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Molecular Survey of the Occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa*, and Amoeba Hosts in Two Chloraminated Drinking Water Distribution Systems

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The spread of opportunistic pathogens via public water systems is of growing concern. The purpose of this study was to identify patterns of occurrence among three opportunistic pathogens (*Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa*) relative to biotic and abiotic factors in two representative chloraminated drinking water distribution systems using culture-independent methods. Generally, a high occurrence of *Legionella* (≥69.0%) and mycobacteria (100%), lower occurrence of *L. pneumophila* (≤20%) and *M. avium* (≤33.3%), and rare detection of *Pseudomonas aeruginosa* (≤13.3%) were observed in both systems according to quantitative PCR. Also, *Hartmanella vermiformis* was more prevalent than *Acanthamoeba*, both of which are known hosts for opportunistic pathogen amplification, the latter itself containing pathogenic members. Three-minute flushing served to distinguish distribution system water from plumbing in buildings (i.e., premise plumbing water) and resulted in reduced numbers of copies of *Legionella*, mycobacteria, *H. vermiformis*, and 16S rRNA genes ($P < 0.05$) while yielding distinct terminal restriction fragment polymorphism (T-RFLP) profiles of 16S rRNA genes. Within certain subgroups of samples, some positive correlations, including correlations of numbers of mycobacteria and total bacteria (16S rRNA genes), *H. vermiformis* and total bacteria, mycobacteria and *H. vermiformis*, and *Legionella* and *H. vermiformis*, were noted, emphasizing potential microbial ecological relationships. Overall, the results provide insight into factors that may aid in controlling opportunistic pathogen proliferation in real-world water systems.

In recent years, opportunistic pathogens, including *Legionella pneumophila*, nontuberculosis mycobacteria (NTM), *Pseudomonas aeruginosa*, and *Acanthamoeba* spp. have become a leading source of waterborne disease in developed countries. A growing incidence of Legionnaires' diseases was reported in the United States from 2000 to 2009 (17) and France from 1998 to 2005 (15). In the United States, *Legionella* has been the single most commonly reported pathogen identified in drinking water-associated outbreaks since its addition to Waterborne Disease and Outbreak Surveillance System in 2001 (13). Multiple studies also linked NTM infection to drinking water systems by employing genetic and epidemiological methods to compare clones isolated from patients and drinking water (see, e.g., references 12, 23, 30, and 32), highlighting drinking water as a potential route of exposure for NTM infection. A study reviewing waterborne nosocomial infection from 1966 to 2001 suggested that outbreaks of nosocomial *P. aeruginosa* infection are commonly related to hospital tap water (4). Recent outbreaks of *Acanthamoeba* keratitis (AK) in the United States were also suspected to be associated with drinking water (16, 62). Further evidence linking AK to tap water demonstrated identical *Acanthamoeba* mitochondrial DNA (mtDNA) profiles for the clinical and home tap water isolates in 75% (6 of 8) of *Acanthamoeba*-positive (8 of 27) patients' homes in the United Kingdom (38). The incidences of waterborne NTM, *P. aeruginosa*, and *Acanthamoeba* infection and their association with drinking water are likely to be underestimated, since they are nonreportable diseases.

Water from plumbing in buildings (i.e., premise plumbing water) is an important reservoir for opportunistic pathogens and represents a direct route for transmission and exposure to humans, typically via inhalation of aerosols or skin contact. The unique characteristics of premise plumbing include a high surface-area-to-volume ratio, long retention times, the presence of reactive pipe materials (e.g., corrosion), and warm temperatures, all of which contribute to low disinfectant residual in household water and therefore promote bacterial colonization and multiplication (57). On the other hand, some waterborne opportunistic pathogens, such as *M. avium* complex, are slow-growing oligotrophs capable of resisting heat and disinfectants, which makes them strong competitors in the drinking water environment (31). The occurrence of *Legionella* and mycobacteria in premise plumbing and potential relationships of occurrence with environmental factors, such as water chemistry (6, 9, 33, 72), temperature (30, 34, 42, 52, 55), water heater capacity and type (48, 55), and premise plumbing characteristics (52), have been previously reported. Water heater temperature is considered to be the most critical determining factor for *Legionella* and NTM colonization in household plumbing (30, 34, 42, 52, 55). A positive relationship between mycobacterial abundance and assimilable organic carbon (AOC) concentration was observed by Falkinham et al. (33) and Torvinen et al. (72) in U.S. and Finnish drinking water distribution systems. The colonization of *Legionella* was also recently found to be associated with trace metals. Negative association of copper levels > 50 mg/liter and *Legionella* colonization and positive association of Zn and Mn and *Legionlla* colonization in hot water systems have

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been proposed in some studies (6, 9). In contrast, a *Legionella* survey of German residences suggested a positive effect of copper pipes with respect to *Legionella* colonization (52).

