Environmental Science Water Research & Technology

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Cite this: *Environ. Sci.: Water Res. Technol.*, 2021, 7, 321

Enumeration and characterization of five pathogenic *Legionella* species from large research and educational buildings†

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Received 30th September 2020,
Accepted 13th December 2020

DOI: 10.1039/d0ew00893a

rsc.li/es-water

Legionella pneumophila is the species that is most often cultured from the natural environment, while disease-relevant *Legionella* species, such as *Legionella micdadei*, *Legionella bozemanii*, *Legionella anisa*, and *Legionella longbeachae* have yet to be extensively explored in premise plumbing systems. This study examined the concentrations of five pathogenic *Legionella* species (listed previously) in the influent and the taps of five different large buildings (BPS, ERC, F, FH, and M), undertaken during the start of two semesters (late summer/fall (August–September) and early winter/spring (January)). A total of 37 large-volume samples to examine building water quality (influents to the buildings and exposure sites (taps)) were collected and analyzed using droplet digital™ PCR. *Legionella* spp. (23S rRNA) were present in all water samples during both seasons. The majority (66%) of the exposure sites (bathroom taps) were positive for at least one target *Legionella* species (listed above). Results showed that pathogenic *Legionella* species were most often detected during the winter/spring sampling event – the percent positives for any one of the pathogenic *Legionella* species at the hot-water taps was 80% in building F and 40% in BPS, M, FH, and ERC. *Legionella pneumophila* and *L. longbeachae* were found in the highest concentrations (2.0 $\log_{10}$ gene copies (GC)/100 mL) at the hot-water taps in buildings F and ERC, respectively. No strong relationships were found with the physical–chemical parameters. Overall, general *Legionella* spp. concentrations increased in the winter/spring samples due possibly to lower water usage (lower occupancy and no use of cooling towers, which led to more water stagnation or time in the system).

Water impact

Legionnaires disease cases have been linked to non-*pneumophila Legionella* species. Pathogenic *Legionella* species were identified in 66% buildings' taps at concentrations that ranged from 1.4 to 2.0 $\log_{10}$ GC/100 mL compared to the influent which ranged from 1.5 to 1.8 $\log_{10}$ GC/100 mL (20%). Significant concentrations of pathogenic *Legionella* spp. are found in buildings and hot water taps with greater levels observed during lower water use.

Introduction

Legionella spp. are Gram-negative, opportunistic waterborne pathogens that reside in premise plumbing (*i.e.*, building) as well as other engineered water systems. *Legionella pneumophila* is the etiologic agent responsible for most Legionnaires disease (LD) with other species identified less frequently causing severe pneumonia and the less-studied Pontiac fever (an acute, but generally milder set of cold-like signs and symptoms).[1] *Legionella* naturally colonizes freshwater and groundwater environments, as well as engineered systems including cooling towers, air conditioners, hot tubs, taps, and showers.[2,3] *Legionella* infections are acquired *via* inhalation of aerosols and air droplets generated from these structures containing the bacteria.[4] The first recognized outbreak of LD, caused by *L. pneumophila*, occurred in 1976.[5] In the United States (US), LD prevalence has increased significantly since 2000, and in 2018 there were approximately 10 000 reported cases.[6] *Legionella* species are difficult to assess and control in the drinking water system because they survive in the biofilm on the surface of the pipes and within amoebae hosts.[7,8] The difficulty in assessing and controlling *Legionella* species makes these bacteria and their associated diseases a paramount public health concern.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0ew00893a

Many outbreaks of LD occur at the community level, as was the case in Flint, Michigan between 2014 and 2015 when Michigan saw a 375% increase in cases, most of which were part of the Flint outbreak.[9,10] During the 2014–2015 Flint outbreak, it was suggested that there were multiple sources of exposure, including the hospital water system, water at home (showers or taps), and residential proximity to cooling towers.[9] Although the LD outbreak during the water crisis in Flint, Michigan, was the largest in the state, there has been an increased number of cases statewide from 2000 to 2016.[10]

Legionella pneumophila, serogroup 1 is the most often diagnosed agent accounting for 90% of identified LD pneumonia cases, perhaps due to the restriction of the urinary antigen test.[11–15] In recent years, other *Legionella* species found in drinking water have also been identified in about 10% of cases.[16–23] *Legionella micdadei*, *L. bozemanii*, *L. longbeachae*, and *L. anisa* have been isolated from human patients.[24,25] There have been five drinking water outbreaks caused by *L. micdadei*,[26–33] one by *L. bozemanii*[34] and two by *L. anisa*[26] in the US. To date, there have not been any reports of *L. longbeachae* related infections associated with building water systems in the US, but there have been outbreaks in Australia, and cases reported in New Zealand, and some parts of Asia [Thailand].[35,36] In Australia, *Legionella* infections are commonly caused by *L. longbeachae*, and one of the exposure pathways was suggested to be potting mixes and compost.[37]

The majority of reported LD outbreaks have occurred in large complex plumbing systems, which are used in hospitals and healthcare facilities.[38] However, 97% of LD cases are sporadic infections,[39] for which the environmental source of exposure is usually unknown. The National Academies report on "Management of *Legionella* in Water Systems" (2019) stated that for every one outbreak case, there are nine more sporadic cases.[40] Despite a substantial amount of research on the molecular virulence mechanisms and ecology of *Legionella*, annual incidence rates of the disease continue to rise along with great uncertainty on how to control the colonization of water systems.

Currently, only a few studies have simultaneously characterized multiple pathogenic *Legionella* species (*L. anisa*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae*) in drinking water systems including the source, distribution system and tap[18,41–45] although most have not evaluated the concentrations and only focused on presence absence. *Legionella bozemanii*, *L. dumoffii*, *L. longbeachae*, *L. anisa*, *L. moravica*, *L. parisiensis*, *L. brunensis*, *L. londinensis*, and *L. hackeliae*, among many others, have been detected in water samples collected from hospitals in Italy,[44] warm water systems in Germany,[18] and in utility drinking water systems in the Netherlands.[41,42] In 2016, a research group in Germany, found *L. pneumophila*, *L. longbeachae*, *L. worsleiensis*, *L. anisa*, and *L. dumoffii*, (among many others) from source water to the cold- and hot-water taps in Germany using genus-specific PCR amplicons (16S rRNA) and single-strand conformation polymorphism fingerprint analyses.[45] *Legionella anisa* was detected in the Netherlands in three of four dental care units (75%) at a concentration of 1×10^2 CFU mL^{-1} using the Dutch *Legionella* standard culture technique, identified by whole-genome sequencing (MALDI-TOF).[43]

The goals of this study were to assess the concentrations of general *Legionella* spp., compared to pathogenic species *L. pneumophila*, *L. anisa*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae*, to understand the microbial quality of the drinking water entering five large research, classroom, and office buildings (all utilizing the same water source) compared to the water quality of the buildings. Utilizing droplet digital PCR, this study addressed the following objectives (i) quantification of *L. pneumophila*, *L. anisa*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae* in the influents and at the bathroom taps (points of use) of five large buildings using large volume composite sampling (ii) exploration of the associations of *Legionella* species with respect to temperature, chlorine, conductivity, pH, and heterotrophic plate count (HPC), and (iii) assessment of whether there were differences between two sampling periods of the year (August/September and January) for five pathogenic *Legionella* species. The quantitative data presented in this study should improve quantitative risk assessment of various specific pathogenic *Legionella* species within a drinking water system. This study gives an estimated concentration of *Legionella* species which may help improve exposure analysis.

