy PowerWater Kit® protocol, when Solution IRS is added to the sample. Extracted DNA was eluted into 100 μL of sterile elution buffer. The concentration and purity of DNA were measured with a NanoDrop 2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a Qubit 2.0 fluorometer using the dsDNA High Sensitivity Assay kit (ThermoFisher Scientific). Each DNA extraction session included a blank (1-L sample of autoclaved Milli-Q water that was extracted alongside the samples), which served as a DNA extraction control.

2.7. dPCR targeting Legionella spp. and L. pneumophila

Legionella spp. and *Lp* were quantified using dPCR with assays targeting the *ssrA* gene, which encodes for the transfer- and messenger-RNA (tmRNA), and the macrophage infectivity potentiator (*mip*) gene, respectively, adapted from Benitez & Winchell for dPCR (Benitez and Winchell, 2013). DNA sequences for all primers and probes, as well as the synthetic DNA used as the positive controls (gBlock™, Integrated DNA Technologies [IDT], Coralville, IA, USA) are provided in Table 2. The sequences used for the synthetic DNA for the *Legionella* spp. and *Lp* assays were consensus sequences of several high-quality genomes from the National Center for Biotechnology Information's (NCBI) Genome Database. To generate the consensus sequences, genomes were downloaded from NCBI, trimmed to the amplified regions of the assays, and aligned using Clustal Omega in the software Jalview (v. 2.11.2.5) (Sievers et al., 2011; Waterhouse et al., 2009). The genomes used to generate the consensus sequence for the *Legionella* spp. assay synthetic DNA positive control included one *Lp*, one *Legionella longbeachae*, and one *Legionella anisa* (NCBI accession numbers AE017354, NZ_CP020412, and NZ_CP029563, respectively). For the synthetic DNA positive control for the *Lp* assay, five *Lp* genomes were used (NCBI accession numbers CP061840, CP048618, LT906452, LR133933, and CP013742). The

Table 2
Sequences for primers, probes, and synthetic DNA used in this study.

Target	Primer/ Probe	Sequence (5′ → 3′)	Gene target	Product Length (bp)	Source
Legionella spp.	Forward Primer	GGCGACCTGGCTTC	*ssrA*	100	(Benitez and Winchell, 2013)
	Reverse Primer	GGTCATCGTTTGCA TTTATATTTA			
	Probe	FAM-ACGTGGGT TGCAA-MGB-NFQ			
	Synthetic DNA	*AGCTGTCAGCACTACTAAC* *TTGCGGTCAGT*GGCGACCTGGCTTCGACGTGG GTTGCAAAACCAGAGGTGCATG CCGAGAATGAGAACTCTCGTAA ATCAGACTCAAACTAAATATAAA TGCAAACGATGA*TGCATGATCTACGTGCGTC* *ACATGCAGTAC*			*
Legionella pneumophila	Forward Primer	TTGTCTTATAGCATTGGTGCCG	*mip*	115	(Benitez and Winchell, 2013)
	Reverse Primer	CCAATTGAGCGCCACTCATAG			
	Probe	Cy5-CGGAAGCAATGGCTAAAGGCATGCA-BHQ2			
	Synthetic DNA	*AGCTGTCAGCACTACTA* *ACTTGCGGTCAGT*TTGTCTTATAGCATTGGTGCC GATTTGGGGAAGAATTTTAA AAATCAAGGCATAGATGTTA ATCCGGAAGCAATGGCTAA AGGCATGCAAGACGCTATG AGTGGCGCTCAATTGG*TGCATGATCTACGTGCGTC* *ACATGCAGTAC*			^

Neutral adaptors before and after the amplicon in the synthetic DNA sequence are indicated by italics and underlining.

* The *Legionella* spp. assay synthetic DNA was designed using the consensus sequence of the target region for three *Legionella* spp. genomes (NCBI accession numbers AE017354, NZ_CP020412, and NZ_CP029563) with 30 bp of neutral adaptors on the 5′ and 3′ ends.

^ The *Lp* assay synthetic DNA was designed using the consensus sequence of the target region for five *Lp* genomes (NCBI accession numbers CP061840, CP048618, LT906452, LR133933, and CP013742) with 30 bp of neutral adaptors on the 5′ and 3′ ends.

synthetic DNA sequences included thirty base pairs of low-complexity, neutral adaptors on the 5′ and 3′ ends. dPCR was performed using QIAGEN QIAcuity 26k 24-well Nanoplates and a QIAGEN QIAcuity dPCR system. Each sample was quantified in duplicate, and each dPCR run included a positive control (*Legionella* spp. or *Lp* synthetic DNA) and negative control (no-template control consisting of sterile, DNase-free water [ThermoFisher Scientific]), which also were quantified in duplicate. dPCR reactions (40 µL) contained 10 µL of template, 10 µL of 4x QIAcuity Probe Mastermix (QIAGEN), 0.4 µL of each primer (final concentration 1 µM, IDT), 0.8 µL of each probe (final concentration 0.2 µM, IDT), 0.5 µL of 20 units/µL EcoRI-HF® (New England BioLabs, Inc., Beverly, MA, USA), and 16.3 µL of sterile, DNase-free water. EcoRI-HF® was used for DNA fragmentation to ensure even distribution of template throughout the nanoplate, leading to more accurate and precise amplification. The cycling conditions were as follows: 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. A single threshold was set for all wells on each dPCR plate that excluded the fluorescence of the negative control and included the fluorescence of the positive control. Five to ten additional cycles were added as needed to increase the separation in fluorescence intensity between positive and negative partitions.

The LLOD for each dPCR assay was determined by performing serial dilutions of the synthetic DNA. For the *Legionella* spp. assay, dilutions ranging from 10^{-2} to 10^{4} gene copies per reaction (gc/reaction) were analyzed. For the *Lp* assay, dilutions from 10^{-2} to 10^{1} gc/reaction were analyzed. For both assays, the synthetic DNA standards were run across at least three plates, with at least ten replicates per standard concentration. The LLOD was defined as the lowest concentration at which at least 95 % of the standard replicates were detected (Bustin et al., 2009), resulting in LLODs of 0.1 gc/reaction (or 40 gc/L of sample, assuming a 1-L sample and 100-µL eluted DNA) for both assays. Samples were considered positive by dPCR if gene copy concentrations were above the LLOD.

2.8. *Legionella*-genus 16S rRNA gene sequencing and analyses

To further characterize the *Legionella* spp. in the water samples, 20 samples (first-draw and flushed) from the summer sampling event (39 DNA extracts from the size-fractionated samples), representing ten of the 19 sites, and one DNA extraction control, were selected for *Legionella*-genus amplicon sequencing (Table S2). Sequencing targeted a 421-bp portion of the V3/V4 region of the *Legionella* spp. 16S rRNA gene (Kahlisch et al., 2010; Pereira et al., 2017). Library construction and sequencing was performed at the Roy J. Carver Biotechnology Center at the University of Illinois Urbana-Champaign. The detailed PCR amplification protocol with conditions and reagents is included in the Supplementary Materials (Text S3; Table S3). Briefly, DNA concentrations in the samples were quantified using a Qubit fluorometer with the dsDNA High Sensitivity Assay kit (ThermoFisher Scientific), and samples were diluted as necessary to 2 ng/µL. Next, PCR amplification using a published assay was performed (Pereira et al., 2017). A second PCR step was then performed using the PCR products, which were diluted 1:70, to barcode the amplicons (Table S4). Concentrations of barcoded amplicons in each sample were quantified using Qubit (ThermoFisher Scientific), and the amplicon lengths were determined using a Fragment Analyzer (Agilent, Santa Clara, CA, USA). Samples were pooled in equal amounts, size selected on a 2 % agarose E-gel (Life Technologies, ThermoFisher Scientific) and extracted from the gel slice using the QIAquick Gel Extraction Kit (QIAGEN). The cleaned, size-selected products were analyzed using a Fragment Analyzer and quantified using quantitative, real-time polymerase chain reaction (qPCR) on a CFX Connect Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). The cleaned, size-selected amplicons were pooled, spiked with 20 % non-indexed PhiX Control (Illumina, Inc., San Diego, CA, USA), and sequenced on an Illumina MiSeq instrument using a PE Nano V2 flow cell, generating paired-end, 250-nucleotide reads.