In general, the driving factors of opportunistic pathogen occurrence and regrowth in premise plumbing remain elusive due to the complexity of premise plumbing and limited knowledge of pathogen transmission and life cycles in engineered water systems. Few studies have considered the influence of microbial ecology, which is likely to be particularly critical in governing occurrence and regrowth of opportunistic pathogens in drinking water systems. For example, the ecological niche of *Legionella* and mycobacteria overlaps with those of amoebae and protozoa (1, 24) and infection of protozoan hosts can enhance reproduction and virulence in these and other pathogens (18, 19). Certain aquatic bacteria have also been reported to exert a negative influence on *L. pneumophila* (35). Therefore, advancing understanding of the microbial ecology of multiple representative opportunistic pathogens is critical to developing appropriate guidance and controls to broadly limit their proliferation. In particular, there is need for a simultaneous, comprehensive molecular examination of multiple opportunistic pathogens. Such a study is of value, considering that the factors that inhibit one pathogen may actually favor the growth of others. For example, in the drinking water distribution system in Pinellas County, FL (PCF), Moore and colleagues (54) previously reported that switching from chlorine to chloramines mitigated *Legionella* colonization but favored mycobacterial colonization. However, the full extent and implications of such phenomena are unknown.

This report provides a comprehensive molecular survey of the occurrence of *L. pneumophila* and other *Legionella*, *M. avium* and nontuberculosis mycobacteria, and *P. aeruginosa*, as well as of two known amoeba hosts (*Acanthamoeba* spp. and *Hartmanella vermiformis*) in two chloraminated drinking water systems in the United States. The first water system, PCF, has been subjected to prior characterization of *L. pneumophila* and *M. avium*, as noted above, and is representative of a warm climate. The second, the Blacksburg-Christiansburg-VPI Water Authority (BCV), located in Virginia, has not been previously characterized and is representative of a temperate climate. Chloramination is of particular interest given that there is a general movement, particularly in the United States, to switch away from chlorine to reduce the risk of disinfection byproduct formation. The present study primarily employed quantitative PCR (qPCR) as a culture-independent approach for pathogen enumeration, the advantages of which include a low detection limit, high specificity, and high throughput. Differing sampling techniques were implemented to estimate the relative influences of premise plumbing (first-draw samples) versus the main water distribution system (after 3 min of flushing) environments. To explore the potential relationships between factors such as water age, opportunistic pathogen numbers, and microbial ecology, the broader microbial community structure in the bulk water and biofilm samples was profiled using terminal restriction fragment length polymorphism (T-RFLP) targeting 16S rRNA genes.

MATERIALS AND METHODS

Site locations and sampling procedures. BCV is located in southwest Virginia and serves a population of about 65,000, treating surface water by chlorination, flocculation, sedimentation, and dual-medium filtration. Chloramines have served as the disinfectant since June 2005, prior to which chlorine was used. Samples were collected from September 2010 to November 2010. The sampling plan was designed based on a water age model provided by the utility. Three to eight houses were selected for each of five water age ranges (3 to 6 days [$n = 4$], 6 to 8 days [$n = 8$], 8 to 10 days [$n = 5$], 10 to 12 days [$n = 6$], and ≥17 days [$n = 3$]; 3 had unknown water age). One-liter samples were collected before flushing the sampled tap (first draw), after flushing for 3 min (postflushing), and from the safety valve and bottom drain valve of corresponding water heaters, if available. The sampling procedure was performed in accordance with the U.S. Environmental Protection Agency (EPA) total coliform sampling guide, except a first-draw sample was included (28). Collected water samples were transported to the laboratory on ice within 2 h.

Legionella spp. and *Mycobacterium* spp. were historically reported to be prevalent in the PCF drinking water distribution system (54, 61), which serves over 640,000 people. Details of PCF were reported in a previous U.S. Centers for Disease Control (CDC) study (54), except that the source water has been adjusted to a blend of surface, ground, and desalinated water (at the time of the previous CDC study, the source water was 100% groundwater). Sampling took place in May 2011, targeting eight sites that were positive and seven sites that were negative for *Legionella* in the previous CDC study (54). Among the eight *Legionella*-positive sites, three were reported positive only when chlorine was used as the disinfectant and negative following the switch to chloramination. Four sites that were *Legionella* positive when chloramine was utilized were also previously *Legionella* positive when chlorine was used. The water sampling procedure was identical to that used for BCV, except that shower water samples were collected when available and biofilm samples were collected from taps and shower heads by swabbing the inner surfaces with sterile cotton. These additional samples facilitated comparison with a previous CDC study (54).