Experimental

Site location and sampling

Water samples were collected during the beginning of two semesters (fall 2018 and spring 2019) from five buildings (F, BPS, M, FH, and ERC) on a large research institution of higher education. This was a large-scale spatial study; samples collected the beginning of the fall semester will be referred to the summer sampling and the beginning of the spring semester will be referred to as the winter sampling. Sample collection was conducted on August 13th and 27th, September 4th, 2018, and January 7th–9th, 14th, and 15th, 2019. This included research buildings F, ERC, and BPS, as well as buildings FH and M containing offices and classrooms. Building age, water use, and distance from the reservoir are shown in Table 1. The buildings are listed based on its pipe mileage from the effluent reservoir. Each building was assessed at an influent point with the sample collected at the most accessible sampling port on each building's influent pipe with the exception of ERC. The ERC influent sampling port was inaccessible; thus, it was decided to sample the nearest valve to the influent pipe, which was an eye-wash station in the mechanical room where the influent pipe entered the building. The building water quality was assessed by composite sampling point of use locations for each building included cold- and hot-water taps (sink faucets and showerheads) located in bathrooms, locker rooms, and breakrooms. All sinks described below were used for sample collection. Building F had two floors, with two sinks on the first floor and three sinks on the second floor; BPS had six

Table 1 Building and sampling site information (sample collection at the start of each semester, August 13th and 27th and September 4th, 2018 January 7th, 8th, 9th, 14th and 15th, 2019). The buildings are listed in order of their increasing of distance from the treated water source

Building[a] (construction year) and pipe material	Volume of water used geomean per month (Aug/Sept) [January] consumption (kGAL)	Distance from reservoir (km)	Building size (m^2)	Floors sampled (# of floors)	# of taps	Volume[b] of water collected from each tap (L) (summer) winter[c]
F (1948)	(94.9)	4.7	7118	First	4	(2.5) 5
75% galvanized 25% copper	[90.9]			Second	6	(1.67) 3.33
BPS (2001)	(4245)	6.8	35 045	First	40	(0.5) 0.25
50% galvanized 50% copper	[382]			Sixth	8	(2.5) 1.2
M (1940)	(11.6)	9.6	5926	First	8	(2.5) 1.25
90% galvanized 10% copper	[60.9]			Second	4	(5) 2.5
FH (1964)	(N/A)	10.2	36 057	First	34	(0.5) 0.29
50% galvanized 50% copper	[100]			Second	20	(1) 0.5
ERC (1986)	(200.8)	19.4	11 896	First	26[d]	(0.77) 0.38
50% galvanized 50% copper	[289.8]					

[a] 10 L composite sample were collected from all buildings; the volume of water from each tap was dependent upon the number of taps per floor per building. [b] For the summer event, hot and cold composite samples were collected as one 10 L sample from each building per floor. [c] Single faucet fixtures with two taps (1/2 were cold and the other 1/2 were hot water pipes). For the winter event, hot and cold composite samples were separated so that two 10 L samples were collected per floor. [d] The numbers of showerheads samples as part of the composite for ERC was two compared to 24 sink faucets.

floors, with 20 sinks on the first floor and four sinks on the sixth floor; M had two floors, with four sinks on the first floor and two sinks on the second floor; FH had two floors, with 17 sinks on the first floor and ten sinks on the second floor; ERC had one floor, with 11 sinks and two showers.

For influent samples, 10 L were collected from each building's influent sampling location. For tap samples, a large-volume (10 L) composite sample was collected to evaluate the water quality of each building's taps rather than the quality of individual taps. The first flush with equal total volumes from each tap was collected and composited into 10 L for the first floor and top floor, respectively. For the summer approach, a cold- and hot-water composite sample was collected to evaluate and compare the water quality on the first and top floors, separately. During the summer sampling, a total of three 10 L samples were collected from each building (influent, first floor taps, and top floor taps). For the winter approach, the cold- and hot-water taps were collected as separate samples to evaluate and compare the water quality of the cold-water taps and the hot-water taps on the first and top floor, respectively. For the winter sampling event, a total of five 10 L samples were collected per building, one influent, one cold- and one hot-water sample from the first and top floor, respectively. The one exception was building ERC, which only had one floor, where three 10 L samples were collected. The volume collected from each tap that was composited was determined by the number of taps on each floor. Table 1 shows the number of taps and the volume collected to construct the composite samples during the summer and winter. The goal was to assess the building water quality *via* composite sampling. Different sampling approaches for the two seasons were chosen initially to evaluate the water quality by floor (summer where cold and hot water were combined) after initial analysis, the decision was made to separate out the samples by hot and cold (winter). The difference between summer and winter at the tap was the volume of water collected (10 L for cold, 10 L for hot, and 10 L for combined); thus, this may have affected the detection limit as one more sample was collected from the buildings in the winter. All samples were collected in carboys (influent and tap samples) with 10% sodium thiosulfate to neutralize residual chlorine. Temperature and chlorine were recorded from each tap to examine the variation by tap and by floor.

Chemical–physical analysis

A 100 mL sample was collected for conductivity, pH, and turbidity analyses. Temperature and residual chlorine (total and free) were measured onsite. Chlorine was measured using the Test Kit Pocket Colorimeter II (HACH®, CO, USA) according to the manufacturer's instructions. Conductivity, pH, and turbidity were measured offsite at the laboratory according to the manufacturers' instructions using a Russell RL060C Portable Conductivity Meter (Thermo Scientific, MA, USA), UltraBasic pH meter (Denver Instrument, NY, USA), and a Turbidity Meter code 1970-EPA (LaMotte Company, MD, USA).

Microbiological analysis

All samples were transported on ice to the laboratory and preserved at 4 °C until processed. While the on-campus water utility tests for coliform bacteria on a routine basis, all samples collected for this study were tested according to the standard methods for coliform bacteria and *E. coli* using Colilert (IDEXX Laboratories, ME, USA) as well as with heterotrophic plate count (HPC) analyses using membrane filters (47 mm diameter, 0.45 μm pore size) (PALL Corporation, NY, USA) on m-HPC agar (Becton, Dickinson and Company, Difco™, MI, USA), incubated for 48 ± 2 h at

35–37 °C, then enumerated for colony-forming units (CFU).[46] Total coliforms were assayed for the summer and winter sampling events, while the HPC analyses were performed only for the winter.

Water sample processing and DNA extraction

The 10 L water samples were processed using a single-use Asahi REXEED-25S dialysis filter (Dial Medical Supply, PA, USA), which was pretreated with 0.01% of sodium hexametaphosphate (used to trap microbial material onto each ultrafilter) and utilized in a dead-end mode. A high-pressure single-use elution fluid canister (Innovaprep LLC, MO, USA) was used to concentrate the 10 L to ~50 mL.

Molecular analysis

Each ultrafiltration concentrate was split into 10 mL subsamples. One 10 mL subsample was further filtered through a 47 mm, 0.45 μm polycarbonate filter (Whatman, Kent, UK) for DNA extraction and analyzed by ddPCR. The remaining subsamples were stored at −80 °C.