Raw fastq files were demultiplexed with the bcl2fastq (v.2.20) Conversion Software (Illumina). Next, primers were trimmed using Snakemake (v.7.32.4) and cutadapt (v.4.6) (Martin, 2011; Mölder et al., 2021). Trimmed sequences were processed using R in RStudio (v.2022.07.2+576) and DADA2 (v.1.26.0) following the Pipeline Tutorial (v1.16; https://benjjneb.github.io/dada2/tutorial.html) (R Core Team 2024; R StudioTeam 2024). Nine DNA extracts were excluded from analysis due to low read counts (<60 reads, Table S2). The NCBI

Table 3
Summary of median physicochemical results by secondary disinfectant, source water type, and sample type.

Parameter*	Median Value (count)						
	Secondary Disinfectant Type		Source Water Type			Sample Type	
	Combined Chlorine	Free Chlorine	Blended	Groundwater	Surface	First-draw	Flushed
Temperature (°C)	21.5 (n=55)	18.9 (n=50)	22.9 (n=15)	19.3 (n=32)	20.3 (n=58)	20.7 (n=54)	20.0 (n=53)
pH	7.8 (n=60)	7.2 (n=50)	7.8 (n=18)	7.5 (n=32)	7.2 (n=60)	7.8 (n=56)	7.2 (n=56)
Total Chlorine (mg/L as Cl_2)	0.50 (n=60)	0.25 (n=50)	0.25 (n=18)	0.25 (n=32)	0.25 (n=60)	0.25 (n=56)	0.50 (n=56)
Total Hardness (mg/L as $CaCO_3$)	250 (n=60)	120 (n=50)	250 (n=18)	120 (n=32)	120 (n=60)	120 (n=56)	120 (n=56)
Total Alkalinity (mg/L as $CaCO_3$)	120 (n=60)	80 (n=50)	180 (n=18)	150 (n=32)	100 (n=60)	120 (n=56)	120 (n=56)

* Free chlorine was excluded from the table because only one sample was positive (summer flushed sample from GF_4, 0.50 mg/L as Cl_2).

Basic Local Alignment Search Tool (BLAST) 16S ribosomal database (downloaded on January 26, 2024) was utilized to identify potential taxonomic matches (v.2.15). The relative abundance and distribution of clinically relevant *Legionella* spp. were examined. Clinically relevant species selected for this analysis included *Lp, L. longbeachae, L. anisa, Legionella bozemanae, Legionella micdadei, Legionella dumoffii, Legionella feeleii,* and *Legionella wadsworthii* (Muder and Yu, 2002). An *L. anisa* amplicon sequence variant (ASV) was found in the negative control (264/264 reads in that sample); however, this ASV was not found in the other DNA extracts. Counts were rarefied to a depth of 1000 reads, leading to seven DNA extracts being removed: SF_5_FD_PA, SF_5_FF_FL, SF_5_FF_PA, SC_2_FD_FL, SC_2_FF_FL, SC_3_FD_FL, and BC_2_FD_FL (FD: first-draw; FF: flushed; FL: free-living; PA: particle-associated). GF_3_FD_FL and SC_4_FD_FL both had *L. anisa* reads that were not retained during rarefaction (read counts < 20 per sample). Permutational Multivariate Analysis of Variance (PERMANOVA) was performed using the adonis2 function in the vegan package (v2.6–4) in R on the Bray Curtis dissimilarities of the full community to test the effects of form (free-living or particle-associated), sample type (first-draw or flushed), and site. Data visualizations were prepared using R in RStudio. The code for assigning taxonomy to ASVs is available on GitHub (https://github.com/HegartyLab). The processed reads are available on NCBI (BioProject ID PRJNA1221278).

2.9. Physicochemical and dPCR data analysis

Data analyses were performed using Python (v.3.10.6) and R (v.4.2.2) in RStudio (v.2022.07.2+576) (R Core Team 2024; R Studio-Team 2024; Python Team 2020). For plotting and statistical analyses, physicochemical results below the lowest value in the measurement range (LLOD) and dPCR results less than the LLOD were set at one-half the LLODs (Antweiler and Taylor, 2008). The ULOD for the physicochemical analyses was the upper limit of the measurement range. Because the physicochemical and dPCR results were not normally distributed (Shapiro Wilk test, $p < 0.05$), the nonparametric Spearman rank correlation analysis was used to determine correlations, and hypothesis testing was conducted using the Mann-Whitney U Test (Wilcoxon Rank Sum Test). A significance level of 0.05 was selected for all analyses.

3. Results

3.1. Preliminary testing demonstrated culturable Lp at several sites

Of the 12 sites sampled during preliminary testing, four tested positive for culturable *Lp*. The positive sites included three surface water sites (SF_1, SF_5, SC_3) and one groundwater site (GF_2; Figure S1). Concentrations of culturable *Lp* in the positive first-draw samples were relatively low, ranging from 1.1 to 15.5 MPN/100 mL. Flushing reduced the concentration of culturable *Lp* at three of the four positive sites: SF_1, SF_5, and SC_3. At GF_2, the concentration of culturable *Lp* increased substantially with flushing, with a result that was above the ULOD (>2272.6 MPN/100 mL). The preliminary testing results showed that, despite the relatively small sample volumes, several sites contained measurable levels of culturable *Lp*, warranting further investigation with larger sample volumes and additional sampling locations.

3.2. Physicochemical results, particularly temperature, differed among site types

Physicochemical parameters measured in samples from the full study included free and total chlorine, pH, temperature, hardness, and alkalinity (Table 3). Median sample temperatures by site ranged from 13 to 28°C (Figure S2). While most sample temperatures were less than 25°C (n=90 of 105), 15 samples had temperatures above this, including three samples with temperatures above 30°C. All but one of these higher temperature samples were collected from sites in the southern U.S., including sites in Texas, Florida, and California. The difference in temperatures was statistically significant for sample pairs between summer and fall (n=35 pairs, p=0.013), fall and winter (n=31 pairs, $p=1.20\times10^{-4}$), and summer and winter (n=34 pairs, $p=1.91\times10^{-5}$). Overall, sample temperatures in combined chlorine systems were significantly higher than those in free chlorine systems ($p<0.001$), likely due to the majority of free chlorine sites in this study being in the northern U.S.

For total chlorine, results were generally higher in combined chlorine systems compared to free chlorine systems. In paired samples (grouped by site and sampling event), flushing increased the total chlorine result in most cases (62 %, n=34 of 55 pairs) from the first-draw value. Total chlorine values were not significantly different ($p>0.05$) by source water type. Overall, pH values were higher in combined chlorine systems than in free chlorine systems. At ten of 19 sites, the median pH was 7.8, including six combined chlorine sites (SC_2, SC_5, GC_1, BC_1, BC_2, and BC_3) and four free chlorine sites (SF_1, GF_1, GF_2, GF_3). The only site with a median pH above 8 was SC_4 (median: 8.4, n=6). However, as 8.4 was the maximum value on the water quality test strip, it is possible the true pH was higher than 8.4. In paired samples, flushing did not change the pH in the majority of cases (56 %, n=31 of 55).

