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Drinking water microbiology in a water-efficient building: Stagnation, seasonality, and physicochemical effects on opportunistic pathogen and total bacteria proliferation

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Abstract.

The rising trend in water conservation awareness has given rise to the use of water-efficient appliances and fixtures for residential potable water systems. This study characterized the microbial dynamics at a water-efficient residential building over the course of one year (58 sampling events) and examined the effects of water stagnation, season, and changes in physicochemical properties on the occurrence of opportunistic pathogen markers. Mean heterotrophic plate counts (HPC) were typically lowest upon entering the building at the service line, but increased by several orders of magnitude at the furthest location in the building plumbing. *Legionella* spp. and *Mycobacterium* spp. were detected in the plumbing, with the highest detection occurring in the summer months. Log-transformed HPC were significantly correlated with total cell counts (TCC) (r_s = 0.714, p<0.01), *Legionella* spp. (r_s = 0.534, p<0.01), and *Mycobacterium* spp. occurrence (r_s = 0.458, p<0.01). Reduced water usage induced longer stagnation times and longer stagnation times were weakly correlated with an increase in *Legionella* spp. (r_s = 0.356, p<0.001), *Mycobacterium* spp. (r_s = 0.287, p<0.001), TCC (r_s = 0.216, p<0.001) and HPC (r_s = 0.395, p<0.001). Interrelationships between seasonal shifts in water chemistry and genus-level genetic markers for opportunistic pathogens were revealed. This study highlights how drinking water microbiology varies seasonally and spatially throughout a low-flow plumbing building and highlights the possible unintended consequences associated with reduced water usage and increases in stagnation.

Water Impact Statement - As trends in water conservation increase, it will become especially important to understand the potential risks of increased microbial and pathogen growth within building plumbing. Insight gained from this study will help building operators to better understand how drinking water in the building can vary by season, fixture location, and sampling time of day.

1. Introduction

As public awareness of water conservation issues is rising, homeowners and consumers are increasingly using water-efficient appliances and fixtures to reduce municipal water usage. Water use in single-family three-person residences declined from 708 L per day (LPD) in 1999 to 500 LPD in 2011.[1] Recent studies have highlighted the economic and environmental benefits of utilizing low-flow water fixtures.[2] Utilization of new water-saving technologies will further catalyze the trend towards lower flows and reduced water usage within buildings. Retrofitted three-person residential buildings that have been designed to reduce water usage, on average, use 405-443 LPD of water.[1]

Despite the benefits associated with reduced water usage, there is a growing concern that the reduction in flow caused by low-flow plumbing fixtures increases the hydraulic retention time (HRT) of the plumbing, exacerbating concerns regarding water quality. Increases in HRT have previously been linked to water quality degradation, including potential for increased microbial growth,[3] loss of disinfectant,[4] metal leaching,[5] as well as taste and odor issues.[6] Compared to centralized distribution systems, building plumbing may face more severe water quality degradation due to higher temperatures, regular intermittent periods of non-use[7,8], and higher surface area to volume ratios[9] in building plumbing.

Legionella pneumophila and *Mycobacterium avium* are two of the most common causative agents of drinking water-associated disease outbreaks.[10,11] They commonly survive and persist in drinking water due to shared properties of disinfectant resistance,

biofilm growth, and ability to thrive in low-nutrient environments.[3] While these opportunistic plumbing pathogens (OPPs)may originate from the drinking water distribution system, they tend to grow more readily in building plumbing due to a lack of disinfectant residual. It is expected that low-flow plumbing may pose a heightened risk for opportunistic pathogen exposure compared to conventional plumbing due to lower flow rates and increased stagnation. While previous studies have evaluated the presence of OPPs in conventional building plumbing[8,12,13] and simulated plumbing systems,[14–17] to the best of our knowledge no studies have holistically evaluated the roles of temporal and spatial physicochemical water quality variation on the occurrence of OPPs in low-flow plumbing over a long period (an entire year).

Since multiple physical, chemical, and microbial water quality parameters change concurrently in building plumbing and during stagnation, it is difficult to determine cause and effect relationships between physicochemical and microbial parameters. By analyzing multiple parameters, one can better determine methods to control these water quality changes that occur as a result of increased stagnation.

In this study, a municipal water supplied a retrofitted water and energy-efficient building which was used to examine drinking water quality changes associated with increased water efficiency. A total of 58 sampling events were completed throughout the 12-month study. The objectives of this study were to (i) determine the impact of stagnation on microbial water quality in a water-efficient building; (ii) examine the seasonality of water physicochemical parameters and its effect on water microbiology over a one year

sampling period; (iii) assess long-term spatial microbiological variations at proximal and distal fixtures in the building; and (iv) to examine the relationship between culturable and total microbial counts with the occurrence of OPPs (*Legionella* and *Mycobacterium* spp.) within a water-efficient building. If increased stagnation in plumbing systems is associated with increased microbial and potential OPPs growth, then control strategies could be needed to prevent waterborne infectious disease.

2. Materials and Methods

2.1 Description of the testing site

A highly water efficient single-family residential building was chosen as the primary test site for this study. In the United States, average water usage in a single-family residential building is 344- 500 L/person/day.[1,18] After renovating the 1920's building with water-saving fixtures, per capita water usage of the building in this study, was reduced to 79 L/person/day.[18] Water entered the three-year old building plumbing from a ¾" (1.90 cm) copper utility line, which feeds into a ¾" (1.90 cm) PEX service line (4.30 m in length). Building plumbing consists of trunk-and-branch PEX plumbing and brass valves and fittings. Fixture information and their respective efficiencies are included in Supplementary Information (Figure S1, Table S1). The municipal water source is groundwater. The groundwater is oxidized, aerated, filtered, chlorinated for secondary disinfection with free chlorine, and a corrosion inhibitor is added before distribution. To monitor water usage, flow meters were installed on all in-building fixtures and a data acquisition system recorded data at each flow meter every second, 24 hrs/day, 365 days/year. Water usage events consisted of periods of flow geater than 3 seconds.

