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

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*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 pneumophilia* and *L. longbeachae* were found in the highest concentrations (2.0 log10 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-*pneumophilia* *Legionella* species. Pathogenic *Legionella* species were identified in 66% buildings’ taps at concentrations that ranged from 1.4 to 2.0 log10 GC/100 mL compared to the influent which ranged from 1.5 to 1.8 log10 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 pneumophilia* 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. pneumophilia*, 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 amoeba hosts.7,8 The difficulty in assessing and controlling *Legionella* species makes these bacteria and their associated diseases a paramount public health concern.
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 miedaedi, 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. miedaedi,26–31 one by L. bozemanii32 and two by L. anisa26 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].33,35 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 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. miedaedi, L. bozemanii, and L. longbeachae) in drinking water systems including the source, distribution system and tap41,44–45 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. miedaedi, 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. miedaedi, 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
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 each floor. Table 1 shows the number of taps and the volumes from each tap was collected and composited into 10 L for the first floor and top floor, respectively. For the winter event, hot and cold composite samples were separated so that two 10 L samples were collected per floor. The numbers of showerheads samples as part of the composite for ERC was two compared to 24 sink faucets.

### 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) and a Turbidity Meter code 1970-EPA (LaMotte Company, MD, USA).
35–37 °C, then enumerated for colony-forming units (CFU). 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-255 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 polyprene 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 (~100 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**

<table>
<thead>
<tr>
<th>Target species</th>
<th>Primer/probe name</th>
<th>Primer/probe sequence</th>
<th>Accession number</th>
<th>Amplicon length (bp)</th>
<th>Ref.</th>
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<td>Legionella species</td>
<td>23SF</td>
<td>5′-CCCCATGAAGCCCGTGTGA-3′</td>
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<td></td>
<td>23SR</td>
<td>5′-ACAATCGCAGATATGGTGATG-3′</td>
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<td></td>
<td>23SP probe</td>
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<td>mipF</td>
<td>5′-AAAGCGATCGAAGCGCTATG-3′</td>
<td>S42595</td>
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<td></td>
<td>mipR</td>
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<td></td>
<td>LmipP</td>
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<td>L. pneumophila (mip gene)</td>
<td>LmipP</td>
<td>5′-GAAACCTTGAAGACCTTGCTTCACTGTT-3′</td>
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<td></td>
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<tr>
<td>L. micdadei</td>
<td>Pan-Legionella F</td>
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<td>Not available</td>
<td>81</td>
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<td>L. anisa</td>
<td>Pan-Legionella R</td>
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<td>L. bozemanii</td>
<td>LmicideaeP</td>
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<td>L. longbeachae</td>
<td>Lbozemanii P</td>
<td>5′-FAM-TAACCGCCTATCAGTTGCAAACGGT-3′</td>
<td>Z30456</td>
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<td></td>
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</tbody>
</table>

* Hexachlorofluorescein (HEX), and fluorescein amidites (FAM), reporter dyes that are added to the 5′ end of an oligonucleotide. 
  
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with pierce 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 \( \geq \) 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 geometric 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
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
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Legionella species in cold compared to hot taps

Legionella species in cold compared to hot taps. 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).

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

Presence of Legionella 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, 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/L (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.

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...
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 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. 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) 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...
HPCs (CFU/100 mL) 2.6 × 10^3 species ranged 1.4 to 2.0 log10 GC/100 mL (average: 1.6 log10 GC/100 mL) at the 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 target) is around 10^3 CFU L\(^{-1}\) to help 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

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 log10 GC/100 mL (average: 1.6 log10 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

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**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