3.3. dPCR detection of total Legionella spp. and total Legionella pneumophila

Total *Legionella* spp. (free-living + particle-associated) were detected by dPCR in 98 % (n=108 of 110) of samples (first-draw and flushed), including all sites and across all three seasons, at concentrations ranging from 6.28×10^{1} to 1.65×10^{5} gc/L. Total *Legionella* spp. concentrations were significantly higher in samples from surface water systems (n=60) as compared to groundwater systems (n=32, p=0.020) and in samples

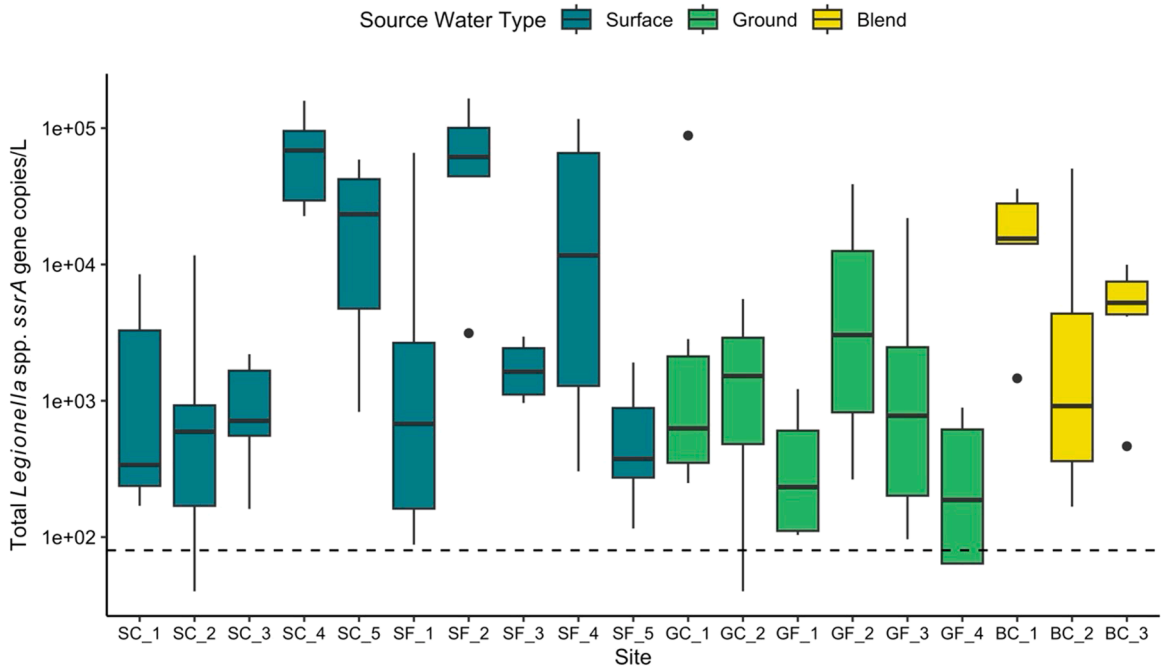

Fig. 1. Total *Legionella* spp. (free-living + particle-associated) dPCR results for the *ssrA* gene (gene copies per liter, gc/L) by site. Boxes show the interquartile range (IQR), with the center line indicating the median. Lower whiskers show 1.5 x IQR or the lowest value greater than 1.5 x IQR. Upper whiskers show 1.5 x IQR or the highest value less than 1.5 x IQR. The horizontal dashed line indicates two times the lower limit of detection (LLOD) of 40 gc/L (80 gc/L) to account for addition of particle-associated and free-living dPCR results. Results less than the LLOD were set at ½ the LLOD for plotting.

from blended water systems (n=18) as compared to groundwater systems (p=0.005) but were not significantly different between surface water and blended water systems (p=0.526; Fig. 1; Figure S3). In samples paired by site and sampling event, total *Legionella* spp. concentrations were significantly higher in first-draw samples as compared to flushed samples (n=55 pairs, p=0.020). In samples paired by site and sample type (first-draw or flushed), total *Legionella* spp. concentrations were significantly higher in fall samples compared to winter samples (n=34 pairs, p=0.007) but were not significantly different between the other seasons. The samples with the highest total *Legionella* spp. concentrations when grouped by site and sample type mostly occurred in summer (n=22) and fall (n=11), though five sites and sample types had the highest concentrations in the winter (GC_2 first-draw, SF_3 flushed, SF_4 first-draw, SF_4 flushed, GF_2 first-draw). Three of these samples were above the median temperature for their sample type (GC_2 first-draw, SF_3 flushed, SF_4 first-draw) (Table 3), and three of these samples were collected from apartment units (SF_3 flushed, SF_4 first-draw, SF_4 flushed). Samples GC_2 first-draw, SF_4 first-draw, and SF_4 flushed had a pH value above the median for their sample type (Table 3).

When comparing results across secondary disinfectant and source water types, total *Legionella* spp. concentrations were not significantly different between the surface water/combined chlorine (SC) samples (n=30) and surface water/free chlorine (SF) samples (n=30, p=0.786) nor the groundwater/combined chlorine (GC) samples (n=12) and groundwater/free chlorine (GF) samples (n=20, p=0.659). The five sites with the highest median concentrations of total *Legionella* spp. (>10^4 gc/L) were clustered in the southern U.S. (SC_4, SC_5, and BC_1) and Mid-Atlantic region (SF_2, SF_4). It is notable that three of these sites are apartment units (SC_5, BC_1, SF_4), since only five (26 %) sampling sites included in the study were located in apartment buildings (Table 1).

For total *Lp* (free-living + particle-associated), twelve of the 110 samples were positive via dPCR. These positive detections were mostly first-draw samples (n=9 of 12), combined chlorine sites (n=10 of 12), and located in systems sourced by surface (n=6 of 12) or blended (n=5

Table 4
Comparison between Legiolert results from 2021 and dPCR results from 2022–2023.

Result Type	Site	Legiolert Result (MPN/ 100 mL)		*Lp* dPCR Results (*mip* gc/L)					
				Summer		Fall		Winter	
		First-Draw	Flushed	First-Draw	Flushed	First-Draw	Flushed	First-Draw	Flushed
Negative by both Legiolert and dPCR	SC_2	Neg	Neg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	SC_4*	Neg	Neg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	GC_1*	Neg	Neg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	GC_2	Neg	Neg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	SF_2	Neg	Neg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
	SF_4	Neg	Neg	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Positive by Legiolert and dPCR	SC_3	**1.1**	**Neg**	**135.2**	< LOD	< LOD	< LOD	< LOD	< LOD
	SF_5	**14.6**	**Neg**	**74.8**	< LOD	< LOD	< LOD	< LOD	< LOD
	GF_2	**2.3**	**>2272.6**	**104.4**	< LOD	< LOD	< LOD	< LOD	< LOD
Positive by dPCR Only	BC_2	Neg	Neg	**73.6**	< LOD	< LOD	< LOD	< LOD	< LOD
	BC_3	Neg	Neg	< LOD	< LOD	< LOD	< LOD	**139.2**	**272**
Positive by Legiolert Only	SF_1*	**15.5**	**2.3**	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD

* The sampling site for the full study was in a different building located in the same DWDS as the preliminary study.