DNA extraction and quantitative detection of *Legionella* by droplet digital PCR

DNA was extracted using QIAamp DNA Mini Kit (Qiagen, CA, USA). Each 10 mL subsample was filtered on a polycarbonate filter (described above) using a sterilized 0.47 mm magnetic filter funnel (PALL Corporation, NY, USA). Immediately afterward, the polycarbonate filter was folded into a 1/8 shape with contents of filter folded to the inside. The filter was then transferred to a 2.0 mL polypropylene screw cap tube (VWR, PA, USA) containing 0.3 g of 212–300 μm acid-washed glass beads (Sigma, MO, USA). DNA extraction was performed by adding 590 μL of AE buffer (Qiagen, CA, USA) to the samples then bead milling using a FastPrep-24™ 5G Instrument MP Biomedicals (VWR, PA, USA). Samples were milled at 6000 rpm for one minute, followed by centrifugation at 12 000 × *g* for one minute. The supernatant (~400 μL) was transferred to a new clean microcentrifuge tube and centrifuged at 12 000 × *g* for an additional three minutes to pellet any remaining debris. Extracted nucleic acid was eluted (~350 μL) into a final clean microcentrifuge tube. The eluted volume was then aliquoted (~60 μL) into several microcentrifuge tubes (~five extraction replicates per sample) for storage at −80 °C – to reduce the need for several freeze/thaw cycles. One aliquot per water sample was later used for PCR analysis (samples were held in −80 °C for up to 30 days before analysis).

Droplet digital PCR (Bio-Rad Laboratories, CA, USA) technology was performed according to the manufacturer's instructions to analyze each sample for general *Legionella* spp. (23S rRNA), *L. pneumophila*, *L. anisa*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae*. The primers and probes used in this study are listed in Table 2. Duplex reactions were performed for three separate assays: the first assay consisted of *Legionella* spp. and *L. pneumophila*, the second assay comprised of *L. micdadei*, and *L. anisa*, and the third assay consisted of *L. bozemanii*, and *L. longbeachae* (Table 2). All primers and probes were ordered from Eurofins (KY, USA).

For each reaction mixture, 2X supermix (no dUTP) (Bio-Rad Laboratories CA, USA) was mixed with a final concentration of 900 nM of forward and reverse primers, 250 nM for each probe (Eurofins Genomics Co., AL, USA), and DNA template (up to 330 ng) to a final volume of 22.0 μL (10% excess), as recommended by Bio-Rad. Exactly 20 μL of each of the samples' reaction mixtures were loaded into a DG8 cartridge (Bio-Rad Laboratories, CA, USA), followed by 70 μl of droplet generator oil (Bio-Rad Laboratories, CA, USA). The samples were then loaded into the QX200 Droplet Generator, and droplets were generated. The droplet emulsion (~40 μl) was then transferred into a 96-well plate using a multichannel pipet. The plate was then heat sealed

Table 2 Primers and probes for target *Legionella* species

Target species	Primer/probe name	Primer/probe sequence[a]	Accession number	Amplicon length (bp)	Ref.
Legionella species	23SF	5′-CCCATGAAGCCCGTTGAA-3′	Not available	92	80
	23SR	5′-ACAATCAGCCAATTAGTACGAG TTAGC-3′			
	23SP probe	5′-HEX[a]-TCCACACCTCGCCTATCAACGTCGTAGT-BHQ1[b]-3′			
L. pneumophila (*mip* gene)	mipF	5′-AAAGGCATGCAAGACGCTATG-3′	S42595	78	
	mipR	5′-GAAACTTGTTAAGAACGTCTTTCATTTG-3′			
	LmipP	5′-FAM[a]-TGGCGCTCAATTGGCTTTAACCGA-BHQ1[b]-3′			
L. micdadei	Pan-*Legionella* F	5′-GTACTAATTGGCTGATTGTCTTG-3′	Z30460	Not available	81
L. anisa	Pan-*Legionella* R	5′-TTCACTTCTGAGTTCGAGATGG-3′	Z30535		
L. bozemanii	LmicdadeiP	5′-FAM[a]-AGCTGATTGGTTAATAGCCCAATCGG-BHQ1[b]-3′	AY883058		
	LanisaP	5′-HEX[a]-CTCAACCTACGCAGAACTACTTGAGG-BHQ1[b]-3′			
L. longbeachae	Lbozemanii P	5′-FAM[a]-TACGCCCATTCATCATGCAAACCAGnT-BHQ1[b]-3′	Z30456		
	LlongbeachaeP	5′-HEX[a]-CTGAGTATCATGCCAATAATGCGCGC-BHQ1[b]-3′			

[a] Hexachlorofluorescein (HEX), and fluorescein amidites (FAM), reporter dyes that are added to the 5′ end of an oligonucleotide. [b] Black Hole Quencher (BHQ1), dark quenchers that does not absorb or emit light and are added to the 3′ end of an oligonucleotide. The temperature for each primer and probe set was 57.1 °C. The sample DNA did not need to be diluted before performing digital PCR as ddPCR is less sensitive to inhibitors. However, the positive control DNA was diluted to minimize the over-saturation of DNA to polymerase and this was identified by performing a dilution series with each positive control sample. The lowest dilution factor used for positive control DNA that yielded consistent quantification was 10^{-5}. The limit of detection for 1.3 log per 100 mL.

with pierceable foil heat seals using a PX1™ PCR Plate Sealer (Bio-Rad, Laboratories, CA USA). The sample reaction mixture was amplified using a Benchmark TC9639 thermal cycler (Benchmark Scientific Inc, NJ, USA) with the following thermocycling parameters: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 57 °C for 1 min, with a final 10 min cycle at 98 °C for 10 min. Droplets were then read using a QX200 droplet reader (Bio-Rad QX200™ Droplet Digital™ PCR System, CA, USA).

The strains of these five species were obtained from American Type Culture Collection (ATCC®) and are listed below in parenthesis. Two negative controls, a filtration blank (phosphate-buffered water) and a non-template control (molecular grade water) were run with each ddPCR plate. Positive controls using DNA from *L. pneumophila* (ATCC No. 33152), *L. micdadei* (ATCC No. 33218), *L. anisa* (ATCC No. 35292), *L. bozemanii*, (ATCC No. 33217) and *L. longbeachae* (ATCC No. 33462) for each assay target were run with each ddPCR plate. Sample results were only considered for analysis when the reader accepted 10 000 or more droplets as part of the quality control. Sample reactions with three or more positive droplets per well were identified as positive for their assay target. Three technical replicates were run for each sample to determine the reproducibility of the assay results. Further detailed ddPCR experimental information is in Table 1 Supplementary material.

Statistical analysis

Descriptive statistics were conducted in GraphPad Prism 8 software (GraphPad Software, CA, USA). Sample concentrations were transformed from gene copies (GC)/100 mL into $\log_{10}$ GC/100 mL for statistical analysis. A geometric mean for each sample was calculated using only the technical replicates that had ≥ three positive droplets. If one technical replicate was positive, only that value was used. The biological data were expressed as geometric means with standard deviations (SD). Correlation analysis was performed between the concentrations of *Legionella* species (23S rRNA) present in samples and water quality parameters tested (temperature, chlorine, turbidity, pH, and conductivity). One-way analysis of variance (ANOVA) was also performed to compare each variable (building influents, taps on the first and top floor (if any), cold- and hot-water taps, and among both sampling events). Statistical results were interpreted at the level of significance $p < 0.05$.