Water-quality analysis. Temperature, pH, total residual ammonia, and total residual chlorine were measured at the time of collection. pH was monitored using a portable pH 110 series meter (Oakton Research, Vernon Hills, IL). Total ammonia was measured using a DR2700 spectrophotometer (Hach, Loveland, CO) according to standard method 4500-NH_3 (2). Total residual chlorine was measured using a Hach chlorine pocket colorimeter according to Hach DPD colorimetric method 8167. NO_3^- was measured using a Dionex (Sunnyvale, CA) DX-120 ion chromatography apparatus according to standard method 4110 (2). Total organic carbon (TOC) was measured on a Sievers 800 portable TOC analyzer (GE, Boulder, CO) using standard method 5310A (2).

Water sample processing and DNA extraction. One liter of water was filtered through 0.22-μm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA), which were fragmented prior to extraction. DNA was extracted directly from cotton swabs for biofilm samples. DNA extraction was carried out using a FastDNA Spin kit (MP Biomedicals, Solon, OH) according to manufacturer protocol. For PCF samples, only half of the membrane was used for DNA extraction, and the other half was reserved for culturing.

qPCR. *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *Acanthamoeba* spp., *H. vermiformis*, *P. aeruginosa*, and total bacteria were enumerated by qPCR using previously published methods (5, 41, 58, 63, 64, 68, 79). All reactions were performed using a Bio-Rad (Hercules, CA) CFX96 real-time system in a final volume of 10 μl. Detailed information about primers, probes, and qPCR programs is provided in Table S1 in the supplemental material. The specificity of all qPCR assays except that for total bacteria was confirmed by cloning and sequencing of qPCR products from selected positive samples collected in this study (see Tables S2 to S8 in the supplemental material). For TaqMan assays, each 10-μl reaction mixture contained 5 μl of 2× SsoFast Probes Supermix (Bio-Rad), 250 nM each primer, 93.75 nM probe, and 1 μl of DNA template. For EvaGreen assays, each 10-μl reaction mixture contained 5 μl of 2×SsoFast EvaGreen Supermix (Bio-Rad), 400 nM each primer, and 1 μl of DNA template. DNA extracts, negative DNA controls

TABLE 1 Physicochemical properties of water in distribution systems BCV and PCF[a]

Water distribution system and source	Temp (°C ± SD)	pH ± SD	NH_4^+ (mg/liter ± SD)	NO_3^- (mg/liter ± SD)	Total Cl_2 (mg/liter ± SD)	TOC (mg/liter ± SD)
BCV						
First draw	20.8 ± 2.8	7.85 ± 0.24	0.58 ± 0.15	0.35 ± 0.05	2.02 ± 0.63	2.56 ± 1.18
Postflushing	19.7 ± 2.4*	7.85 ± 0.23*	0.63 ± 0.14	0.34 ± 0.05	2.21 ± 0.63	2.36 ± 2.96
Water heater	37.3 ± 8.4	8.02 ± 0.32	0.64 ± 0.10	0.37 ± 0.05	1.81 ± 0.62	2.87 ± 3.00
PCF						
Postflushing	26.8 ± 0.9*	7.63 ± 0.21*	0.45 ± 0.36	0.25 ± 0.21	2.15 ± 1.13	3.51 ± 2.68
Water heater	43.2 ± 6.0	7.66 ± 0.24	0.47 ± 0.33	0.24 ± 0.22	1.27 ± 0.93	5.84 ± 2.51

[a] The first draw data from PCF was not available. *, significant difference with 95% confidence ($P < 0.05$) in postflushing sample results between BCV and PCF.

(template DNA replaced by sterile Nanopure water), and 10-fold serial dilutions of standard DNA were included in triplicate in each qPCR run. Based on a serial dilution analysis, a sample dilution of 1:5 was determined to be effective for elimination of qPCR inhibition. Melt curve analysis was implemented on EvaGreen qPCR assays in order to verify specificity by ramping the temperature from 65 to 95°C at a rate of 0.5°C/5 s. The limit of quantification (LOQ) for all qPCR assays ranged from 1 to 10 gene copies/reaction and was implemented as appropriate for each specific run. For samples with a gene copy concentration near the LOQ (0 to 5 gene copies/μl), only samples that yielded a detectable threshold cycle (C_T) in all three triplicate experiments were considered to represent positive results. To determine the effective LOQ and recovery efficiency corresponding to upstream sample processing (i.e., membrane filtration and DNA extraction), *L. pneumophila*, *Legionella* spp., *M. avium*, *Mycobacterium* spp., *P. aeruginosa*, *H. vermiformis*, and *Acanthamoeba* spp. were spiked at defined concentrations in 500 ml of water and analyzed. Using this approach, LOQs were determined to be 32, 32, 170, 170, 114, 2.4, and 0.85 CFU or cells/ml, respectively. Linear models providing conversion between CFU and qPCR for targeted organisms as assayed in this study were established (see Fig. S1 and Table S9 in the supplemental material).