Additional information regarding the flow data analysis was conducted as previously reported.[19] The volume used per event and mean stagnation time, (mean defined as the mean time elapsed between water use events) at the same fixture, were determined to help uncover any relationships between increased HRT and water quality.

2.2 Sampling regime and water chemistry analysis

Water sampling events were evenly distributed to examine spatial and temporal variations of water quality throughout the building. Beginning in October 2017 and ending in October 2018, a minimum of 12 sampling events occurred per season. These sampling events were conducted at three time points during the day (7:30 a.m., 12:00 p.m., and 3:00 p.m.) with three to four sampling events for each time point in each season 58 total sampling dates in fall (n=13), winter (n=17), spring (n=12), summer (n=16). Potable cold water fixtures were sampled sequentially at three locations, starting with the service line, kitchen sink cold water line, and the distal-end bathroom sink cold water line. Hot water lines were then sampled at four locations: water heater tank, kitchen sink hot water line, bathroom sink hot water line (distal-end), and the shower (distal-end). At each fixture, approximately one-liter first-flush samples were collected for physicochemical analysis. Water samples for heterotrophic plate counts (HPC) and molecular microbial analysis were collected in two autoclaved 1-L HDPE bottles with sodium thiosulfate added to inactivate any chlorine in the water.

Temperature, pH, and total chlorine were measured onsite immediately after sample collection. The pH was analyzed with an Oakton 450™ pH probe. Total chlorine was

quantified using N,N-diethyl-phenylenediamine (DPD) reagent and Hach Pocket Colorimeter™ chlorine test kits. The limit of detection for the Hach Pocket Colorimeter™ is 0.02 mg/L as Cl_2. However, the minimum state-suggested level of total chlorine is set at 0.20 mg/L as Cl_2 and chlorine levels should not be below this limit for more than 5% of samples per month. Metal concentrations (Al, As, Be, Cd, Co, Cr, Mn, Ni, Se, Pb, Zn, Fe, Cu) were quantified by inductively coupled plasma-optical emission spectrometry (iCAP 7400 Duo ICP-OES, Thermo Scientific) and an autosampler (ASX-280, CETAC Teledyne). Ion concentrations were quantified by ion chromatography (Metrohm 940 Professional IC Vario) immediately after sample collection, as previously described.[19] Total organic carbon (TOC) was measured using a Shimadzu TOC-L CPH/CPN in accordance with USEPA method 415.1.[20]

2.3 Microbiological analysis

HPC and flow cytometry (FCM) methods were used to analyze the culturable cell population and the total cell count (TCC), respectively. Depending on the concentration of colony forming units (CFU) in each HPC sample, dilutions were prepared such that each plate yielded 20-200 colonies. Dilutions were filtered through a sterile 0.45 µm membrane filter (Millipore), and the membrane filter was plated and incubated on m-HPC agar (Difco™) at 35° C for 48 hours. Following the 48-hour incubation period, CFU were counted.

FCM analysis was conducted to quantify the total number of microbial cells in each water sample using SYBR Green I dye, which binds specifically to nucleic acids (Swiss

Research method 366.1).[21] Each water sample was stained 1:100 with SYBR-Green I nucleic acid gel stain diluted in filtered dimethylsulfoxide (DMSO). The samples were incubated in a 96-well plate in the dark at 37° C for 13 minutes. Triplicate samples from each fixture were analyzed using FCM (CytoFLEX, Beckman-Coulter Inc., Brea, CA, USA). A constant and uniform gating strategy was applied to all samples.

2.4 Quantitative PCR (qPCR) OPPs analysis

For molecular detection, approximately one liter of water sample was filtered through 47mm diameter, 0.4 µm pore size polycarbonate filters (Millipore #HTTP04700). DNA extractions were performed as described in EPA Methods 1611.[22] Briefly, the filter was transferred to a two mL semi-conical screw cap microcentrifuge tube containing 0.3 g of acid-washed, 212-300 mm glass beads (Sigma-Aldrich, #G-1277) and 600 mL AE buffer (Qiagen, Valencia, CA, USA) added. The tubes were sealed, bead milled at 5,000 reciprocations/min for 60 s and centrifuged at 12,000 × g for one min to pellet silica beads and debris. The supernatants were transferred to clean, low retention micro-centrifuge tubes and centrifuged for an additional five min. The resulting clarified supernatants were transferred to another clean, low retention micro-centrifuge tubes and stored at -80^{o} C until qPCR analysis.

Legionella spp. (targeting 23S rRNA), *Legionella pneumophila (mip* gene*)*, *Mycobacterium* spp. (the internal transcribed spacer sequence) and *Mycobacterium avium* (16S rRNA) were enumerated by qPCR using previously published methods.[23–25] All qPCR reactions were performed using a StepOne Plus™ real-time PCR sequence

detector (Applied Biosystems, Foster City, CA). For each assay, a 10-fold diluted standard curve of at least six points, a non-template control, and samples were tested in triplicate. Genomic DNA of *Legionella pneumophila* strain Philadelphia-1 (ATCC 33152D-5) and *Mycobacterium avium* (ATCC 25291), respectively, was used to generate standard curves. For *Mycobacterium*, amplification reaction mixtures (final total volume of 25 µl) contained five µl template DNA, 12.5 µl of 2 × Perfecta qPCR ToughMix (QuantaBio), 400 nM of each primer and 200 nM of probe. The thermal cycling protocol was as follows: 15 min at 95°C for initial denaturation, followed by 45 cycles of three steps consisting of 30 s at 95° C, 40 s at 55° C, and 30 s at 72° C. A duplex qPCR assay was used for *Legionella* detection. Amplification reaction mixtures (final total volume of 25 µl) contained five µl template DNA, five µl of 5 × Perfecta Multiplex qPCR ToughMix (QuantaBio), 500 nM of each primer and 200 nM of probe. The thermal cycling protocol was as follows: 15 min at 95°C for initial denaturation, followed by 45 cycles of two steps consisting of 15 s at 95° C and 60 s at 60° C. qPCR parameters have been included. qPCR amplification efficiencies for the quantification of the *Legionella* 23S rRNA, *Legionella pneumophila mip* gene, *Mycobacterium* spp. and *Mycobacterium avium* 16S rRNA assays were 98.6 ± 1.7%, 100.9 ± 3.2%, 97.5 ± 2.5% and 86.5 ± 5.3%, respectively, and the correlation coefficients of the standard curves were 0.998 ± 0.003, 0.996 ± 0.003, 0.999 ± 0.002 and 0.993 ± 0.003, respectively. Standard precautions were applied when conducting the qPCR, such as UV sterilization of PCR equipment and the working environment, using aerosol-resistant tips, separate locations for sample preparation and amplification. Negative controls using PCR-grade water were included in each reaction set.