Results

Characterization and concentrations of *Legionella* 23S rRNA and five pathogenic *Legionella* species

Overall, a total of 37 large volume composite samples from five buildings were analyzed during this study: 14 from the beginning of the summer sampling event (fall term) and 23 from the beginning of the winter term. *Legionella* species (23S rRNA) were found in all water samples at concentrations ranging from 1.4 to 4.5 $\log_{10}$ GC/100 mL, and 54% of the samples were positive for at least one of the target species: *L. pneumophila* (2/37), *L. anisa* (5/37), *L. micdadei*, (1/37), *L. bozemanii*, (16/37), and *L. longbeachae* (11/37) at average geomean concentrations of 1.7, 1.6, 1.7, 1.6, and 1.6 $\log_{10}$ GC/100 mL, respectively (Fig. 1).

Five *Legionella* species detected in the influent and tap water samples in five different buildings

Two of the five influent samples were positive for at least one of the target pathogenic *Legionella* species in the summer sampling and no pathogenic species were detected in the influents to the buildings during the winter sampling event. *Legionella bozemanii* and *L. longbeachae* were detected in the influent of the BPS building at concentrations of 1.6 and 1.5 $\log_{10}$ GC/100 mL, respectively. *Legionella pneumophila*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae* were detected in the influent (eyewash site) of the ERC building at concentrations of 1.5, 1.7, 1.8, 1.5 $\log_{10}$ GC/100 mL, respectively (Fig. 2).

In the taps of the buildings, *L. pneumophila* was detected in 3.7% of the composite samples (1 of 27; January 7th, 2019). *Legionella anisa*, *L. bozemanii* and *L. longbeachae* were detected in 18.5, 51.8, and 33% respectively (during both the summer and winter sampling events). *Legionella micdadei* was not detected in the composite tap water samples (Fig. 2).

Potential amplification of general (23S rRNA) and five pathogenic *Legionella* species between the influent and the taps

Fig. 2 compares the influent concentrations to the taps for buildings F, BPS, M, FH, and ERC and demonstrates the potential for amplification of general (23S rRNA) and pathogenic *Legionella* species in the premise plumbing. In the summer sampling event, 80% of the buildings (BPS, M, FH, and ERC) had higher *Legionella* (23S rRNA) concentrations at the exposure sites (taps) compared to influent water samples (Fig. 2A). In the winter sampling

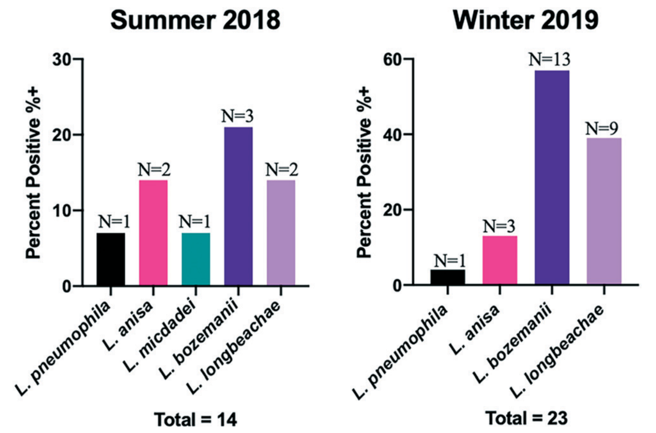

Fig. 1 *Legionella* species in all water samples collected during summer and winter sampling events. For the summer and winter, the *N* values are the number of samples in which the species were detected.

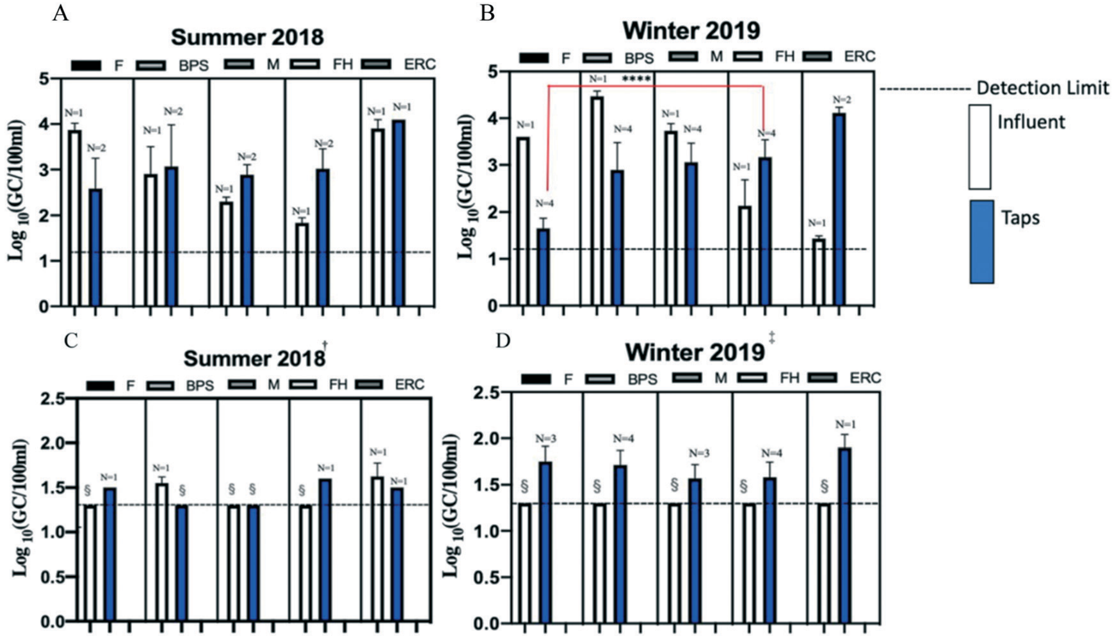

Fig. 2 *Legionella* spp. (23S rRNA) and pathogenic *Legionella* species concentrations at the influent and the tap during both sampling events. A) *Legionella* spp. 23S rRNA: summer, influent $N = 1$, taps $N = 2$. B) *Legionella* spp. 23S rRNA: winter, influent $N = 1$, taps $N = 4$. C) Average concentration of detected pathogenic *Legionella* species: summer, influent $N = 1$, taps $N = 1$. D) Average concentration of detected pathogenic *Legionella* species: winter, influent $N = 0$, taps $N = 1$, 3 and 4. The asterisks (****) below represent the significance for F tap *vs.* FH tap; $p = <0.0001$; one-way ANOVA. Average (if applicable) concentration of detected target species. Error bars are indicative of technical replicate and/or more than one target species detected per sample. [†]F and FH buildings, positive for *L. anisa* in the composite cold and hot-water tap; BPS building, positive for *L. bozemanii* and *L. longbeachae* in the influent water sample; M building, samples with no detection; ERC building, positive for *L. pneumophila*, *L. micdadei*, *L. bozemanii* and *L. longbeachae* in the influent water sample and positive for *L. bozemanii* in the composite cold and hot-water tap. [‡]Target *Legionella* species were below detection limit in the influent water sample. F building, positive for *L. anisa* and *L. bozemanii* in the cold water tap and positive for *L. pneumophila*, *L. anisa*, *L. bozemanii*, and *L. longbeachae* in the hot water tap; BPS and M buildings, positive for *L. bozemanii*, and *L. longbeachae* in both the cold and hot water taps; FH building, positive for *L. bozemanii*, and *L. longbeachae* in the cold water taps and positive for *L. bozemanii* and *L. anisa* in the hot water taps; ERC building, positive for *L. bozemanii*, and *L. longbeachae* in the hot water taps. [§]Dotted line is the detection limits with results without a standard deviation correspond to the non-detect samples.

event, 40% of the buildings (FH, and ERC) had higher *Legionella* (23S rRNA) concentrations observed at the taps compared to influent water samples (Fig. 2B). The concentration of general *Legionella* spp. (23S rRNA) in building FH was significantly higher than the concentration detected in building F (Fig. 2B). Two buildings (F and FH) showed suspected amplification for *L. anisa* in the summer samples (Fig. 2C). All buildings in the winter showed potential amplification, *L. pneumophila*, *L. anisa*, *L. bozemanii*, and *L. longbeachae* (Fig. 2D). Overall, there were higher *Legionella* concentrations (23S rRNA and target pathogenic species) seen at these exposure sites (taps) compared to influent samples (Fig. 2).