***Legionella* cultivation.** *Legionella* bacteria in PCF water samples were enumerated by colony count on buffered charcoal yeast extract (BCYE) agar (27) following a 30-min pretreatment at 50°C (49). Heat pretreatment instead of acid pretreatment was selected based on preliminary experiments demonstrating impairment of *Legionella* culturability by acid pretreatment. After 10 days of incubation at 37°C, the identity of *Legionella*-like colonies was verified by qPCR performed with both *Legionella* spp. and *L. pneumophila* (see Table S1 in the supplemental material).

T-RFLP. 16S rRNA genes were amplified on a Bio-Rad C1000 thermal cycler via nested PCR using fluorescently labeled primer 27f (5′-6-carboxyfluorescein [FAM]-AGAGTTTGATCMTGGCTCAG-3′) (66) and 907r (5′-CCGTCAATTCCTTTRAGTTT-3′) (39) with an annealing temperature of 50°C. The first round- and second-round amplification cycles were optimized to 15 and 30, respectively, in order to reach a balance between increasing T-RFLP profile resolution and minimizing PCR bias (59, 66). PCR products were purified using a GeneClean spin kit (MP Biomedicals, Solon, OH). Purified PCR products (10 μl) were digested with 20 U of HhaI (Promega, Madison, WI). Digested PCR products (1 μl) were mixed with 8.75 μl of formamide and 0.25 μl of GeneScan 500 LIZ size standard (Applied Biosystems [ABI], Foster, CA) and denatured at 95°C for 5 min followed by snap cooling in an ice bath prior to electrophoresis on an ABI 3130 genetic analyzer. T-RFs of between 50 to 500 bp with peak heights of ≥50 fluorescence units were identified using GeneMapper V 4.0 (ABI).

Statistical analysis. The Shapiro-Wilk test was used to test the normality of data sets. The Student *t* test was used to compare means of physiochemical parameter values. Since the log-transformed gene copy numbers were not normally distributed, the nonparametric Wilcoxon rank sum test was used to compare numbers of gene copies of targeted organisms. An equal- or given-proportion test was used to compare the detection rates. Analyses of correlations between different targeted organisms and physiochemical parameters were conducted using Spearman rank correlation analysis. The differences in Shannon diversity index values between samples with different water ages were compared using one-way analysis of variance (ANOVA) followed by pairwise comparison (pairwise *t* test). The differences in chloramine concentrations for samples with different water ages were compared using the nonparametric Kruskal-Wallis rank sum test followed by a multiple comparison test (kruskalmc). All of statistical tests named above were implemented by R (http://www.r-project.org/). Primer-E (Plymouth, United Kingdom) was employed to retrieve univariate indices (i.e., evenness, richness, and Shannon diversity index) and perform multivariate statistical analysis of T-RFLP profiles. The similarity of T-RF profiles, including accounting for T-RF peak heights, was examined using Bray-Curtis analysis (20), which generated bacterial community resemblance matrices for cluster analysis, multidimensional scaling (MDS), and analysis of similarity (ANOSIM). Global R values, generated by ANOSIM, fall between 0 and 1, indicating the degree of discrimination of sample groups. R = 1 indicates that all samples within the group are more similar to each other than to any sample from other groups. An R value of 0 indicates that the similarity between the groups and the similarity within the groups are the same on average (22). Analysis of >999 random permutations tested the null hypothesis that the bacterial community structures were similar. Biota and/or environmental matching (BEST) analysis was used to conduct correlation analyses of environmental parameters and microbial community data (21). Significance was set at a *P* value of ≤0.05.

RESULTS

Water quality characteristics. The water quality characteristics of the two distribution systems are presented in Table 1. The average temperature of the BCV water samples collected after flushing was approximately 7°C lower than that of the PCF samples ($P < 0.001$). Except for pH ($P = 0.002$), no other significant differences in water quality constituents were found between the two systems in postflushing water. However, a higher variance of the total chlorine concentration was noted in the PCF distribution system.