215

216 *2.5 Statistical analysis*

217 Ranges and mean values for physicochemical and microbial data were determined. The
218 data were tested for normality using the Kolmogorov-Smirnov test.[26,27] Nonparametric
219 Spearman rank correlation analysis was used to determine correlations between non-
220 normal distributed chemical properties and microbial metrics (TCC, HPC, *Legionella*
221 spp., and *Mycobacterium spp.).* Correlation analysis was applied using IBM ® SPSS ®
222 version 26 statistical software to explore the relationship between stagnation,
223 physicochemical factors (pH, temperature, total chlorine), and log-transformed microbial
224 metrics (HPC and FCM), where the type I error at a significance level of 0.05 was
225 considered significant. Holm's correction method was tried to check for error rates for
226 multiple hypothesis tests. This method reduces type I errors when multiple tests are
227 performed.

228

229 **3. Results & Discussion**

230 *3.1 Physicochemical water quality variation by season.*

231 While seasonal variations in pH, temperature, and total chlorine concentration were
232 observed at the service line, much greater variations were found inside the building
233 (Table 1). The service line water pH was similar across seasons, with the mean pH of
234 7.74 and 7.51 in the winter and summer months, respectively. However, the pH at other
235 fixtures often increased throughout the building (range: 7.2-9.4). The pH levels were
236 likely higher in the building compared to the service line, due to increased stagnation
237 and contact with calcium deposits in the pipe scale. Increased stagnation time and
238 temperature were correlated with increased pH (r_s = 0.229-0.303, $p<0.001$). It is

239 recommended to keep water pH between 6.5-8.5 in accordance with the EPA
240 secondary maximum contaminant level (SMCL) to avoid pipe corrosion, deposit release,
241 and drinking water taste issues.[28]
242
243 Water temperature at the service line varied from 21.6-23.6° C in the summer months
244 and ranged from 11.5-19.0° C in the winter months. The total chlorine in the service line
245 varied by season (spring mean: 0.6 mg/L; range: non-detectable (ND) to 2.1 mg/L as
246 Cl_2). Service line mean total chlorine levels were the greatest in the winter (Mean: 0.9
247 mg/L, range: ND-1.6 mg/L as Cl_2) and lowest in the summer (mean: 0.4 mg/L, range:
248 ND-0.8 mg/L as Cl_2) and fall (mean: 0.4 mg/L, range: 0.2-0.8 mg/L as Cl_2). At the
249 service line in the summer months, chlorine was only detected for 75% of the samples
250 (n=12 of 16), whereas in the winter, chlorine was detected at the building entry for 94%
251 of the samples (n=16 of 17). After entering the plumbing system in the summer months,
252 chlorine was only detectable (state law ND is <0.2mg/L) at 3% of fixture samples (n=3
253 of 96) during the summer (June-September) months. Whereas, in the winter months,
254 chlorine was present at detectable levels (>0.2 mg/L) for 32.4% of building fixture
255 samples collected in winter (n= 33 of 102). Chlorine concentrations in drinking water of
256 conventional plumbing systems and green buildings have been compared previously for
257 one to two discrete sampling events.[4] Lower (0.04 mg/L) chlorine concentrations were
258 consistently detected in stagnant green building fixtures than in stagnant conventionally
259 plumbed buildings (0.71 mg/L),[4] and chlorine was not detected in the green building
260 until after thirty minutes of flushing. Without flushing, low-flow buildings are likely to
261 have lower chlorine concentrations than conventionally plumbed buildings.[4] Seasonal

temperature shifts were significantly correlated with changes in total chlorine concentrations (r_s= -0.41, p<*0.001)*. Overall, water temperature and total chlorine varied seasonally and spatially, even after entering the building plumbing. This phenomenon of lower chlorine residual detection in warmer months has been previously reported and is due to the relationship between total chlorine concentration and temperature.[29]

Copper, lead, and zinc levels were higher in the building plumbing than at the entry point of the building (Table S2). Metal and ion concentrations for each fixture are provided in Table S2 and Table S3. Mean copper concentrations at the building entry were lowest in the summer (mean: 32.2 µg/L) and increased substantially after exiting the water heater (mean: 92.5 µg/L). The mean copper concentrations were well below the acceptable EPA Lead and Copper Rule copper limit of 1300 µg/L.[30] Our concurrent study indicated that copper and zinc concentrations were higher in the morning sampling events compared to the afternoon sampling events.[27] Magnesium and calcium concentrations decreased significantly after treatment through the water softener, while sodium concentrations increased after water softening (Table S3). Increases in copper and zinc concentrations are likely due to contact with brass valves and fittings.

3.2 Microbial metrics varied by fixture: HPC and TCC

Microbial growth increased in the distal fixtures in relation to the entry point of the building, underscoring that the indoor building environment is more favorable for microbial growth than the service line. Significant differences in HPC and TCC levels were observed across fixtures (Table S5, Table S6), but service line water consistently

had lower mean values of HPC compared to the other fixtures in the building for the spring (mean: 2.74 log CFU/100 mL), summer (mean: 2.88 log CFU/100 mL) and fall months (mean: 3.16 log CFU/100 mL), but not winter. Surprisingly, mean winter HPC levels at the service line (mean: 2.08 log CFU/100 mL) were higher than the HPC levels at the kitchen sink cold water line (mean: 1.89 log CFU/100 mL) and the 2nd floor bathroom sink cold water line (mean: 1.74 log CFU/100 mL) in the winter. The higher HPC levels detected at the service line compared to the kitchen sink cold water line may be due to the low level of use for the service line sampling tap.