Comparison of five targeted *Legionella* species in summer and winter semesters

In the summer sampling event, all five specific *Legionella* spp. (*L. pneumophila*, *L. anisa*, *L. micdadei*, *L. bozemanii* and *L. longbeachae*) were detected. In the winter, four species were detected as *L. micdadei* was not found. Overall, *L. bozemanii* (43%, 16/37) and *L. longbeachae* (29.7%, 11/37) accounted for the majority of the *Legionella* positive samples detected in both sampling events. In the summer for the composite samples buildings F and FH only had one species detected (*L. anisa*) and building M had no detects. Building BPS was positive for both *L. bozemanii*, and *L. longbeachae* and ERC was positive for *L. pneumophila*, *L. bozemanii*, *L. longbeachae* and *L. micdadei*. In the winter, hot water taps were positive in F, BPS, M, FH, and ERC for *L. pneumophila* (detected once), *L. anisa*, *L. bozemanii*, and *L. longbeachae*. The cold-water taps were positive in F, BPS, M, and FH for *L. anisa*, *L. bozemanii*, and *L. longbeachae* species (see Fig. 1 in ESI†).

Fig. 1 compares the presence and absence of the pathogens in tap water samples from the summer and winter seasons. *Legionella pneumophila*, *L. anisa*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae* were present in low quantities (near the detection limit, $1.3 \log_{10}$ GC/100 mL) throughout the buildings drinking water system. The concentrations of these species ranged from 1.5 to $1.8 \log_{10}$ GC/100 mL in the summer samples, and from 1.4 to $2.0 \log_{10}$ GC/100 mL in the winter samples (Fig. 2). More specifically, during the summer, 7% (1/14) of the composite samples were positive for *L. pneumophila* and *L. micdadei*, 14% (2/14) for *L. anisa*, and *L. longbeachae*, and 21% (3/14) for *L. bozemanii* (Fig. 1). Collectively, five target *Legionella* species were detected in

36% (5/14) of the summer samples. During the winter, 65% (15/23) samples were positive with one or more of the target *Legionella* species. *Legionella bozemanii* had the highest occurrence at 57% (13/23), followed by *L. longbeachae* at 39% (9/23), *L. anisa* at 13% (3/23), and *L. pneumophila* at 4% (1/23) (Fig. 1). *Legionella micdadei* was not detected in any of the winter samples (Fig. 1).

Legionella species in cold compared to hot taps

Fig. 3 compares the $\log_{10}$ gene copies of the most prevalent species, *L. bozemanii* and *L. longbeachae* in the cold- and hot-water taps. *Legionella bozemanii* concentrations were higher in the hot-water samples (geomean of 1.7) than in the cold-water samples (geomean of 1.6) in BPS, M, FH, and ERC. *Legionella longbeachae* concentrations were also higher in the hot-water samples (geomean of 1.8) compared to the cold-water samples (geomean of 1.6) in F, BPS, and ERC buildings. Concentrations of *L. bozemanii* and *L. longbeachae* in the cold-water taps were significantly different (unpaired *t*-test, $p = 0.03$) than the hot-water taps. Overall, the five target *Legionella* species were more prevalent in hot-water samples (39% positive, 9/23) compared to the cold-water samples (26% positive, 6/23). Within the hot-water samples, there appeared to be more diversity of the target *Legionella* species (*L. pneumophila*, *L. anisa*, *L. bozemanii*, or *L. longbeachae*) present, compared to the cold-water tap samples, where *L. pneumophila* was not detected (Fig. 3).

Chemical–physical water quality

For the summer sampling event, the cold- and hot-water taps were composite samples; thus, the chemical–physical parameters are reflective of this as the interest was determining the difference of water quality by floors. In August and September, water temperatures in the influent of all buildings ranged from 12.6 to 20.2 °C, with an average of 16.5 °C (Table 3). The temperatures of the composite cold- and hot-water samples were similar in range among both floors but slightly different across buildings. The water temperature across the buildings ranged from 25.8 to 34.2 °C and 27.1 to 36.7 °C on the first floor and top floor, respectively. Free chlorine ranged from 0.04 mg L^{-1} to 0.52 mg L^{-1}, with an average of 0.3 mg L^{-1} in the influents. The buildings' average free chlorine on the first floor was 0.09 mg L^{-1} and increased on the top floor to 0.21 mg/ (Table 3). This may be due to a higher occupancy on the top floor and greater use of the bathrooms due to research labs and office space being active at the start of the semester, compared to the first floor with more classrooms which were not in use at the time of sampling. The conductivity ranged from 750 to 867 μS cm^{-1}, with an average of 802 μS cm^{-1} in the buildings' influent. The buildings' average conductivity was 915.8 μS cm^{-1} on the first floor and then decreased on the top floor (827.8 μS cm^{-1}). Turbidity ranged from 1.3 to 66.2 NTU (this groundwater sources being notorious for high iron content with many "red water" alerts), with an average of 19.5 NTU in the buildings' influent. The buildings' average turbidity dropped to 3.2 NTU on the first floor and slightly increased on the top floor (5.9 NTU). The mean pH was 7.4 in the influents, first floors, and the top floors.

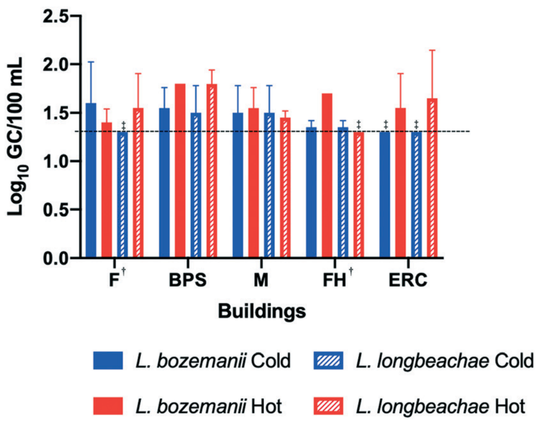

Fig. 3 Presence of *Legionella bozemanii* and *L. longbeachae* in cold- and hot-water composite samples from taps in the five buildings. Bars reflect all measurements collected at each tap. Dashed line represents the detection limit (1.3 $\log_{10}$ GC/100 mL). Samples with no signal are reported as the detection limit. †F building, positive for *L. pneumophila* in the hot tap; F building, positive for *L. anisa* in the cold- and hot-water taps. FH building, positive for *L. anisa* in the hot tap. *L. micdadei* was not detected in the winter sampling event. ‡Dotted line is the detection limits with results without a standard deviation correspond to the non-detect samples.