Occurrence of *Legionella*, mycobacteria, *P. aeruginosa*, and two species of amoeba. The frequencies of detection (FOD) and densities of *Legionella*, mycobacteria, *P. aeruginosa*, and two species of amoeba for the two water systems are presented in Tables 2 and 3. The highest FOD in BCV was seen with *Mycobacterium* spp. (94% of samples), followed by *Legionella* spp. (30% of samples). *L. pneumophila* was detected in 4 out of 27 (15%) *Legionella*-positive samples. *M. avium* was detected in 8 of 85 (9%) mycobacterium-positive samples. The average proportions of *L. pneumophila* and *M. avium* were approximately 15% and <0.1% of the total *Legionella* spp. and *Mycobacterium* spp., respectively, as determined on the basis of the assumption that all *Legionella* species, including *L. pneumophila*, carry 3 genome copies of 23S rRNA gene and that all mycobacteria, including *M. avium*, carry 1 genome copy of 16S

TABLE 2 Opportunistic-pathogen survey of drinking water distribution system BCV

Target organism	Occurrence rate (%)		Concn (no. of gene copies/ml ± SD)	
	Sites ($n = 29$)	Samples ($n = 90$)	Highest	Avg for positive samples
Legionella spp.	69.0	30.0	$2.3 \times 10^3 \pm 9.7 \times 10^2$	186.6 ± 458.2
L. pneumophila	13.7	4.4	13.7 ± 5.1	9.8 ± 4.4
Mycobacterium spp.	100	94.4	$1.8 \times 10^5 \pm 9.6 \times 10^4$	$1.4 \times 10^4 \pm 3.7 \times 10^4$
M. avium	24.1	8.9	1.9 ± 0.3	1.1 ± 0.6
H. vermiformis	27.6	14.4	$7.1 \times 10^4 \pm 4.4 \times 10^3$	$1.2 \times 10^4 \pm 2.0 \times 10^4$
Acanthamoeba spp.	13.7	6.7	6.8 ± 2.9	2.2 ± 2.4
P. aeruginosa	3.4	1.1	1.8 ± 0.3	1.8

rRNA gene (http://rrndb.mmg.msu.edu/search.php). The FOD of *H. vermiformis* was noted to be twice that of *Acanthamoeba* spp., with a significantly higher average density ($P < 0.001$). Only one sample was positive for *P. aeruginosa*, a water heater sample with a very low number of gene copies of 1.8 ± 0.3/ml.

In the PCF distribution system (Table 3), all 15 sites yielded positive detection results for *Legionella* spp. and *Mycobacterium* spp. The FOD of *L. pneumophila* and the FOD of *M. avium* were 20% and 33% of sites sampled, respectively. *Acanthamoeba* spp. were not detected in any water sample and were detected in only one biofilm sample. As observed in BCV samples, *H. vermiformis* was more prevalent than *Acanthamoeba* ($P < 0.001$). *P. aeruginosa* was detected at 2 of 15 sites in 1 biofilm and 3 water samples. Generally, no preference of biofilm versus bulk water was observed for any of the bacterial groups monitored ($P = 0.11$).

Detection of *Legionella* by cultivation. Colonies of *Legionella* spp., as confirmed by qPCR, were recovered from only 1 of 56 water samples (2%), at a density of 2 CFU/ml, in PCF.

Effect of 3-min flushing. Figure 1 compares the average gene copy numbers of *Legionella* spp., *Mycobacterium* spp., *H. vermiformis*, and total bacteria measured in first-draw and postflushing samples from BCV. The average densities of the targeted genes postflushing were 6- to 45-fold lower than those seen with the corresponding first-draw samples. These differences were significant for *Legionella* spp. ($P = 0.002$), *Mycobacterium* spp. ($P < 0.001$), *H. vermiformis* ($P = 0.018$), and total bacteria ($P < 0.001$). However, a significant effect of flushing was observed only for total bacterial 16S rRNA genes in PCF ($P = 0.032$), where it was not possible to impose an 8-h stagnation period prior to sampling. It was also noted that none of the postflushing samples were positive for *L. pneumophila* or *M. avium* in BCV. In PCF, the only site positive for *M. avium* in the first-draw sample was no longer positive after flushing; about a 10-fold reduction was observed for the only positive sample for *P. aeruginosa*.

Associations with biotic factors. Moderate to strong correlation between numbers of *Mycobacterium* spp. and total bacterial 16S rRNA gene copies were observed in BCV water samples ($\rho = 0.6216$ to 0.7729, $P < 0.001$) (Table 4); however, at PCF this same correlation was observed only in biofilm samples ($\rho = 0.7308$, $P < 0.001$). Low to moderate correlations between numbers of *H. vermiformis* and total bacterial 16S rRNA genes were displayed in water heater samples and biofilm samples in both distribution systems ($\rho = 0.3863$ to 0.6911, $P < 0.05$). Low to moderate positive correlations were found between *Legionella* spp. and *H. vermiformis* ($\rho = 0.3550$ to 0.5907, $P < 0.05$) in BCV but not PCF. *Mycobacterium* spp. also displayed weak to moderate correlations ($\rho = 0.3697$ to 0.5560, $P < 0.05$) with *H. vermiformis* in some sample types at both BCV and PCF. The only observed correlations for *Legionella* spp. were weak to moderate correlations with total numbers of bacterial 16S rRNA genes ($\rho = 0.4593$, $P = 0.008$) and *Mycobacterium* spp. ($\rho = 0.3786$, $P = 0.032$) in BCV water heater samples (Table 4).