The highest mean HPC levels were found in water samples collected from the water exiting the water heater during the fall, spring, and summer months (range: 6.37-7.37 log CFU/100 mL). Fixture HPC levels exceeded a recommended maximum level of 4.69 log CFU/100 mL (5.0×10^4 CFU/100 mL).[31] In this study, HPC and TCC were significantly correlated ($r_s = 0.714$, $p<0.01$), in other studies the correlation between HPC and TCC was similar (Pearson $\rho=0.57$).[32] A strong correlation between HPC and TCC may have been observed due to generally favorable growing conditions in the building, including low chlorine concentration and longer stagnation times.

In this study, HPC and TCC also varied by season due to shifting water temperatures and total chlorine concentrations. Both the HPC and TCC were lower in the winter and higher in the summer. Thus, this seasonal trend in microbial growth may, in part, be due to variation in outdoor temperatures, which vary considerably by season. Log-transformed HPC concentrations were significantly correlated with temperature of water

samples (r_s = 0.460, *p<0.01)* (Table 2). Given the significant negative correlation between HPC and total chlorine (r_s = -0.294**, *p<0.01)* (Table 2), it is likely that the lower disinfectant concentrations in water exiting the water heater contributed to the higher degree of microbial growth in the hot water fixtures. Indoor air temperature is also a driving factor for increased microbial growth in building plumbing.[33] All of the samples collected from the water heater during the spring, summer, and fall exceeded the suggested HPC guideline, and many of the other hot water fixtures exceeded the guideline in the spring and summer months. The seasonality of HPC growth is consistent with findings of other studies evaluating seasonal water quality variation in premise plumbing.[15,34] Although the HPC growth in drinking water often exceeds the recommended maximum level of 4.69 log CFU/100 mL (5.0 x 10^4 CFU/100 mL), HPC bacteria do not explicitly impose a health threat. This suggests that the HPC guideline may not be a suitable or informative metric of the water quality.

The maximum first-flush temperature observed at the sampling outlet of the energy-efficient water heating system was 26° C. Thus the water heater may have acted as an incubator facilitating microbial growth in the warm water fixtures throughout the building, as the HPC concentrations were always greater after exiting the water heater. A relatively large volume of in-building water storage is required to support the solar thermal-photovoltaic system[19]. Due to the energy-efficient design of the building, the water heating storage volume (1360 L) is not typical for a single-family home (typically 150-420 L/home). Conventional residential water heaters have a hydraulic retention time (HRT) of 1 day.[4] In this study, HRT in the water heating system (Table S7-b) varied

by season as determined by dividing the volume of the water heating system (1378 L) by the mean total volumetric flow rate for each season (Table S7-b). The lowest mean HRT in the water heater occurred in the fall (14.4 days) and the highest HRT occurred in the summer (29.2 days). These high HRT values indicate that it took two to four weeks for a complete water turnover to occur in the water heater, as compared to conventional water heaters that have an average HRT of one day. To counteract these long hydraulic residence times, the importance of properly controlling the temperature in water heaters should be considered,[35] so as to prevent the growth of OPPs. More specifically, a temperature-based risk assessment tool indicates that temperatures exiting water heaters should be maintained above 55-60°C to prevent the growth of OPPs such as *Legionella pneumophila.*[36]

3.3 Occurrence of Potential OPPs

Mycobacterium spp. and *Legionella* spp. gene copy numbers were lowest at the building entry and increased in water after entering building plumbing (Table 3, Table S5). *Legionella pneumophila* was not detected in any samples, but *Mycobacterium avium* was detected in two fall samples out of 259 total water samples measured throughout the study. Both *Mycobacterium* spp. and *Legionella* spp. concentrations were low at the service line, but increased substantially in building plumbing. *Mycobacterium* spp. concentrations were also lowest at the service line, but the concentrations increased by 1-3 logs in the building plumbing. This indicates that the building plumbing was more favorable for OPP growth as compared to the centralized distribution system.

Seasonal variation in Legionella and Mycobacterium spp. *detection.* In the summer, all water samples collected from the building's water fixtures tested positive for both *Legionella* spp. and *Mycobacterium* spp. (Table 3), except the service line where municipal water entered the building. Across the seasons, *Legionella* spp. concentrations at the building entry were similar (range: 1.43-1.77 log gene copy/100 mL). However, the seasonal trends in *Legionellla* spp. concentrations were more evident at all fixture locations, where *Legionella* levels followed the trend: summer > fall > winter, except at the shower (*Legionella* spp. concentrations were not measured in the spring). Interestingly, mean *Legionella* concentrations in the shower were the highest in the winter months (mean: 4.71 log gene copy/100 mL), followed by summer (mean: 3.81 log gene copy /100mL) and fall (2.78 log gene copy/100mL). These levels are low compared to a previous study of *Legionella* spp. growth in green buildings, where mean *Legionella* spp concentrations were reported as 6.949 log gc/100 mL.[4] *Legionella spp* concentrations may have been higher in the shower during the winter months due to higher ambient temperatures in the building and higher water use in the shower (Table S6-a). Alternatively, the higher water use in the shower in the winter months may have allowed for a more continuous supply of nutrients, which may have led to an increase in *Legionella* spp concentrations during winter.