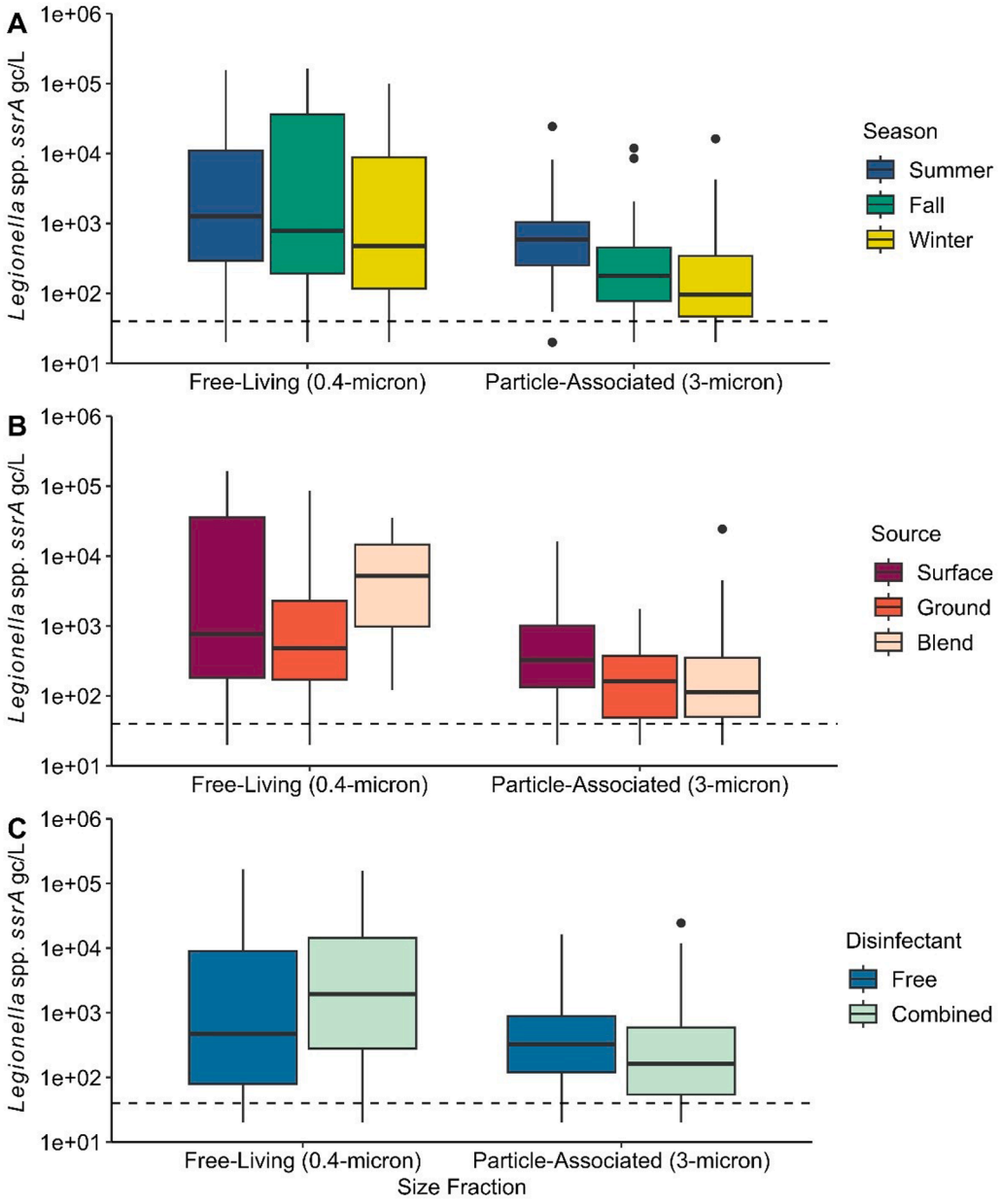

Fig. 2. Free-living (0.4-μm) and particle-associated (3-μm) *Legionella* spp. *ssrA* gene dPCR results grouped by (A) season, (B) source water type, and (C) secondary disinfectant type. Boxes show the interquartile range (IQR), with the center line indicating the median. Lower whiskers show 1.5 x IQR or the lowest value greater than 1.5 x IQR. Upper whiskers show 1.5 x IQR or the highest value less than 1.5 x IQR. The horizontal dashed line indicates the lower limit of detection (LLOD) of 40 gene copies (gc) per L. Results less than the LLOD were set at ½ the LLOD for plotting.

of 12) water. *Lp* detections were sporadic, where *Lp* was detected only once at four of the seven *Lp*-positive sites. The site with the most *Lp*-positive detections and the highest concentrations of total *Lp* was SC_5, with four of six samples positive and concentrations ranging from 1.48×10^{2} gc/L to 1.83×10^{4} gc/L. Of the nine sites that were tested for *Lp* during both the preliminary testing (culture-based; 2021) and the full study (dPCR; 2022–2023), three sites were positive by culture and dPCR in at least one sample each (Table 4). Four sites were negative by both methods in all samples.

3.4. Free-living and particle-associated Legionella spp.

Next, the *Legionella* spp. dPCR results for the 3-μm membrane filters (particle-associated) were compared to those for the 0.4-μm filters (free-living) (Figure S4). For paired samples with results from both filters, *Legionella* spp. concentrations were significantly different between the free-living and particle-associated fractions (n=107, $p=2.86\times10^{-9}$), with 75 % (n=81) of the samples having higher free-living than particle-associated *Legionella* spp. concentrations.

Overall, seasonal trends were similar for free-living and particle-associated *Legionella* spp., with higher concentrations in the warmer seasons (Fig. 2a). When paired by site and sample type (first-draw or flushed), fall concentrations of free-living *Legionella* spp. were significantly higher than those in winter (n=33 pairs, p=0.004), but there was not a significant difference in the free-living *Legionella* spp. concentrations between the other seasons. When paired by site and sample type (first-draw or flushed), particle-associated *Legionella* spp. concentrations were significantly higher in the summer than in the fall (n=36 pairs, p=0.030) and winter (n=36 pairs, $p=7.64\times10^{-4}$) but were not significantly different between fall and winter (n=34 pairs, p=0.231).

When comparing *Legionella* spp. concentrations by source water type, free-living *Legionella* spp. concentrations were significantly higher in

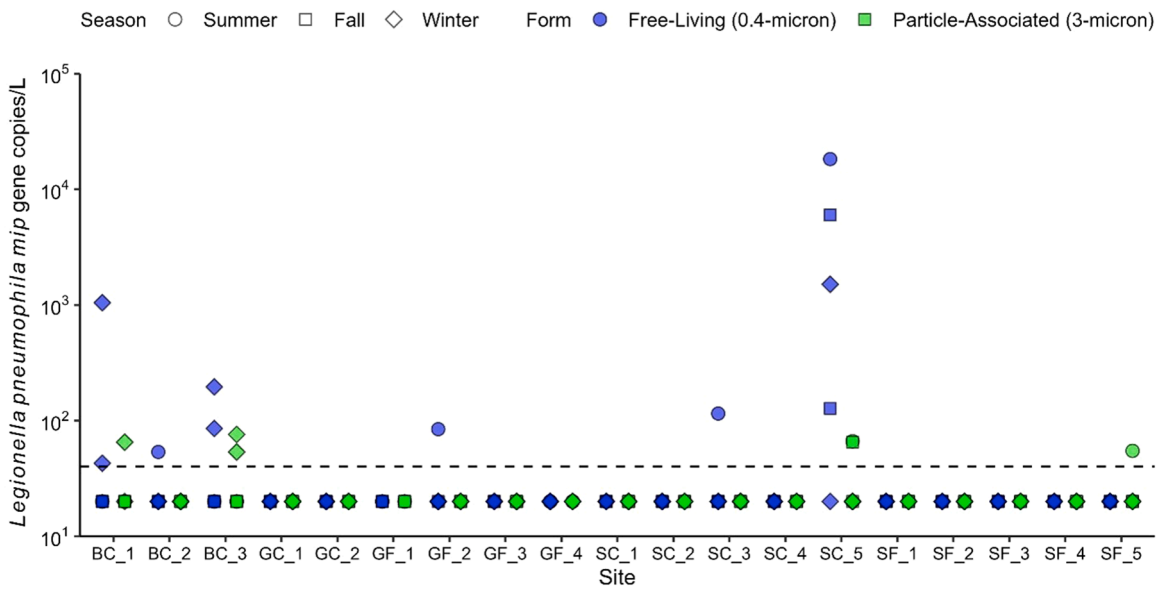

Fig. 3. Free-living (0.4-μm) and particle-associated (3-μm) *Legionella pneumophila mip* gene dPCR results by site and season. The horizontal dashed line indicates the lower limit of detection (LLOD) of 40 gene copies (gc) per L. Results less than the LLOD were set at ½ the LLOD for plotting.

blended water (n=18, median: 5.22×10^3 gc/L) as compared to groundwater (n=31, median: 4.83×10^2 gc/L, p=0.016, Fig. 2b). There was not a significant difference between the free-living *Legionella* spp. concentrations in blended water and surface water (n=58, median: 7.67 x10^2 gc/L) or surface water and groundwater. In contrast, particle-associated *Legionella* spp. concentrations were significantly higher in surface water (n=60, median: 3.27x10^2 gc/L) as compared to blended water (n=18, median: 1.14x10^2 gc/L, p=0.025) and groundwater (n=32, median: 1.62x10^2 gc/L, p=0.008).