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>BPS</th>
<th>M</th>
<th>FH</th>
<th>ERC(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composite cold-water</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Influent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature °C</td>
<td>14.5</td>
<td>23.6</td>
<td>13.3</td>
<td>11.2</td>
<td>26.9</td>
</tr>
<tr>
<td>Conductivity μS</td>
<td>914</td>
<td>794</td>
<td>931</td>
<td>799</td>
<td>797</td>
</tr>
<tr>
<td>Turbidity NTU</td>
<td>18.6</td>
<td>155</td>
<td>6.33</td>
<td>4.6</td>
<td>106.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
<td>7.5</td>
<td>7.3</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>Total (free) chlorine residual mg L(^{-1})</td>
<td>0.24(0.23)</td>
<td>1.16(1.46)</td>
<td>0.09(0.18)</td>
<td>0.05(0.17)</td>
<td>1.19(1.33)</td>
</tr>
<tr>
<td>Coliforms MPN/100 ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. coli MPN/100 ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>HPCs (CFU/100 mL)</td>
<td>8.8 × 10^3</td>
<td>7.40 × 10^3</td>
<td>2.70 × 10^2</td>
<td>3.00 × 10^3</td>
<td>4.00 × 10^5</td>
</tr>
<tr>
<td>1st floor from composite samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature °C (cold and hot taps)</td>
<td>17.8</td>
<td>22.8</td>
<td>23.3</td>
<td>21.8</td>
<td>21.1</td>
</tr>
<tr>
<td>Conductivity μS (cold and hot taps)</td>
<td>37.6</td>
<td>31.3</td>
<td>36.3</td>
<td>28.2</td>
<td>24.7</td>
</tr>
<tr>
<td>Turbidity NTU (cold and hot taps)</td>
<td>793</td>
<td>1161</td>
<td>913</td>
<td>865</td>
<td>924</td>
</tr>
<tr>
<td>pH (cold and hot taps)</td>
<td>7.4</td>
<td>7.4</td>
<td>7.6</td>
<td>7.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Total (free) chlorine residual mg L(^{-1}) (cold and hot taps)</td>
<td>0.04(0.03)</td>
<td>0.14(0.1)</td>
<td>0.14(0.13)</td>
<td>0.05(0.16)</td>
<td>0.07(0.09)</td>
</tr>
<tr>
<td>Coliforms MPN/100 ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. coli MPN/100 ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>HPCs (CFU/100 mL)</td>
<td>2.6 × 10^3</td>
<td>1.02 × 10^3</td>
<td>3.53 × 10^3</td>
<td>1.37 × 10^3</td>
<td>8.1 × 10^3</td>
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<tr>
<td>Top floor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature °C (cold and hot taps)</td>
<td>19.9</td>
<td>22</td>
<td>24.2</td>
<td>23.3</td>
<td>N/A</td>
</tr>
<tr>
<td>Conductivity μS (cold and hot taps)</td>
<td>38.9</td>
<td>31.1</td>
<td>44.9</td>
<td>29.6</td>
<td>36.1</td>
</tr>
<tr>
<td>Turbidity NTU (cold and hot taps)</td>
<td>786</td>
<td>977</td>
<td>882</td>
<td>916</td>
<td>N/A</td>
</tr>
<tr>
<td>pH (cold and hot taps)</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.5</td>
</tr>
<tr>
<td>Total (free) chlorine residual mg L(^{-1}) (cold and hot taps)</td>
<td>0.05(0.07)</td>
<td>0.05(0.05)</td>
<td>0.4(0.4)</td>
<td>0.03(0.05)</td>
<td>N/A</td>
</tr>
<tr>
<td>Coliforms MPN/100 ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>N/A</td>
</tr>
<tr>
<td>E. coli MPN/100 ml</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>N/A</td>
</tr>
<tr>
<td>HPCs (CFU/100 mL)</td>
<td>7.00 × 10^4</td>
<td>1.14 × 10^4</td>
<td>5.3 × 10^2</td>
<td>2.4 × 10^3</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\(^{a}\) ERC, only has one floor.
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, but hot-water taps are known to be a major source for their amplification. 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. 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. 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, 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. 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). 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. The impacts of increased water age are increased water temperature and a loss of chemical residual in the building water system. These changes in combination influenced the occurrence (presence/absence) of _Legionella_ species. In addition, several studies have shown that the concentrations of general _Legionella_ spp. (23S rRNA) and _L. pneumophila_ increased during the summer season, which was presumed to be due to water temperature. 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.
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.18,23,41–47,55,76 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-cultureable 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 others79 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

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