In the winter, 37.5% (n=8) of the samples collected from the service line tested positive for *Mycobacterium* spp., whereas in the summer, 87.5% (n=16) of the samples collected from the service line tested positive for *Mycobacterium* spp. On average,

Mycobacterium spp at the service line were detected at similar concentrations each season ranging from 3.9-4.13 log gc/100 mL, these numbers are som lower than the *Mycobacterium* spp. levels that have been detected in water systems from a similar climate (6.15 log gc/100 mL).[37] *Mycobacterium* gene copy numbers increased throughout the building and fixture levels were higher in the summer and fall as compared to the winter. Similar seasonal trends in *Legionella* spp. detection have been observed at the centralized distribution system level, where *L. pneumophila* is more likely to be detected in summer months and when temperatures are >18°C and chlorine residuals are <0.1 mg/L.[38] However, in this study, *Legionella* spp. concentrations were similar across seasons at the building entry but varied seasonally at the building taps.

In this study, *Legionella* spp. and *Mycobacterium* spp. were also found with high frequency in warm water fixtures and distal ends. *Legionella* spp. gene copy numbers increased with water temperature, where all of the hot water fixtures had a higher *Legionella* spp. detection rate (Table 3). Of all the fixtures, the shower and the distal end bathroom hot water line typically had the highest frequency of *Legionella* spp. detection with mean detection frequencies ranging from 87.5%-100%. A previous study by Ling *et al.* (2018) indicated that *Legionella* spp. may proliferate in the distal ends in the plumbing that experience long stagnation times.[12] A similar spatial trend occurred in this study, as a higher frequency of detection of *Legionella* spp. occurred in both the 2nd floor bathroom shower and 2nd floor bathroom sink hot water line, due to their similar distal locations in the plumbing and long stagnation times. This finding highlights that building water sampling and testing for potential opportunistic pathogens should not

occur only at the building entry where water quality is highest. Instead, water samples should be collected at hot and cold water taps throughout the building to gain an accurate assessment of the microbial populations throughout the building.

3.4 Role of water chemistry changes in microbial growth and potential OPPs

Seasonal water chemistry variation affected HPC concentrations.

Seasonal variations in water chemistry may help explain variation in microbial growth throughout the domestic drinking water system. Log-transformed HPC concentrations were negatively correlated with chlorine (r_s = -0.294, $p<0.01$). As expected, total chlorine and temperature are negatively correlated (r_s = -0.413, $p<0.01$) with each other. In addition, HPC concentrations were positively significantly correlated ($p<0.01$) with many metals and nutrients quantified in this study, including Cu, Zn, Br, Cl, Na and Mg (Table S8-S9). Copper and transition metals play important biological roles as redox cofactors.[39] Iron and potassium were negatively correlated with HPC concentrations (Table S8-Table S9). Complete tables for metals and ion concentrations measured in this study can be found in SI (Table S2 and Table S3). Changes in water chemistry can affect biofilm stability.[40] Biofilm detachment during stagnation cannot be completely discounted,[12] as detachment was not distinguished from planktonic growth in this study.

HPC concentrations were significantly correlated with shifts in water physicochemical properties (temperature, DO, and pH) ($p<0.01$), indicating that seasonality in water chemistry may contribute to seasonal shifts in microbial concentrations (Table 2). Mean total chlorine concentrations were significantly and negatively correlated ($p<0.01$) with

HPC during both fall and summer sampling periods. This indicates that the lack of chlorine was an important contributing factor to increased microbial growth. Microbial growth in domestic drinking water systems fluctuates seasonally[15] with higher HPC concentrations in the summer compared to the winter. Factors that contribute to higher microbial growth in the summer may be lower chlorine concentrations, seasonal source water variations in total organic carbon (TOC),[41] and variations in temperature.[42]

Impacts of water chemistry on total cell counts as determined by FCM. When comparing the physicochemical parameter correlation with TCC data, the log-transformed TCC concentrations were significantly correlated with temperature (r_s = 0.463, $p<0.01$) and TOC (r_s = 0.512, $p<0.01$), while TCC were negatively and weakly correlated with total chlorine (r_s = -0.225, $p<0.01$).The correlation between TCC and ion concentrations were determined (Table S8). Significant correlations were found between log-transformed TCC and several aqueous ions (Br, Cl, Cl, Na, Mg, and Zn) ($p<0.01$). Significant negative correlations were shown between log-transformed TCC and Fe, K, and NO_3 ($p<0.01$). Since HPC and TCC were significantly correlated, many of these metal and nutrient relationships that occurred with TCC are also likely to occur with HPC.

Relationships between physicochemical water quality and OPPs. Legionella spp. log-transformed gene copy numbers were significantly correlated with temperature (r_s = 0.344, $p<0.01$), and chlorine (r_s = -0.417, $p<0.01$) (Table 2). Similar physicochemical relationships were observed for the detection of *Mycobacterium* spp. with temperature (r_s = 0.380, $p<0.01$) and total chlorine (r_s = -0.369, $p<0.01$), but not with pH. The lack of

correlation between *Mycobacterium* abundance and pH could be due to the fact that *Mycobacteria* species can grow at a wider pH range[43] (5.4-7.4) as compared to *Legionella*, which grows best at a pH range of 6.5-7.5.[44] Also, pH affects the speciation of copper, which *Legionella* spp. is highly sensitive to, thus secondary effects of copper exposure could also account for *Legionella's* sensitivity to pH.[45]

Aqueous ion concentrations were significantly correlated with log-transformed concentrations of *Legionella* spp. and *Mycobacterium* spp. ($p<0.01$). Ion concentrations of Br, Cl, Na and Pb were positively significantly correlated with the occurrence of both *Legionell*a and *Mycobacterium* spp. (Table S8-S9). Negative correlations ($p<0.01$) were observed for both bacterial species and the ion concentrations of K, Mn, NO_3, and Fe. The inverse correlation between nitrite, nitrate, and the detection of *Mycobacterium* spp. are likely due to the role that *Mycobacterium* plays in nitrate and nitrite reduction.[46] The ability of *Mycobacteria* to reduce nitrate may enable the species to rapidly assimilate to oxygen and nitrogen changes, allowing for better survival in the low-nutrient water, such as drinking water.[47]

Microbial interrelationships. *Legionella* spp. and HPC concentrations were significantly correlated ($r_s = 0.600$, $p<0.01$). *Mycobacterium* spp. and HPC concentrations were also significantly correlated, but to a lesser extent ($r_s = 0.458$, $p <0.01$) (Table 2). Interestingly, the correlation of *Mycobacterium* spp. and *Legionella s*pp. concentrations as determined by qPCR was strong ($r_s = 0.758$, $p<0.01$). The correlation between the two genera may indicate that the presence of either species may serve as a better

predictor of potential OPPs occurrence as compared to HPC or TCC. Several drinking water microbial ecology studies have found significant relationships between the qPCR signals of *Legionella* and *Mycobacterium.*[48,49] This may be due to an overlap in niches for the two genera.[49] Neither HPC or TCC metrics constitute a strong predictor of OPPs growth, but a variety of microbial metrics may be required to accurately portray the microbial community within a drinking water system.