Free-living *Legionella* spp. concentrations were higher in combined chlorine systems (n=59, median: 1.94×10^3 gc/L) than free chlorine systems (n=48, median: 4.73×10^2 gc/L; Fig. 2c), though this difference was just above the threshold for significance (p=0.072). The opposite trend was observed for particle-associated *Legionella* spp., where concentrations were lower in the combined chlorine systems (n=60, median: 1.62×10^2 gc/L) than free chlorine systems (n=50, median: 3.27×10^2 gc/L), though this result also was not statistically significant (p=0.090).

For *Lp*, free-living *Lp* was detected in 11 of 12 samples that tested positive for total *Lp* (Fig. 3; Table S5). Most of these positive detections were in systems using combined chlorine as the secondary disinfectant (n=10 of 11) and in first-draw samples (n=8 of 11). There was at least one positive free-living *Lp* detection in all three source water types, although the greatest *Lp* concentrations were seen in a system sourced by surface water (SC_5, which is in an apartment building). Three sites had a positive free-living *Lp* detection for both the first-draw and flushed samples (SC_5, fall; BC_1, winter; BC_3, winter), all of which were located in apartment buildings. Flushing substantially reduced free-living *Lp* concentrations in fall and winter samples from SC_5 (98 % and >97 % decrease, respectively) as well as the winter sample from

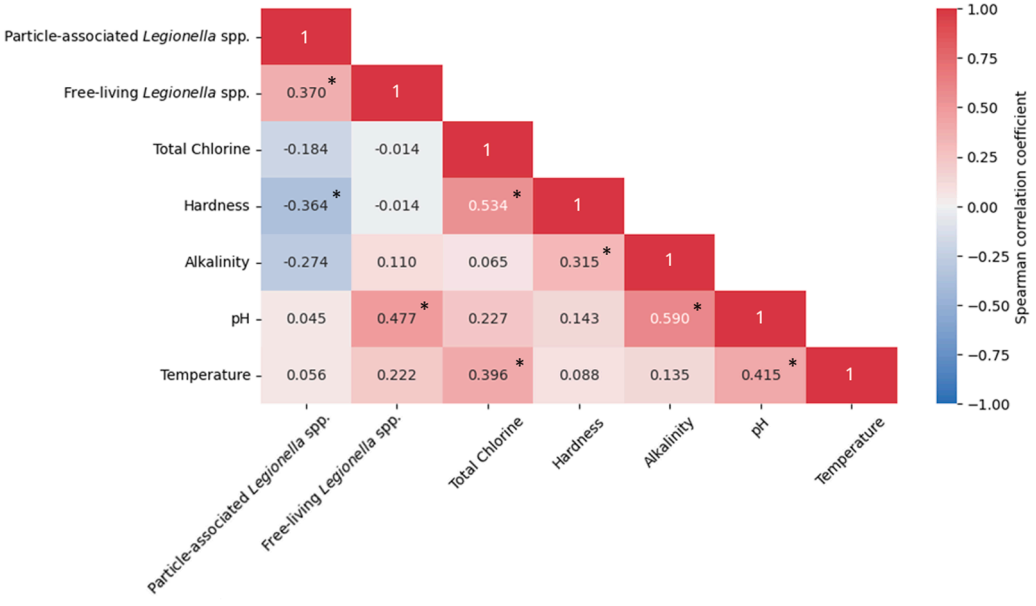

Fig. 4. Spearman rank correlation for water quality parameters and *Legionella* spp. concentrations for the size-fractionated flushed water samples. * Correlations are significant at the 0.05 level.

BC_1 (96 % decrease). In the winter BC_3 samples, flushing increased the free-living *Lp* concentration from 8.56×10^{1} gc/L to 1.96×10^{2} gc/L, despite the total chlorine concentration increasing from non-detectable in the first-draw sample to 4 mg/L as Cl_2 in the flushed sample.

Particle-associated *Lp* was only detected in six samples, five of which were first-draw samples and all of which were from surface water or blended water systems (Fig. 3). Only one particle-associated *Lp*-positive sample was from a free chlorine system (SF_5, summer, first-draw). Particle-associated *Lp* concentrations were low in positive samples, with the highest result being only 7.60×10^{1} gc/L (BC_3, winter, flushed). The particle-associated *Lp* concentration exceeded that of free-living in only one of 12 samples (SF_5 first-draw: 5.48×10^{1} gc/L). Flushing decreased particle-associated *Lp* concentrations in four of five instances. Flushing slightly increased the particle-associated *Lp* in the winter BC_3 sample, as was seen in the free-living results, though the concentrations for the particle-associated results were similar (first-draw: 5.36×10^{1} gc/L; flushed: 7.60×10^{1} gc/L).

3.5. Relationship between physicochemical parameters and Legionella spp. results

Correlation analysis was performed to determine whether physicochemical parameters (temperature, pH, total chlorine, alkalinity, and hardness) correlated with *Legionella* spp. dPCR concentrations. When evaluating only the first-draw samples, significant correlations were observed between hardness and alkalinity and between particle-associated *Legionella* spp. and free-living *Legionella* spp. (Figure S5). For flushed samples, the physicochemical parameters found to correlate significantly with *Legionella* spp. varied between the free-living and particle-associated forms (Fig. 4). For particle-associated *Legionella* spp. in flushed samples, a significant moderate negative correlation existed with hardness (r_s = -0.36, p = 0.009) and a nearly significant negative correlation was observed with alkalinity (r_s = -0.27, p = 0.051). For free-living *Legionella* spp. in flushed samples, a significant positive correlation was observed with pH (r_s = 0.477, p=3.96×10^{-4}). Notably, hardness was also positively and significantly correlated with total chlorine (r_s= 0.53, p = 5.43×10^{-5}). There was a negative, though insignificant correlation between total chlorine and particle-associated *Legionella* spp. (r_s = -0.18, p = 0.197). Thus, lower concentrations of particle-associated *Legionella* spp. in samples with high hardness might have been a result of higher chlorine concentrations.

Limited significant correlations existed between physicochemical parameters and *Lp* concentrations, likely due to the small number of *Lp*-positive samples (Figure S6). However, hardness had a significant, positive correlation with free-living *Lp* concentration (r_s = 0.237, p = 0.016), as did alkalinity (r_s = 0.198, p = 0.046). There were no significant correlations observed with particle-associated *Lp*, but as mentioned previously, particle-associated *Lp* was only detected in six samples and in low concentrations.