For the winter sampling event, the cold- and hot-water taps were collected as separate composite samples; thus, the chemical–physical parameters are reflective of this. In the winter, building influent water temperature ranged from 11.2 to 26.9 °C, with an average of 17.9 °C (Table 4). The average (21.4 °C for the first floor and 22.4 °C on the top floor) cold-water temperature for the buildings did not differ between floors; however, the buildings' hot-water was slightly warmer, on average, on the top floor (36.1 °C) compared to the first floor (31.6 °C). Free chlorine ranged from 0.17 to 1.46 mg L^{-1} (influent of FH to influent of BPS) with an average of 0.6 mg L^{-1}. The buildings' mean for free chlorine (first and top floors) differed between the cold- (0.07 mg L^{-1}) and hot-water taps (0.04 mg L^{-1}) (Table 4). The buildings' average conductivity in winter ranged from 794 to 931 μS cm^{-1} in the influent with an average of 847 μS cm^{-1}. The conductivity of the cold- (947 μS cm^{-1}) and hot-water taps (931 μS cm^{-1}) on the first floor of the buildings were only slightly different. The conductivity on the top floors varied more between the cold- (890 μS cm^{-1}) and hot-water taps (918 μS cm^{-1}). Turbidity ranged from 4.6 to 155 NTU (influent of FH Hall to influent of BPS) with an average of 58.3 NTU (may be due to high iron content). The mean turbidity for the cold-water taps was 7.6, and 2.4 for the hot-water taps on the buildings' first floors. However, the mean turbidity slightly increased on the top floor for both taps, 20.6 NTU (cold-water tap), and 2.81 NTU (hot-water tap). The pH was approximately the same as

Table 3 Chemical–physical and microbial data for influents and composite cold and hot-water samples August 13th, 27th and September 4th, 2018

Composite cold and hot-water sample						
	F	BPS	M	FH	ERC[a]	Building average
Influent						
Temperature (°C)	15.7	12.6	18.3	15.9	20.2	16.5
Conductivity (μS)	750	867	755	780	858	802
Turbidity (NTU)	24.5	3.58	1.3	1.89	66.2	19.5
pH	7.3	7.5	7.5	7.5	7.6	7.48
Total (free) chlorine residual (mg L^{-1})	0.58 (0.52)	0.52 (0.32)	0.39 (0.3)	0.53 (0.43)	0.04 (0.04)	0.4 (0.3)
Coliforms (MPN/100 mL)	<1	<1	<1	<1	<1	<1
E. coli (MPN/100 ml)	<1	<1	<1	<1	<1	<1
1st floor composite samples[b]						
Temperature[b] (°C)	33.4	29.2	34.2	31.9	25.8	30.9
Conductivity (μS)	814	857	807	1256	845	915.8
Turbidity (NTU)	0.55	0.98	6.36	3.44	4.43	3.2
pH	7.3	7.4	7.5	7.2	7.6	7.4
Total (free) chlorine residual[b] (mg L^{-1})	0.16 (0.13)	0.16 (0.02)	0.12 (0.03)	0.24 (0.17)	0.17 (0.1)	0.17 (0.09)
Coliforms (MPN/100 mL)	<1	<1	<1	<1	<1	<1
E. coli (MPN/100 mL)	<1	<1	<1	<1	<1	<1
Top floor[b]						
Temperature[b] (°C)	32	27.1	36.7	35	N/A	32.7
Conductivity (μS)	794	904	794	819	N/A	827.8
Turbidity (NTU)	2.06	5.07	13.1	3.46	N/A	5.9
pH	7.3	7.5	7.5	7.4	N/A	7.4
Total (free) chlorine residual[b] (mg L^{-1})	0.29 (0.22)	0.33 (0.21)	0.1 (0.05)	0.1 (0.04)	N/A	0.2 (0.21)
Coliforms (MPN/100 mL)	<1	<1	<1	<1	N/A	<1
E. coli (MPN/100 mL)	<1	<1	<1	<1	N/A	<1

[a] ERC, has one floor. [b] Composite cold and hot taps.

the summer sampling, ranging from 7.3 (influent) to 7.6 (first-floor hot-water tap) and 7.5 (top-floor hot-water taps).

Relationship between the presence of *Legionella* and water quality parameters

During the summer sampling event, there was not a relationship (positive or negative) between temperature, HPCs, chlorine, turbidity, pH, or conductivity with respect to general *Legionella* spp. (23S rRNA). Fig. 4 shows the correlation ($R = 0.5$ to 0.6) between *Legionella* spp. 23S rRNA and three water quality parameters (free residual chlorine concentration, conductivity, and turbidity) in the hot water taps during the winter semester events. While not statistically significant the positive trend between turbidity, pH, and HPCs, was driven more by the characteristics of the building with F being low and ERC being high with the other buildings in between.

Chlorine residuals were very low in the building taps and no chlorine residual was detected when *L. pneumophila* and *L. anisa* were both detected in the hot-water taps on the first floor of building F. For the *L. anisa* positive sample (in the hot-water tap on the second floor on building FH), the residual chlorine was 0.04 mg L^{-1}. *Legionella bozemanii*, and *L. longbeachae* occurred in hot-water taps on both floors (except ERC) of all five buildings; in the positive samples, the free residual chlorine ranged from 0 to 0.32 mg L^{-1}. Free residual chlorine concentrations were below the US Centers for Disease Control and Prevention (US CDC) (under the safe water system) minimal 0.2 mg L^{-1} threshold[47] in all *Legionella*-positive samples, except the BPS influent sample, which was 0.32 mg L^{-1}. This sampling port (BPS influent) is located at the point water that enters into the building where turbidity levels were 3.58 NTU and water temperature was 12.6 °C. There was no significant correlation between pathogenic *Legionella* species and any water quality parameter (water temperature, residual chlorine, turbidity, pH, HPCs, or conductivity) tested; thus, this suggests the need for further investigation with larger data sets.

Discussion

This study revealed new quantitative information about the distribution of general *Legionella* and five pathogenic species in a complex of five buildings on the same community drinking water system. *Legionella* spp. (23S rRNA), *L. pneumophila*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae* were found in the influent water pipes and four target species (excluding *L. micdadei*) were found at distal points of use. *Legionella* and pathogenic species are part of the water microbiome as reported previously.[48,49] However, these studies did not provide concentrations on the pathogenic species other than *L. pneumophila* and the hospital systems are often the primary focus. In Schwake *et al.* (2016)[49] 20 single-story buildings were examined, and no pathogenic *Legionella* spp. were detected. This work presented herein examined large volume samples of the influent to buildings compared to composite large volume tap water samples and

Table 4 Chemical–physical and microbial data for influents and composite cold and hot-water tap samples, January 7th, 8th, 9th 14th and 15th, 2019