3.5 Stagnation and water usage patterns influenced microbial growth

Total volume and mean stagnation times were determined using hydraulic data captured from the flow meters (Table S7-a). Total water usage varied slightly by season (range: 19.7-25.5 m^3), with the highest water usage occurring during the fall. More specifically, the stagnation times at the building entry point were very low (90^{th} percentile: 0-1 hours) based on the season. However, stagnation times were often the highest at the distal ends in the plumbing for the bathroom sink cold water line (90^{th} percentile range: 7.3-11.6 hours) and the shower (90^{th} percentile range: 3.6-15.6 hours).

To determine the relationship between stagnation and increased microbial growth for each sample tap, the correlations between mean stagnation time and the microbial metrics were determined (Tables S4-a: Table S4-e). At the service line building entry point, stagnation time was not significantly correlated with any of the microbial metrics (HPC, TCC, *Legionella* spp., *Mycobacterium* spp.). However, mean stagnation times were positively and significantly correlated with TCC (r_s = 0.601, $p<0.001$), HPC (r_s = 0.564, $p<0.01$), and *Legionella* spp. gene copy numbers (r_s = 0.545, $p<0.01$) at the distal

492 end bathroom sink (Tables S4-d), indicating that microbial growth increased with longer
493 stagnation times. Water stagnation played an elevated role in microbial proliferation
494 within this water-efficient building, especially due to the variation in water age
495 throughout the building.
496
497 A previous study demonstrated that water-efficient buildings with elevated water age
498 might exhibit higher levels of microbial and OPPs growth in premise plumbing.[4]
499 Molecular estimation methods such as qPCR may overestimate the actual OPPs risk,
500 as the method does not differentiate between live and dead cells.[50] Most molecular
501 microbial results presented in this study are for the genus level (not species level), but it
502 is possible that *Legionella* spp. and *Mycobacterium* spp. exhibit similar growth
503 characteristics to the opportunistic pathogens as these genera contain, *Legionella*
504 *pneumophila* and *Mycobacterium avium.*
505
506 *3.6 Implications and moving forward in green building design to protect water quality*
507 Although previous studies have compared water quality of low-flow plumbing for a few
508 sampling events,[4,16] this study consisted of 58 sampling events over a one year period,
509 which included over 2.4 billion online monitoring data points (fixture flow and
510 temperature) to further evaluate the (i) seasonality of water physicochemical parameters
511 and its effect on drinking water microbiology; (ii) spatial stagnation and microbiological
512 variation at low-flow fixtures in the building; and (iii) relationship between low-flow water
513 use hydraulics with water microbiology and chemistry.
514

Elevated microbial activity, as indicated by HPC, TCC, and increased levels of potential OPPs genus-level genetic markers were significantly correlated with seasonal fluctuations in water pH, chlorine, and temperature. Levels of disinfectant concentrations were generally low in all samples in this study, which may account for a consistent proportion of cultivable cells relative to total cells. A detachment of viable bacteria from biofilm may have also contributed to this trend. Thus, if preventative measures (i.e., flushing) are taken to avoid OPP proliferation in building plumbing, the effects of seasonal changes in water chemistry on microbial growth may need to be considered.

In this study, distal fixture locations and warm water fixtures exhibited higher degrees of microbial growth compared to cold water fixtures. Elevated microbial growth was significantly correlated with increased stagnation time, low chlorine levels, and temperature variations throughout the building. In the event of suspected waterborne disease water samples should be collected throughout the building, not just at the entry point of the building, where water quality is more likely to be in compliance with drinking water standards. The detection of *Legionella* spp. occurred more frequently in the summer months (June-September) than in any of the other seasons.

Operators for buildings with low-flow water systems may need to consider the use of additional disinfection measures for the control of microbial growth and potential OPPs in drinking water in the summer months, especially in buildings with high occupancy of the elderly and the immunocompromised. The *National Academy of Science* (NAS)

Engineering and Medicine Water Science and Technology Board has recommended that the *Centers for Medicare & Medicaid Services* memo should require monitoring for *Legionella* in water samples for all hospitals.[51] Alternatively, it has also been recommended that the use of low-flow fixtures should be completely avoided altogether in hospitals to protect at-risk populations.[51]

Lower flows and reduced water usage led to increased stagnation, which was correlated with elevated *Legionella* and *Mycobacterium* spp. concentrations, microbial growth, and lower chlorine concentrations. While the use of water-efficient fixtures is expanding, the unintended consequences of reduced water usage need to be better evaluated to control and prevent OPP proliferation in green buildings. Additionally, flushing of taps or onsite disinfection may be useful to reduce the risk of waterborne OPPs disease in green buildings with low-flow plumbing.