3.6. Three clinically-relevant Legionella spp. were detected with genus-specific sequencing

Amplicon sequencing targeting a portion of the *Legionella*-genus 16S rRNA gene in 20 of the summer samples (first-draw and flushed, n=39 size-fractionated samples) and one control (n=1) was used to investigate *Legionella* spp. diversity (Hozalski et al., 2024; Kahlisch et al., 2010; Pereira et al., 2017). Of these size-fractionated DNA extracts, nine were excluded from downstream analysis due to insufficient reads and another seven were removed during the rarefaction process, leaving 23 size-fractionated DNA extracts representing eight sites. The *Legionella* spp. detected via sequencing varied greatly among the sites. The most abundant *Legionella* spp. identified include *Legionella lytica, Legionella qingyii, Lp, L. anisa, Legionella erythra,* and *L. longbeachae* (Fig. 5). Relative abundances of *Legionella* spp. varied greatly by site, sample type (first-draw [FD] vs. flushed [FF]), and form (particle-associated [PA] vs. free-living [FL]). For example, the sequences from some size-fractionated samples were nearly or entirely *Legionella* spp. (relative abundances near 100 %), such as the particle-associated, first-draw sample from BC_2, the particle-associated samples from SC_4, and the free-living, first-draw sample from SF_3 (Figure S7). In contrast, *Legionella* spp. relative abundances in other size-fractionated samples

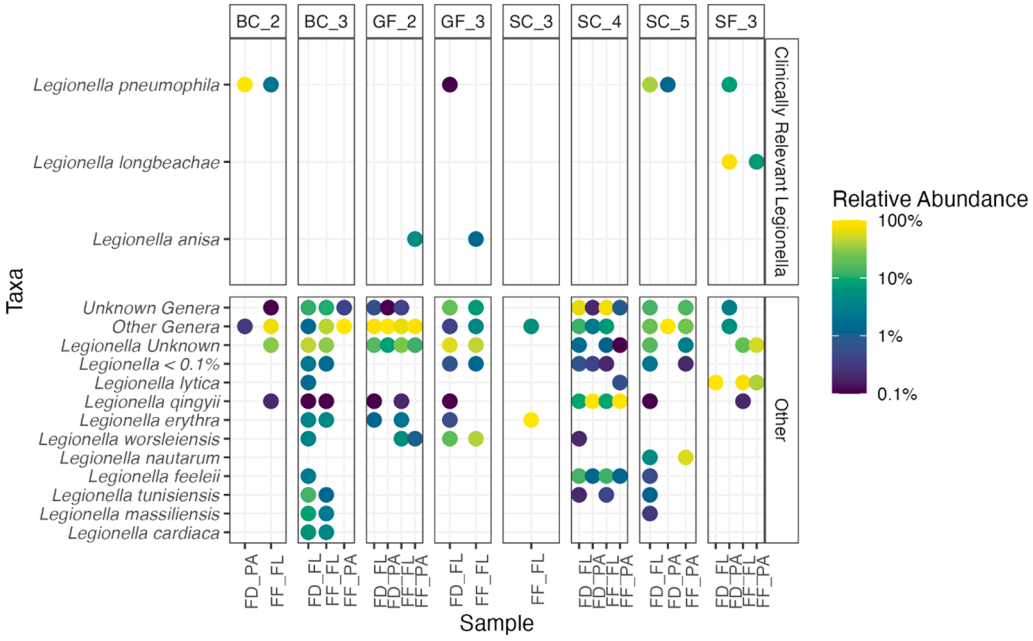

Fig. 5. *Legionella* genus amplicon sequencing relative abundances per size-fractionated DNA extract. Twenty-three size-fractionated DNA extracts from eight sites yielded sufficient reads for analysis after rarefaction. *Legionella* spp. with a relative abundance under 0.1 % were grouped together (*Legionella* < 0.1 %), as were the non-*Legionella* genera with known taxonomy (Other Genera), those *Legionella* with unknown species identification (*Legionella* Unknown), and the unknown taxa (Unknown Genera). Bubble color represents the relative abundance. *Legionella* species were separated by clinical relevance and listed in order of decreasing overall abundance (top to bottom). FD: first-draw; FF: flushed; FL: free-living; PA: particle-associated.

were dominated by other taxa (e.g., samples from GF_2). Most of the samples discarded due to insufficient reads also were dominated by non-*Legionella* taxa (e.g., SC_2 and SF_5 samples). At some sites, clear differences between the particle-associated and free-living *Legionella* spp. were observed, as illustrated by the results for SF_3 and SC_4, where similar *Legionella* spp. were seen by form (Figure S7). The sequencing results showed strongly significant clustering by site and less strong, but still significant, clustering by form and sample type (Figure S8, Table S6). We also observed interaction effects on the Bray-Curtis dissimilarities (Table S6). The interactions were much stronger for site:sample type and site:form than for form:sample type. These results suggest the effects of site, form, and sample type are dependent on each other.

Clinically relevant species of *Legionella* that were found include *pneumophila, anisa*, and *longbeachae* (Fig. 5). *Lp* was the most prevalent, being found at four sites (BC_2, SC_5, GF_3, and SF_3). At Sites BC_2 and SC_5, *Lp* was detected by both dPCR and sequencing in at least one summer sample, whereas *Lp* was never detected by dPCR at Site GF_3 or SF_3. *L. anisa* was detected at low relative abundances at two sites (GF_2 [4.7 %] and GF_3 [1.6 %]). Other studies have found *L. anisa* to be the most frequently detected *Legionella* spp. after *Lp* (Crook et al., 2024; Doleans et al., 2004; Van der Mee-Marquet et al., 2006). Sites SF_3 and GF_3 were the only sites where more than one clinically-relevant species of *Legionella* was detected.

Detection of *Legionella* spp. and *Lp* by sequencing did not necessarily correlate with concentrations of total *Legionella* spp. and *Lp* measured by dPCR. For example, SF_2, the site with the second highest median concentration of *Legionella* spp. by dPCR (median: 6.45×10^4 gc/L) returned insufficient reads for sequence processing. However, SC_4, the site with the highest median concentration of *Legionella* spp. by dPCR (median: 6.89×10^4 gc/L), was found by sequencing to have a high relative abundance of *Legionella* spp., specifically *L. qingyii,* in the particle-associated fractions of the first-draw and flushed samples. While *Lp* was detected by sequencing at four sites, it was detected at seven sites by dPCR. The increased detection of *Lp* by dPCR compared to amplicon sequencing is likely a function of dPCR's sensitivity, as discussed previously (Wood et al., 2019). Non-target amplification and insufficient sequencing depth also could have contributed to *Lp* not being detected in as many samples with DNA sequencing (Barlow et al., 2020; Salipante and Jerome, 2020). Non-target amplification was evident in several of the size-fractionated DNA extracts, such as the particle-associated, first-draw sample from SC_5, with *Pseudomonas* spp. being one of the most abundant non-*Legionella* genera that could be identified (Figure S7).

4. Discussion

This study uncovers the free-living and particle-associated portions of *Legionella* spp. and *Lp* in drinking water samples collected in residential buildings across the U.S., as well as the varying occurrence and concentration of *Legionella* spp. among source water type, secondary disinfectant, and physicochemical parameters.

4.1. Source water, disinfectant type, and seasonality

Significantly higher concentrations of total *Legionella* spp. were found in surface water than in groundwater systems in the current study (Fig. 1). This was expected because higher concentrations of *Legionella* spp. have been reported previously in drinking waters sourced from surface water as compared to groundwater (Boer et al., 2008; Kim et al., 2023; Zacheus and Martikainen, 2011) and because *Lp* (Fliermans et al., 1981) and their FLA hosts (Rodriguez-Zaragoza, 1994) are known to thrive in surface water. Furthermore, a study on legionellosis outbreaks in the U.S. found that 54 % of the outbreaks were associated with building water systems served by surface water systems, while only 34 % were associated with building water systems served by groundwater (Holsinger et al., 2022). While several studies have reported that secondary disinfectant type influences *Legionella* spp. and *Lp* occurrence in drinking water systems (Donohue et al., 2019; LeChevallier, 2019; Xi et al., 2024), the current study found that total *Legionella* spp. concentrations were not significantly different by secondary disinfectant when comparing within the same source water type, suggesting that *Legionella* spp. concentrations are more strongly influenced by source water than secondary disinfectant.