Composite cold-water Composite hot-water	F	BPS	M	FH	ERC[a]	Building average
Influent						
Temperature °C	14.5	23.6	13.3	11.2	26.9	17.9
Conductivity μS	914	794	931	799	797	847
Turbidity NTU	18.6	155	6.33	4.6	106.9	58.3
pH	7.3	7.5	7.3	7.3	7.3	7.3
Total (free) chlorine residual mg L^{-1}	0.24(0.23)	1.16(1.46)	0.09(0.18)	0.05(0.17)	1.19(1.33)	0.5(0.6)
Coliforms MPN/100 ml	<1	<1	<1	<1	<1	<1
E. coli MPN/100 ml	<1	<1	<1	<1	<1	<1
HPCs (CFU/100 mL)	8.8×10^1	7.40×10^3	2.70×10^2	3.00×10^1	4.00×10^5	8.16×10^4
1st floor from composite samples						
Temperature °C (cold and hot taps)	17.8	22.8	23.3	21.8	21.1	21.4
	37.6	31.3	36.3	28.2	24.7	31.6
Conductivity μS (cold and hot taps)	793	1161	913	865	924	931
	801	1220	904	895	914	947
Turbidity NTU (cold and hot taps)	2.4	6.44	16.4	4.22	8.55	7.6
	0.1	1.44	2.39	4.36	3.78	2.4
pH (cold and hot taps)	7.4	7.3	7.6	7.6	7.7	7.5
	7.7	7.4	7.7	7.8	7.6	7.6
Total (free) chlorine residual mg L^{-1} (cold and hot taps)	0.04(0.03)	0.14(0.1)	0.14(0.13)	0.05(0.16)	0.07(0.09)	0.09(0.1)
	0.03(0)	0.03(0.05)	0.03(0.02)	0.08(0.12)	0.09(0.11)	0.05(0.06)
Coliforms MPN/100 ml	<1	<1	<1	<1	<1	<1
E. coli MPN/100 ml	<1	<1	<1	<1	<1	<1
HPCs (CFU/100 mL)	2.6×10^1	1.02×10^5	3.53×10^3	1.37×10^3	8.1×10^3	2.3×10^4
	1.31×10^3	3.0×10^4	6.8×10^2	2.8×10^3	9.20×10^4	2.54×10^4
Top floor						
Temperature (°C) (cold and hot taps)	19.9	22	24.2	23.3	N/A	22.4
	38.9	31.1	44.9	29.6		36.1
Conductivity μS (cold and hot taps)	786	977	882	916	N/A	890
	802	1062	895	914		918
Turbidity NTU (cold and hot taps)	4.04	3.73	71	3.64	N/A	20.6
	0.32	1.62	7.14	2.16		2.81
pH (cold and hot taps)	7.5	7.3	7.4	7.5	N/A	7.4
	7.6	7.2	7.8	7.6		7.5
Total (free) chlorine residual mg L^{-1} (cold and hot taps)	0.05(0.07)	0.05(0.05)	0.4(0.4)	0.03(0.05)	N/A	0.13(0.14)
	0.07(0.03)	0.02(0.01)	0.09(0.09)	0.02(0.04)		0.05(0.04)
Coliforms MPN/100 ml	<1	<1	<1	<1	N/A	<1
E. coli MPN/100 ml	<1	<1	<1	<1	N/A	<1
HPCs (CFU/100 mL)	7.00×10^1	1.14×10^4	5.3×10^2	2.4×10^3	N/A	3.6×10^3
	1.31×10^3	1.56×10^3	1.54×10^2	1.04×10^3		9.72×10^2

[a] ERC, only has one floor.

suggests amplification occurs of pathogenic species of *Legionella* in the system which is observable at the taps (Fig. 2). In addition, it was found that possible amplification can occur immediately at locations where the water enters the building as shown by the eye wash station at the ERC building. The reasons for this possible amplification are not completely clear however residence time, stagnation, water age could all be involved as the ERC building is furthest away from the water source (discussed below). However, all five buildings showed evidence of colonization with pathogenic *Legionella* species at the taps in the winter sampling event.

The concentrations of the specific *Legionella* pathogenic species ranged 1.4 to 2.0 $\log_{10}$ GC/100 mL (average: 1.6 $\log_{10}$ GC/100 mL) at the exposure taps. The maximum contaminant level goal (MCLG) for *Legionella* is zero as established in the 1989 Surface Water Treatment Rule; however, this target is not federally regulated and thus no maximum contaminant level has been established.[50] Moreover, the CDC provides a toolkit, which is a step-by-step guidance from ASHRAE Standard 188; this helps building owners evaluate the water system in their buildings to determine if a water management program is required[51] however no numerical level for PCR exists at which action should be taken to remediate the building's water. Previous risk assessments suggest that the level which equates to the 10^{-4} annual infection risk target for drinking water safety (analysis of acceptable risk levels used a 1 in 10 000/person-year as a target) is around 10^3 CFU L^{-1} (10^2 CFU/100 mL) for faucets.[52] While the referenced acceptable risk level (described previously) was evaluated using CFU (culture method) and early studies suggested a ratio of around 7 CFU to 3 GC (PCR method) this assumed that all cells were culturable.[53] There

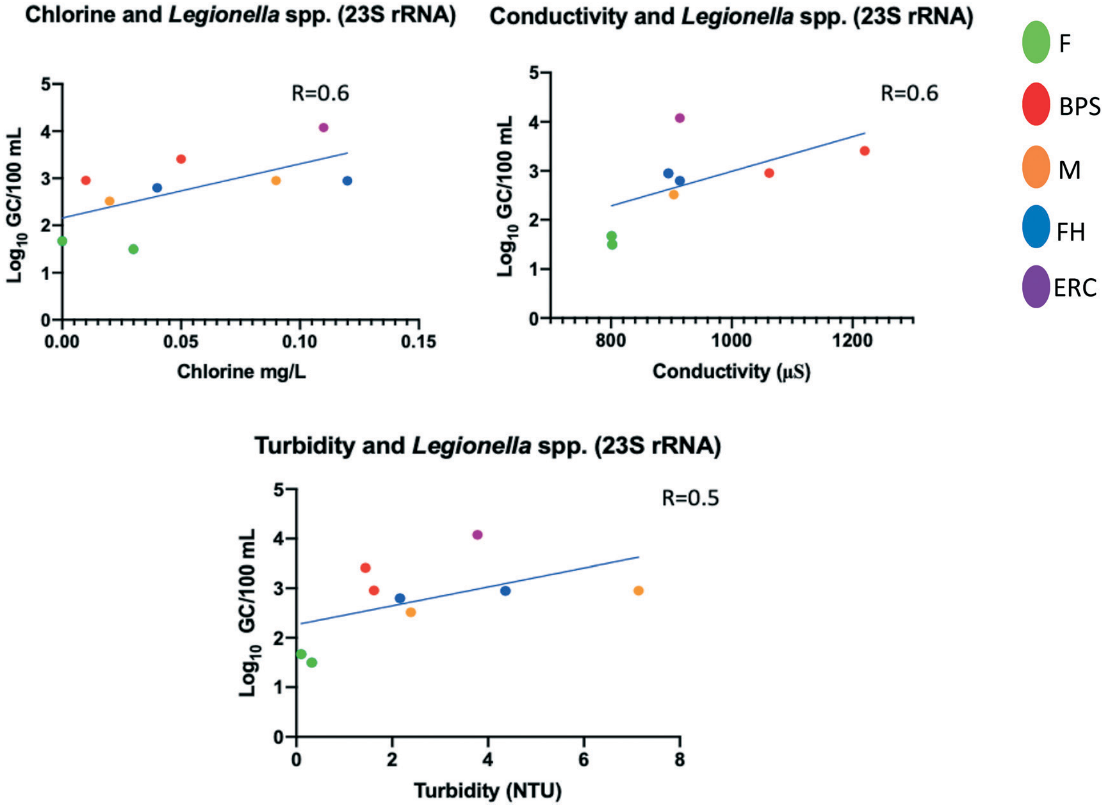

Fig. 4 Correlation between three water quality parameters (chlorine, conductivity, and turbidity) and *Legionella* spp. 23S rRNA in the hot water taps during January 7th, 8th, 9th, 14th and 15th, 2019 sampling event. The color coding for each building is as follows: green: F; red: BPS; orange: M; blue: FH; purple: ERC.

is a need for a greater comparison examining live and dead as well as viable- but- non-culturable cells to ultimately address risk *via* molecular tools. These concentrations presented herein at the taps are nearing the level of the acceptable annual risk – assuming that these species are 100% cultivatable and have similar dose-response characteristics (for example, the expression of virulence factors) as *L. pneumophila*.