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560 ***TABLES & FIGURES***

561

562 **TABLE 1:** Service line water temperature, pH, and total chlorine concentration by

563 season

Parameter	*Season (number of sampling events)*			
	Fall (n=13)	*Winter (n=17)*	*Spring (n=12)*	*Summer (n=16)*
Water Volume (m^3)	25.5	23.6	19.7	23.8
Water pH	7.66 - 7.72 Mean: 7.76	7.65 - 7.81 Mean: 7.74	7.66 - 7.78 Mean: 7.72	7.24 - 7.67 Mean: 7.51
Temperature (°C)	20.0 - 22.6 Mean: 21.6	11.5 - 19.0 Mean: 14.6	19.5 - 22.5 Mean: 21.1	21.6 - 23.6 Mean: 22.9
Total Chlorine (mg/L as Cl_2)	0.2 - 0.8 Mean: 0.40	ND - 1.6 Mean: 0.88	ND - 2.1 Mean: 0.58	ND - 0.8 Mean: 0.41

564 *nd = Level was not found above 0.1 mg/L concentrations*

565

566

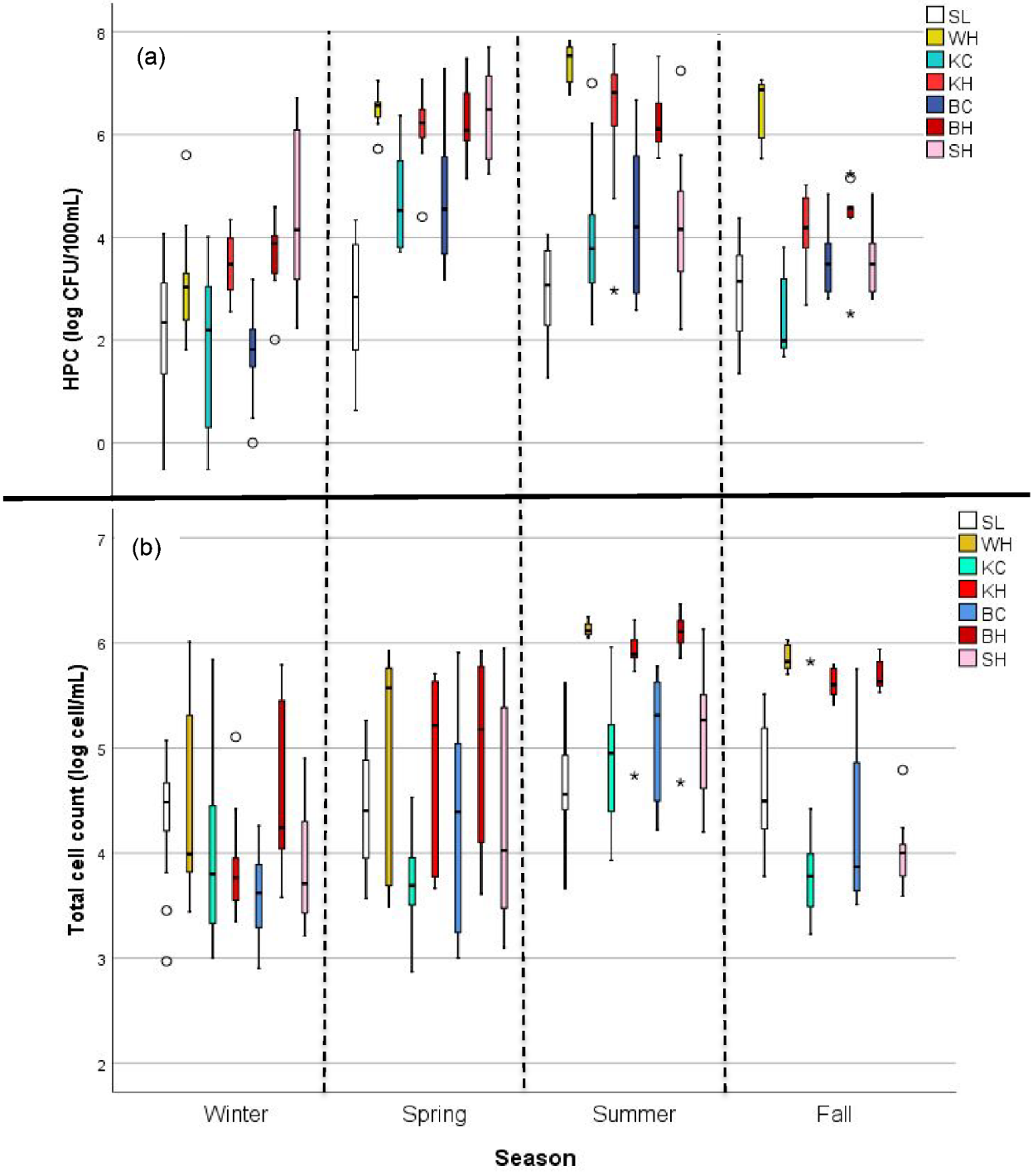

FIGURE 1: (a) Heterotrophic plate counts (HPC) (CFU/100mL) and (b) total cell counts (TCC) (cells/mL) vary by water fixture and season. Fixture abbreviations: service line

570 (SL), water heater (WH), kitchen sink cold (KC), kitchen sink hot (KH), bathroom sink
571 cold (BC), bathroom sink hot (BH), and shower (SH)

572

573

574 **TABLE 2**: Spearman correlation analysis for physicochemical and microbial properties

575

	pH	**Temp**	**Chlorine**	**TOC**	**DO**	**Log TCC**	**Log HPC**	***Log Leg* spp.**	***Log Myco* spp.**
pH (n = 406)	1								
Temp (n = 390)	NS	1							
Chlorine (n = 406)	-0.206**	-0.413**	1						
TOC (n = 406)	0.179**	0.491**	-0.343**	1					
DO (n = 406)	-0.302**	-0.398**	0.327**	-0.426**	1				
Log TCC (n = 406)	NS	0.463**	-0.225**	0.512**	-0.236**	1			
Log HPC (n = 406)	0.187**	0.460**	-0.294**	0.622**	-0.351**	0.714**	1		
Log *Legionella* spp. (n=258)	0.133*	0.344**	-0.417**	0.503**	NS	0.534**	0.6**	1	
Log *Mycobacterium* spp. (n=258)	NS	0.38**	-0.369**	0.434**	0.147*	0.430**	0.458**	0.751**	1

+	0.00-0.19	Very weak	-	0.00-0.19	Very weak
+	0.20-0.39	Weak	-	0.20-0.39	Weak
+	0.40-0.59	Moderate	-	0.40-0.59	Moderate
+	0.60-0.79	Strong	-	0.60-0.79	Strong
+	0.80-1.0	Very strong	-	0.80-1.0	Very strong

576 TCC: total cell counts, HPC: heterotrophic plate counts, TOC: total organic carbon, temp:
577 temperature, and DO: dissolved oxygen. Leg: Legionella. Chlorine: total chlorine.
578 ** Correlation is significant at the 0.01 level.*Correlation is significant at the 0.05 level.