Legionella spp., like many bacteria, are detected at an increased concentration (Huo et al., 2021; Liu et al., 2019a) and frequency (Johnson et al., 2018) in drinking water samples collected in warmer months than in colder months. *Legionella* spp. grow best in the temperature range of 77–113°F [25–45°C] (CDC 2023). Their FLA hosts typically tolerate temperatures between 50–86°F [10–30°C] (Rodriguez-Zaragoza, 1994), although the optimal temperature range depends on the FLA genus. *Acanthamoeba* spp., for example, show the highest levels of detection in the summer in natural waters (John and Howard, 1995; Ren et al., 2018) and DWDSs (Hoffmann and Michel, 2001; Liu et al., 2019a; Stockman et al., 2011). Overall, total *Legionella* spp. concentrations were significantly, positively correlated with temperature (r_s=0.263, p=0.007) in the current study. Surprisingly, despite *Legionella* spp. being generally associated with warmer seasons (Huo et al., 2021; Johnson et al., 2018; Liu et al., 2019a), the summer total *Legionella* spp. concentrations were not significantly higher than the fall or winter concentrations in paired samples. However, the fall results were significantly higher than winter results. This is likely because the highest concentration samples for some sites and sample types were in the fall, rather than summer. At five sites, the highest *Legionella* spp. concentrations were in winter samples, and sites BC_1 and BC_3 tested positive for *Lp* only during the winter sampling event (Fig. 3). A potential explanation for these winter detections might be that both sites are located in the southern U.S., where drinking water temperatures in the winter are typically warmer than in the northern U.S. The water temperatures of the first-draw and flushed winter samples at Site BC_3 were 24°C and 21.5°C, respectively, which are notably warmer than the median winter sample temperature of 19°C for first-draw and 14°C for the flushed samples (Table 3; Figure S2). (Temperature was not measured for the winter samples at Site BC_1 due to an error.) Additionally, lower water use in winter might have contributed to the winter *Legionella* spp. detections, because lower water use can increase water age in DWDSs and stagnation in building plumbing (Opalinski et al., 2020) and has been previously linked to increased opportunistic pathogen concentrations (Zhang et al., 2021).

Total *Lp* was detected by dPCR in only twelve of 110 samples (Fig. 3). Most of these detections were in systems sourced by surface (n=6 of 12) or blended (n=5 of 12) water. The higher frequency of *Lp* detection in surface water systems than groundwater systems has been reported previously (Boer et al., 2008; Kim et al., 2023; Zacheus and Martikainen, 2011). The majority of *Lp*-positive samples (n=10 of 12) were from systems using combined chlorine as the secondary disinfectant, possibly linked to significantly higher sample temperatures in combined chlorine systems than those in free chlorine systems (Section 3.2). Donohue et al. reported a significantly higher *Lp* detection frequency by qPCR in samples with combined chlorine as the secondary disinfectant compared to free chlorine, which the authors note is a change from their previous study that found similar detection frequencies between the two disinfectants (Donohue et al., 2023). These findings warrant further investigation into the role of secondary disinfectant on *Lp* presence and concentration.

4.2. Total Legionella spp. were detected at all sampling sites while total Legionella pneumophila was detected only sporadically

Our findings are consistent with previous research showing that *Lp* is sporadically detected in DWDSs, although some sites might have more persistent colonization (Donohue et al., 2019). Donohue et al. found that systems using surface water with free chlorine as the secondary

disinfectant had the highest average percent *Lp* of total *Legionella* spp. (48 %) as compared to groundwater with free chlorine, surface water with combined chlorine, and groundwater with combined chlorine (32 %, 15 %, and 7 %, respectively) in water samples taken after a brief 15-s flush (Donohue et al., 2023). Our study found the greatest percent *Lp* (for first-draw and flushed samples with detectable concentrations of *Legionella* spp.) in systems using groundwater with free chlorine (17 %), followed by surface water with combined chlorine, surface water with free chlorine, and groundwater with combined chlorine (8 %, 7 %, and 5 %, respectively), although it is important to note the smaller size of our study (n=110 samples) compared to Donohue et al. (n=209).

Various studies have shown decreases in *Lp* occurrence and concentration after flushing (Angert et al., 2023; Cristina et al., 2014; Grimard-Conea et al., 2022; Ra et al., 2023), although the effects of flushing can vary. For example, flushing based on a set time, as done in some studies, does not account for differences in building plumbing volumes and fixture flow rates. In the current study, the citizen scientists were asked to flush until the temperature stabilized to approximate when distribution system water is reached. Flushing significantly reduced total *Legionella* spp. concentrations between first-draw and flushed samples paired by site and sampling event (n=55 pairs, p=0.020). Flushing was more effective at reducing total *Legionella* spp. concentrations for combined chlorine systems (n=30 pairs, p=0.016) than free chlorine systems (n=25 pairs, p=0.442), and for surface water systems (n=30 pairs, p=0.014) than groundwater systems (n=16 pairs, p=0.669). Flushing also was more effective at reducing the median total *Legionella* spp. in houses as compared to apartment buildings (46 % and 31 % reduction, respectively), although there was no statistically significant difference between the *Legionella* spp. concentrations in the first-draw and flushed samples for either building type. For total *Lp*, flushing reduced the concentration in eight of nine samples that had detectable *Lp* in the first-draw sample, and this reduction was effective to below the LLOD in six of eight samples. Further, there were no occasions where *Lp* was detected in the flushed sample but not the first-draw sample, consistent with previous findings that *Lp* have a tendency to grow in stagnant pipes (Angert et al., 2023; Mathews et al., 2025).

4.3. Free-living Legionella spp. significantly outnumbered particle-associated

The dPCR results in the current study showed that free-living *Legionella* spp. and *Lp* concentrations exceeded particle-associated in most samples (Fig. 2). One possible reason for *Legionella* spp. and *Lp* primarily occurring in the free-living phase is that there was not substantial sloughing of biofilm. It has been suggested that *Lp* grows within hosts (i.e., FLA) associated with biofilm (Paniagua et al., 2020), where not only is *Lp* shielded from disinfection, but biofilm stiffness also might prevent sloughing and release of *Lp* into the bulk water. Biofilms have been reported to be stiffer and release less *Lp* in simulated biofilms with long-term disinfection as compared to untreated simulated biofilms (Shen et al., 2016; Shen et al., 2017). Additionally, *Lp* might show spatial preference within a biofilm: a study observing *Lp* within a *Pseudomonas fluorescens* biofilm observed *Lp* migration to the base of the biofilm, which might be less susceptible to sloughing events compared to the outer regions (Silva et al., 2024).

In the current study, while the median free-living *Legionella* spp. concentration was greater at combined chlorine sites than free chlorine sites, the median particle-associated *Legionella* spp. concentration was greater in free chlorine sites than combined chlorine sites (Fig. 2c). This difference might be linked to disinfectant reactivity. For example, Buse et al. reported that free-living *Lp* were more rapidly inactivated and that *Lp* nucleic acids degraded more rapidly when exposed to free chlorine as compared to combined chlorine (Buse et al., 2019). Similarly, other studies have reported that free chlorine is more effective at inactivating free-living *Lp* than is combined chlorine (Zhang and Lu, 2021), based on Ct values of free chlorine (1–5 mg-min/L; room temperature-30°C) (Cervero-Aragó et al., 2015; Dupuy et al., 2011) vs. combined chlorine (16–65 mg-min/L; 25–35°C) (Dupuy et al., 2011; Jakubek et al., 2013) against free-living *Lp*. Therefore, in our results, the lower proportion of free-living to total *Legionella* spp. in free chlorine systems might be due to their more rapid inactivation by free chlorine, and the higher proportion of free-living to total *Legionella* spp. in combined chlorine systems might be linked to their slower inactivation by combined chlorine. Also, protozoan priming coupled with the choice of secondary disinfectant is a potential explanation for our finding that particle-associated *Lp* was detected mostly in systems using combined chlorine as the secondary disinfectant because *Lp* passaged through FLA have been shown to exhibit greater combined chlorine resistance than do free-living *Lp* grown in standard media (Cambronne et al., 2023).