Associations with abiotic factors. Moderate negative correlations were noted between gene copy numbers of *Mycobacterium* spp. and total chloramines ($\rho = -0.52$, $P = 0.004$) and between total bacterial 16S rRNA gene copy numbers and total chloramines ($\rho = -0.49$, $P = 0.007$) for the first-draw BCV samples. For PCF samples, no correlations of either of these groups with total chloramines were found. In BCV first-draw samples, numbers of *Mycobacterium* spp., *H. vermiformis*, and total bacterial 16S rRNA gene copies displayed low to moderate correlations with TOC ($\rho = 0.4$, $P < 0.05$). However, in PCF, only *Legionella* spp. were found to correlate with TOC and only in water heater samples

TABLE 3 Opportunistic-pathogen survey of drinking water distribution system PCF

Target organism	Occurrence rate (%)				Concn (± SD)			
					Highest		Avg for positive samples	
	Sites ($n = 15$)	Samples ($n = 80$)	Water ($n = 54$)	Biofilm ($n = 26$)	Water (no. of gene copies/ml)	Biofilm (no. of gene copies/swab)	Water (no. of gene copies/ml)	Biofilm (no. of gene copies/swab)
Legionella spp.	100	67.5	83.3	34.6	759.6 ± 285.7	$1.5 \times 10^6 \pm 1.8 \times 10^5$	100.8 ± 184.2	$2.2 \times 10^5 \pm 4.7 \times 10^5$
L. pneumophila	20	5.0	5.6	3.8	219.4 ± 23.8	$1.9 \times 10^4 \pm 1.1 \times 10^4$	90.4 ± 111.9	1.9×10^4
Mycobacterium spp.	100	93.7	98.1	84.6	$2.1 \times 10^4 \pm 4.2 \times 10^3$	$2.9 \times 10^7 \pm 8.1 \times 10^5$	$1.4 \times 10^3 \pm 3.5 \times 10^3$	$3.8 \times 10^6 \pm 8.1 \times 10^6$
M. avium	33.3	10	11.1	7.7	850.1 ± 458.7	$4.3 \times 10^5 \pm 3.9 \times 10^4$	38.4 ± 166.1	$9.1 \times 10^4 \pm 1.9 \times 10^5$
H. vermiformis	73.3	28.7	29.6	26.9	$5.1 \times 10^3 \pm 2.2 \times 10^2$	$4.6 \times 10^6 \pm 2.3 \times 10^5$	$781.7 \pm 1,408.0$	$1.8 \times 10^6 \pm 1.9 \times 10^6$
Acanthamoeba spp.	6.7	1.25	0	3.8	N/A[a]	$3.0 \times 10^4 \pm 5.2 \times 10^4$	N/A	3.0×10^4
P. aeruginosa	13.3	5.0	5.6	3.8	700.3 ± 158.7	$5.3 \times 10^4 \pm 5.5 \times 10^3$	340.6 ± 363.0	5.3×10^4

[a] N/A, not available.

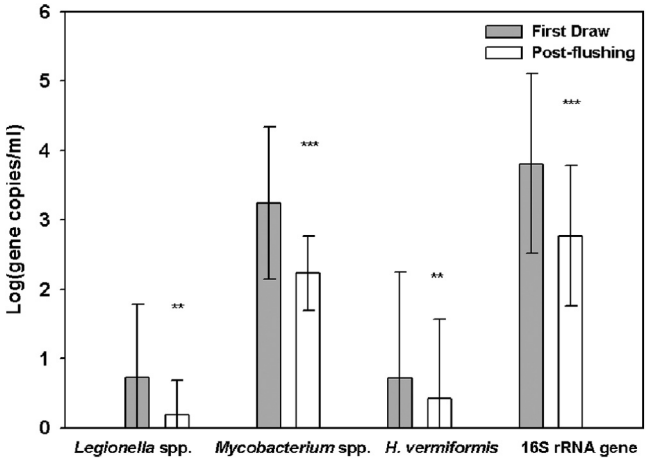

FIG 1 Average copy numbers of *Legionella* spp., *Mycobacterium* spp., *H. vermiformis*, and the 16S rRNA gene in first-draw and postflushing samples. Error bars represent the standard deviations of 29 log-transformed qPCR measurements [log(x + 1)] of target organisms in all first-draw and postflushing samples collected from BCV. ** and ***, significant differences according to paired Wilcoxon rank sum testing at the $P < 0.01$ and $P < 0.001$ levels, respectively.