TABLE 3*:* Occurrence of *Legionella* spp. and *Mycobacterium* spp. in different water fixtures in a water-efficient building as determined by qPCR

	***Legionella* spp. (log gene copy/ 100mL)**			***Mycobacterium* spp. (log gene copy/ 100 mL)**		
	Summer % Positive Min ($\bar{x}$) max	**Fall** % Positive Min ($\bar{x}$) max	**Winter** % Positive Min ($\bar{x}$) max	**Summer** % Positive Min ($\bar{x}$) max	**Fall** % Positive Min ($\bar{x}$) max	**Winter** % Positive Min ($\bar{x}$) max
Service Line	12.5% 1.39 (1.65) 2.9	30.8% 1.12 (1.77) 3.6	14.3% 1.37 (1.43) 1.81	87.5% 1.87 (4.13) 5.00	38.5% 1.60 (3.9) 4.96	37.5% 1.85 (3.97) 4.87
Water Heater	100.0% 4.16 (4.63) 4.86	100.0% 1.36 (3.56) 4.6	50.0% 1.29 (1.67) 2.85	100.0% 6.54 (7.14) 7.61	92.3% 1.57 (7.1) 7.94	87.5% 1.85 (5.5) 6.19
Kitchen cold	100.0% 1.69 (3.06) 4.19	61.5% 1.03 (1.89) 3.4	62.5% 1.37 (1.49) 1.64	100.0% 4.14 (5.78) 6.21	69.2% 1.48 (5.36) 6.41	87.5% 1.85 (3.66) 4.05
Kitchen hot	100.0% 4.29 (4.57) 5.08	84.6% 1.12 (3.19) 4.2	75.0% 1.29 (1.62) 2.10	85.7% 1.85 (6.71) 7.20	76.9% 1.60 (6.87) 7.37	75.0% 1.85 (4.75) 5.58
Bathroom cold	100.0% 2.51 (3.19) 4.29	69.2% 1.12 (2.14) 2.9	50.0% 1.37 (1.63) 2.11	100.0% 4.96 (6.48) 7.35	69.2% 1.60 (5.78) 6.52	75.0% 1.85 (3.54) 4.28
Bathroom hot	100.0% 3.15 (4.78) 5.26	92.3% 1.12 (3.38) 4.6	87.5% 1.37 (2.12) 2.62	100.0% 1.60 (6.71) 8.43	69.2% 1.60 (6.87) 7.90	87.5% 1.60 (4.81) 5.29
Shower	100.0% 2.33 (3.81) 5.3	92.3% 1.12 (2.78) 4.8	100.0% 2.52 (4.71) 5.72	100.0% 5.29 (6.77) 7.42	76.9% 1.60 (6.86) 7.62	100.0% 4.79 (5.91) 6.38

** Legionella and Mycobacterium* spp. *detection limits are 13 and 70 gene copies /100 mL, respectively. Number of samples collected by season varied (Winter: n=8; Summer: n=16; Fall, n=13). % Positive is the number of positive detection events divided by total sampling events per season at each fixture.*

TABLE 4: *Spearman correlation between Legionella and Mycobacterium* spp. *occurrence with microbial and physicochemical metrics*

		Log HPC	*Log TCC*	*Temperature*	*Stagnation*	*Chlorine*
Legionella spp.	Correl. R_s	0.600	0.534	0.344	0.356	-0.417
	p	<0.001	<0.001	<0.001	<0.001	<0.001
	N	242	257	256	246	258
Mycobacterium spp.	Correl. R_s	0.458	0.430	0.380	0.287	-0.369
	p	<0.001	<0.001	<0.001	<0.001	<0.001
	N	240	255	256	244	256

	+	0.00-0.19	Very weak		-	0.00-0.19	Very weak
	+	0.20-0.39	Weak		-	0.20-0.39	Weak
	+	0.40-0.59	Moderate		-	0.40-0.59	Moderate
	+	0.60-0.79	Strong		-	0.60-0.79	Strong
	+	0.80-1.0	Very strong		-	0.80-1.0	Very strong

TCC: total cell counts, HPC: heterotrophic plate counts

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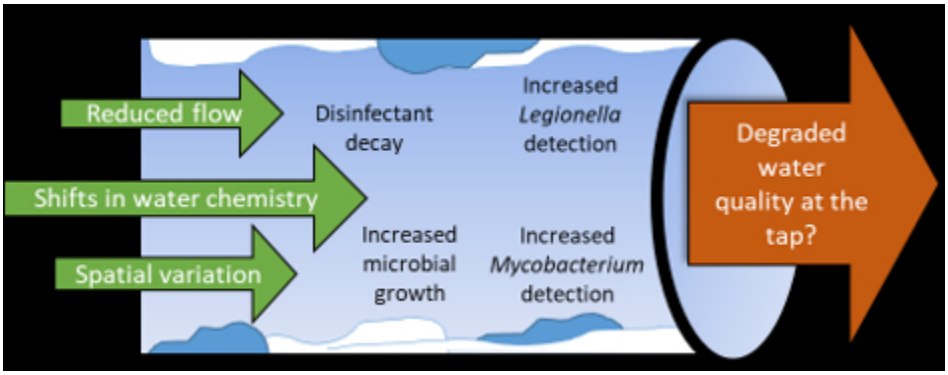
Reduced flow
Shifts in water chemistry
Spatial variation
Disinfectant decay
Increased microbial growth
Increased *Legionella* detection
Increased *Mycobacterium* detection
Degraded water quality at the tap?