The higher concentrations of free-living and particle-associated *Legionella* spp. in warmer seasons as compared to cooler seasons (Fig. 2A) support the increased persistence and replication of *Legionella* spp. in warmer waters (Shaheen et al., 2019). Particle-associated *Legionella* spp. concentrations were more strongly associated with summer than were free-living *Legionella* spp.: the difference between particle-associated *Legionella* spp. concentrations in summer and fall samples was significant (n=36 pairs, p=0.030), but there was no significant difference between fall and winter concentrations (n=34 pairs; p=0.228). For free-living *Legionella* spp., there was no significant difference in concentration between the summer and fall samples (n=33 pairs, p=0.426), but a significant difference existed between the fall and winter samples (n=33 pairs, p=0.004). As mentioned previously, there were significantly higher water temperatures in paired samples between the summer and fall and fall and winter sampling events (Section 3.2), leading to greater concentrations of free-living and particle-associated *Legionella* spp. in warmer seasons.

4.4. Correlations with physicochemical parameters were affected by the form of Legionella spp.

Hardness was significantly, negatively correlated with particle-associated *Legionella* spp. concentrations but was not significantly correlated with free-living *Legionella* spp. (Fig. 4). Groundwater sources typically have higher hardness than do surface waters, and as discussed in Section 3.4, lower concentrations of *Legionella* spp. were detected in DWDSs sourced from groundwater. A study of hotel water systems in Greece found that hardness $\geq$321 mg/L as $CaCO_3$ increased risk of *Legionella* spp. colonization in hotel cold-water systems (Kyritsi et al., 2018). Further, high water hardness can lower detachment of particle-associated *Legionella* spp.; a study of biofilms grown on PVC coupons found that biofilms developed from hard groundwater were stiffer and had less detachment compared to biofilms developed from soft groundwater (Shen et al., 2018).

Free-living *Lp* was significantly, positively correlated with both hardness and alkalinity (Figure S6). A higher *Lp* detection frequency has been reported in hard waters than soft waters, with a median hardness of 250 mg/L as $CaCO_3$ in *Lp*-positive samples (Donohue et al., 2023). In the current study, the median hardness of the *Lp*-positive samples was also 250 mg/L as $CaCO_3$, and the sample with the highest total *Lp* concentration (SC_5, first-draw, summer) had very high hardness (425 mg/L as $CaCO_3$). Free-living *Lp* was more strongly correlated with total chlorine, hardness, alkalinity, and pH than was particle-associated *Lp* (Figure S6), perhaps due to protection conferred to particle-associated *Lp* by biofilm or FLA. However, relatively few samples were positive for particle-associated *Lp* and positive samples generally had low concentrations; thus, caution must be exercised with respect to interpreting these data.

In the flushed samples, free-living *Legionella* spp. and pH were positively correlated (Fig. 4), which might be related to the higher concentrations of free-living *Legionella* spp. in systems with secondary disinfection by combined chlorine than by free chlorine. Higher pH can

be used to reduce nitrification in combined chlorine systems (TCEQ, 2024), and lower pH is needed to drive HOCl dominance in free chlorine systems. In a study that quantified *Legionella* spp. and *Lp* in drinking water samples across the U.S., the highest detection of *Legionella* spp. via qPCR (59 %) was in drinking water with pH 8.1–8.5 (Donohue et al., 2023), which the authors suggest is due to higher detection frequency in combined chlorine than free chlorine systems.

4.5. Limitations

One study limitation was that we did not confirm that the *Legionella* spp. DNA detected on the 3-μm filter was truly particle-associated or that the *Legionella* spp. DNA detected on the 0.4-μm filter was truly free-living. However, sequential filtration has been employed extensively in the literature to separate free-living from particle-associated bacteria in environmental freshwater samples (Liu et al., 2019b; Yan et al., 2024) and drinking water samples (Mai et al., 2023), and method validations were performed as described in Text S2.

Citizen-science studies have grown in popularity with the number of published articles strongly increasing since 2010 (Kullenberg and Kasperowski, 2016). In the current study, water sampling at sites across the U.S. was made possible by citizen scientists. However, since we were not present for sample collection, it cannot be guaranteed that all the sample collection methods were followed exactly. This limitation was mitigated by providing citizen scientists with detailed written and video instructions and maintaining communication throughout the study. Further, physicochemical parameters from samples in the same DWDS were compared for data quality assurance, as were water sample temperatures before and after flushing (Text S4).

Several limitations exist with regard to the *Legionella* spp. 16S rRNA gene sequencing. One limitation is that several of the size-fractionated DNA extracts returned insufficient reads for analysis. This is common in samples with low DNA yields, which is often true for drinking water samples that are 1 L or less. Future studies should consider collection of larger volumes to increase total DNA and improve chances for successful sequencing of *Legionella* spp. Additionally, the assay amplified some non-target genera, including *Pseudomonas* spp. and *Sphingomonas* spp., which are commonly found in drinking water systems (Douterelo et al., 2018; Zhang et al., 2024). This non-targeted amplification might overwhelm the *Legionella* spp. signal in systems with significant populations of these genera. Therefore, a more specific *Legionella* spp. assay must be developed. Lastly, a number of ASVs could not be identified to the species level, limiting determination of clinical relevance. Potential reasons for this include that current sequence databases often lack sequences for environmental and non-clinically relevant species and strains and that there might be insufficient variation within the sequenced region to distinguish between species.

5. Conclusions

This is the first study to quantify particle-associated (e.g., associated with biofilm, FLA, or other particles) and free-living *Legionella* spp. and *Lp* in drinking water samples. In 75 % of samples, free-living *Legionella* spp. concentrations were higher than particle-associated *Legionella* spp. concentrations. Significantly greater concentrations of free-living *Legionella* spp. were found in samples sourced from surface water than from groundwater and in systems using combined chlorine than free chlorine as the secondary disinfectant. However, particle-associated *Legionella* spp. concentrations were greater in free chlorine than combined chorine systems, although this difference was not statistically significant. *Lp* was detected sporadically and mostly in the free-living form. Three clinically relevant *Legionella* spp. were detected*: Lp, L. anisa*, and *L. longbeachae*, and the detected species of *Legionella* were site dependent. Together, these results suggest that users at the tap are primarily exposed to the free-living form of *Legionella* spp. rather than the particle-associated form. Further, the study emphasizes the need to investigate the influence of source water type and site on *Legionella* spp. in DWDSs and building plumbing, with particular focus on differing treatment responses by free-living and particle-associated *Legionella* spp.

CRediT authorship contribution statement

Danielle M. Angert: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Katherine S. Dowdell:** Software, Formal analysis, Investigation, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Lan Nguyen:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Chaehyun Park:** Writing – review & editing, Visualization, Software, Formal analysis. **Jess Brown:** Writing – review & editing, Funding acquisition, Conceptualization. **Caroline Russell:** Writing – review & editing, Funding acquisition, Conceptualization. **Melina Bautista:** Writing – review & editing, Funding acquisition, Conceptualization. **Bridget Hegarty:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Project administration, Formal analysis, Data curation. **Eric D. Cambronne:** Writing – review & editing, Project administration, Methodology, Conceptualization. **Mary Jo Kirisits:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.124258.

Data availability

Data will be made available on request.

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