TABLE 5 ANOSIM analysis of microbial community structures in different sample groups[a]

Factor	Water sample or system or pairwise test comparison	Global R value	*P* value
Site	BCV, PCF (postflushing samples)	0.298	**0.001**
Water age (days)	3 to 6, 6 to 8, 8 to 10, 10 to 12, ≥17	0.097	**0.003**
	3 to 6, 6 to 8	0.147	**0.041**
	6 to 8, 8 to 10	0.095	**0.028**
	8 to 10, 10 to 12	0.118	**0.01**
	10 to 12, ≥17	0.078	0.148
	3 to 6, 8 to10	0.103	0.054
	3 to 6, 10 to 12	0.222	**0.002**
	6 to 8, 10 to 12	0.06	0.09
	3 to 6, ≥17	0.12	0.057
	6 to 8, ≥17	0.069	0.205
	8 to 10, ≥ 17	−0.031	0.603
Sample type	Water sample, biofilm sample	−0.016	0.636
	First draw sample, flushing sample, water heater sample	0.009	0.297

[a] Paired date ranges represent pairwise tests. Bold values indicate significant difference with 95% confidence ($P < 0.05$).

(ρ = 0.73, P = 0.01). No correlations with temperature were found for either system.

Characteristics of the broader bacterial community. The broader bacterial communities of all samples were profiled by T-RFLP. Postflushing samples from BCV and PCF were pooled for ANOSIM, which demonstrated that the bacterial community compositions were significantly different between the BCV and PCF systems (global R = 0.298, P = 0.001) (Table 5). Three-dimensional (3D) multidimensional scaling (MDS) plots illustrated the separation of samples by location (Fig. 2). No significant clustering was observed among samples pooled into the categories of first draw, postflushing, and water heater (global R = 0.009, P = 0.297). Of the 26 biofilm samples collected from PCF taps and showerheads, no significant differences from corresponding water samples in the T-RFLP patterns were observed (P = 0.636).

The average values of the Shannon diversity index increased from 1.0 ± 0.8 at a water age of approximately 3 to 6 days to 2.1 ± 0.3 at a water age of >17 days. Significantly lower values of the Shannon diversity index were observed in samples with water ages of approximately 3 to 6 days and approximately 6 to 8 days compared to >17 days ($P < 0.05$). ANOSIM indicated a weak water age effect on bacterial community structure (global R = 0.097, P = 0.003) (Table 5). Further, pairwise tests revealed weak separation between samples with water ages of approximately 3 to 6 days and approximately 6 to 8 days, approximately 6 to 8 days and approximately 8 to 10 days, approximately 8 to 10 days and approximately 10 to 12 days, and approximately 3 to 6 days and approximately 10 to 12 days (R = 0.091 to 0.222, $P < 0.05$), which was also confirmed by MDS (see Fig. S2 in the supplemental material). BEST analysis was applied to determine the correspondence of bacterial community profiles to the environmental parameters reported in Table 1; however, no relationships were identified.

The wide variability observed in MDS plots (Fig. 3) indicates that the microbial community compositions of the main distribution systems differed dramatically from location to location. For all sampled BCV and PCF sites, the differences in bacterial community composition between first-draw and postflushing samples ranged from 16%

TABLE 4 Correlation analysis of relationship between different potential opportunistic pathogens in BCV and PCF[a]

Water distribution system and source	Spearman's rank correlation (*P*)					
	Legionella spp. vs 16S rRNA gene	*Mycobacterium* spp. vs 16S rRNA gene	*H. vermiformis* vs 16S rRNA gene	*Legionella* spp. vs *Mycobacterium* spp.	*Legionella* spp. vs *H. vermiformis*	*Mycobacterium* spp. vs *H. vermiformis*
BCV						
First draw	0.3243 (0.086)	**0.7729 (<0.001)**	0.2650 (0.165)	0.3619 (0.054)	**0.5907 (<0.001)**	**0.4020 (0.031)**
After flushing	0.0756 (0.699)	**0.6216 (<0.001)**	0.3089 (0.103)	0.2932 (0.123)	**0.4356 (0.018)**	**0.3697 (0.048)**
Water heater	**0.4593 (0.008)**	**0.7401 (<0.001)**	**0.3863 (0.029)**	**0.3786 (0.033)**	**0.3550 (0.046)**	0.2283 (0.209)
PCF						
First draw	0.4372 (0.103)	0.5107 (0.054)	0.4633 (0.082)	0.0860 (0.760)	0.4124 (0.127)	**0.5560 (0.031)**
After flushing	0.2901 (0.294)	−0.0036 (0.995)	−0.2017 (0.471)	0.0090 (0.975)	0.3839 (0.158)	0.0733 (0.795)
Water heater	0.4231 (0.152)	0.5494 (0.055)	**0.6911 (0.009)**	0.5549 (0.052)	0.3195 (0.287)	0.4644 (0.110)
Biofilm	0.2516 (0.215)	**0.7308 (<0.001)**	**0.5107 (<0.001)**	0.1972 (0.334)	0.3541 (0.076)	**0.4853 (0.012)**

[a] Correlation results were presented in the form of Spearman's rank correlation (ρ). *P* values were indicated in parentheses. Bold values indicate significant difference with 95% confidence ($P < 0.05$).