Both hot- and cold-water taps can be colonized, and *Legionella* species can survive in cold-water taps,[3] but hot-water taps are known to be a major source for their amplification.[54] Many studies report prevalence but not concentrations. *Legionella pneumophila* was detected in 82% of samples from a hot-water system at a university hospital located in Sherbrooke, Canada, by culture.[55] In 2019, Bédard and colleagues found *L. pneumophila* SG1 positive in 41%, and *L. pneumophila* serogroups 4 and 10 in 91% of the water samples in hot-water taps and connecting pipes in an undisclosed Canadian hospital by culture followed by sequence-based typing.[56]

This study detected the presence of specific *Legionella* species in both cold-water and hot-water taps with slightly higher concentrations seen in hot-water; this is in agreement with previous studies[3,57–61] but these were not statistically significant. Recently, Donohue and colleagues found a difference in concentrations for *L. pneumophila* in hot-water taps compared to cold-water taps collected from 46 states across the US served by public water utilities.[62] The concentrations were higher (median concentrations: 2.6 $\log_{10}$ GC/100 mL) for *L. pneumophila* in the hot-water taps than in the cold-water taps (median concentrations: 1.5 $\log_{10}$ GC/100 mL).[62] In this study, *L. pneumophila* in the hot-water tap at a concentration of 2.0 $\log_{10}$ GC/100 mL was similar to these studies. These results suggest that when examining the water quality of the building, hot water taps can be composited particularly from floors that are in less use and this will provide information on *Legionella* colonization.

Increased water age in the distribution system has been suggested to have an adverse downstream effect within the building water system.[63] The impacts of increased water age are increased water temperature and a loss of chemical residual in the building water system.[64] These changes in combination influenced the occurrence (presence/absence) of *Legionella* species.[57,64] In addition, several studies have shown that the concentrations of general *Legionella* spp. (23S rRNA) and *L. pneumophila* increased during the summer season[65–67] which was presumed to be due to water temperature.[68–72] However, in this study, *L. pneumophila*, *L. anisa*, and *L. longbeachae* increased in concentration during the winter sampling event. All three species (listed above) increased in the hot-water taps in buildings F, FH, and ERC. Hot-water taps are a source for *Legionella* amplification;[58]

thus, there is likely a need for a building monitoring approach that includes sampling the hot water taps separately from the cold-water taps.

Our study examined these buildings at the beginning of two semesters (summer/fall Aug/Sept compared to winter/January). Each building except M had flow meters and water usage could be evaluated. The data on water use showed key buildings in the complex use less water in January compared to August due to cooling towers (BPS and ERC) which are used in the summer. Prior to classes beginning the bottom floor bathrooms of some of the buildings which housed the classrooms and not the research laboratories showed less use. Seasonal effects in this study on water temperature were not observed. The water temperature averages on the first and top floors for all the buildings were close to the Legionella's optimal growth temperatures (25 to 45 °C).[71] Turbidity values were high in BPS, M, and ERC building samples. There was a moderate correlation between turbidity and *Legionella* colonization in hot water tap samples—this relationship may be a direct result of iron.[73] The water quality on the various floors looked only slightly different with respect to higher chlorine residuals on the top floors perhaps due to greater water usage as the research laboratories and offices were consistently occupied and water was used and replaced at the taps. However, buildings were different in their physical chemical quality. Similar to previous studies, higher chlorine residuals observed in the BPS building (0.32 mg L^{-1}) showed little to no effect on disinfecting *Legionella* in the influent sample.[74] Interestingly, the influent of the ERC building had the greatest variety of *Legionella* species (*L. pneumophila*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae*) and the detection of these species appears to be related to the lower levels of residual chlorine (0.04 mg L^{-1}) at this site. This could be due to the ERC building having increased water age as its influent water pipe is the furthest away from the water source (reservoir) at 19.4 km. These data suggest that the influent water is seeding the system and water quality of the building including microbial aspects (*Legionella*) was affected more by water stagnation (low water use) and that understanding water age as it plays a role in the occurrence of *Legionella* species warrants further exploration as monitoring moves forward.

Health departments should consider the role of other *Legionella* species (*L. anisa*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae*) in the presentation of pneumonia as they may pose a equal to or greater risk than *L. pneumophila* as they are widely distributed in the environment.[18,23,41–45,75,76] The detection method used for routine sampling of water in hospitals and cooling towers is the culture-dependent method, however, there are some limitations to this practice: the inability to rapidly and precisely identify specific *Legionella* species and the presence of viable-but- non-culturable species, however PCR methods are incapable of distinguishing live and dead cells.[77,78] Yet quantitative assessment using PCR can provide important information on concentrations and the approach used in this study to assay composites means that the "building" water quality can be examined and not just individual taps. Digital droplet PCR was very useful and could be run with duplex assays for the various species in this study, overcoming time delay from sampling to quantitative results as reported by others[79] compared to the "gold standard" culture method for *Legionella* detection and LD diagnosis.[77] These types of data provide information for building operations that could improve water quality such as more flushing and increasing flows through the system.

Conclusions

Legionella are a part of the water microbiome and were found 100% of the time. Yet pathogenic species beyond *L. pneumophila*, including *L. anisa*, *L. micdadei*, *L. bozemanii*, and *L. longbeachae* are also important in describing "building" water quality in a community drinking water system in the US. Our results provide evidence that pathogenic species in addition to *L. pneumophila* are increasing and potentially amplifying when comparing presence and concentrations between the influent and the points of use (taps) in various large educational buildings. More *Legionella* species were found under conditions where water stagnation (water age) or longer retention times in the pipes (low water use) were observed. By monitoring pathogenic species *L. pneumophila*, *L. anisa*, *L. bozemanii*, *L. longbeachae* and *L. micdadei* changes to building management can be made to address potentially different risks at different times of the year. This supports the need for a water management plan for various building types to reach optimization which includes monitoring.

The examination of large volume (10 L) water samples using ultrafiltration increased the detection limits for specific *Legionella* species. A monitoring scheme that includes composite, large-volume sampling, and rapid assessment by ddPCR could lead to better control of *Legionella* in building drinking water systems.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the U.S. EPA Grant No. (FAIN): 83689001 and U.S. EPA agreement number R836890. The contents of this publication are solely the responsibility of the authors and do not represent the official views of the EPA. AL-J thanks MF, Parker Kelly, and Rebecca Ives for their assistance on sampling. AL-J thanks the Infrastructure Plant Facility Staff at MSU for their assistance sampling the buildings.

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