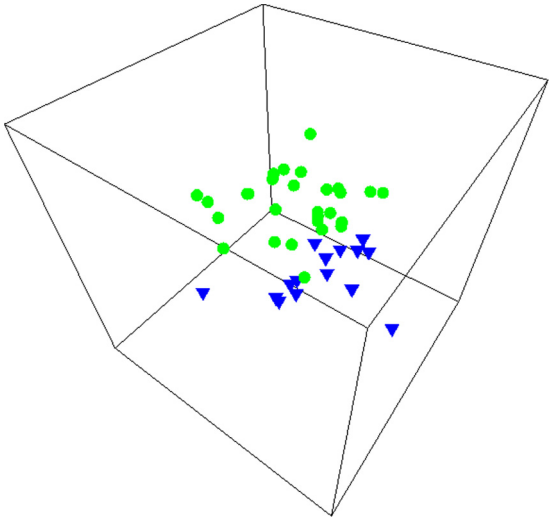

FIG 2 Multidimensional scaling analysis of bacterial community composition (T-RFLP profiles) for postflushing samples from BCV and PCF. Green round symbols represent samples from BCV. Blue inverted triangle symbols represent samples from PCF. Note: one sample from BCV was excluded from analysis due to an absence of T-RFLP peaks.

to 100%. More than half (55%) of the sampled sites demonstrated a greater than 50% change after flushing (Fig. 3).

DISCUSSION

Water quality characteristics. The relatively lower average temperature in BCV postflushing samples was as expected, considering the difference in seasons and latitudes (10°) and may account for some of the differences observed between the two systems. Water heater temperatures in both systems were also lower on average than the recommended range for pathogen control (49 to 60°C) (50). Temperature stratification has recently been noted to be a widespread phenomenon in water heaters, typically resulting in colder temperatures at the bottom of the water heater (11), highlighting a key issue of concern for better protecting public health.

Occurrence of *Legionella*, mycobacteria, *P. aeruginosa*, and two species of amoebas. It is interesting that the two distribution systems shared similar patterns of targeted organism occurrence, despite their geographic separation. Both systems were characterized by high FODs of *Legionella* spp. and *Mycobacterium* spp., low FODs of *L. pneumophila* and *M. avium*, and rare detection of *P. aeruginosa.* It is not clear whether the season contributes to the pattern similarity, since sampling at both locations occurred during warm seasons (spring and fall). A seasonal effect on *Legionella* diversity or distribution has been found in one location of a French pristine river (60) and in cooling towers (74). It is possible that seasonal factors could have been at play in the present study by influencing the bacterial growth environment (e.g., temperature, humidity, and water chemistry) and operation of premise plumbing (e.g., water heater usage).

In addition to *L. pneumophila*, several members of *Legionella* such as *L. longbeachae*, *L. micdadei*, *L. bozemanii*, and *L. dumoofffii* are documented human pathogens (56). High diversities of *Legionella*, including pathogenic species, have been observed in drinking water treatment plants and treated drinking water in the Netherlands (80, 81). In these studies, *L. pneumophila* accounted for only 0.1% to 1% of the total *Legionella* spp.; instead, *L. bozemannii* predominated in the clone library, which is in agreement with the finding of the present work. However, *L. pneumophila* has been observed to be the most frequently isolated species in several other premise plumbing field surveys (6, 10, 45, 52). For example, *L. pneumophila* accounted for 93% of *Legionella*-positive samples in a culture-based survey of hot water systems in Italy (6). The physiologic and genetic basis for the survival and persistence of *Legionella* species in drinking water systems remains elusive. Many factors such as water source, water treatment processes and conveyance, and the characteristics of the premise plumbing likely play a role in permitting certain *Legionella* species to persist.

Similarly, a high diversity of mycobacteria, some of which, such as *M. avium*, *M. intracellulare*, *M. kansasii*, *M. abscessus*, and *M. chelonae* (33, 47, 65, 73, 77), are known to be opportunistic pathogens, has been demonstrated to be characteristic of the drinking water environment; among those species, *M. avium* is currently on the U.S. EPA candidate contaminant list. In the present study, a considerable number of mycobacteria were detected, accounting for 0.1% to 68% of total bacterial estimate, assuming

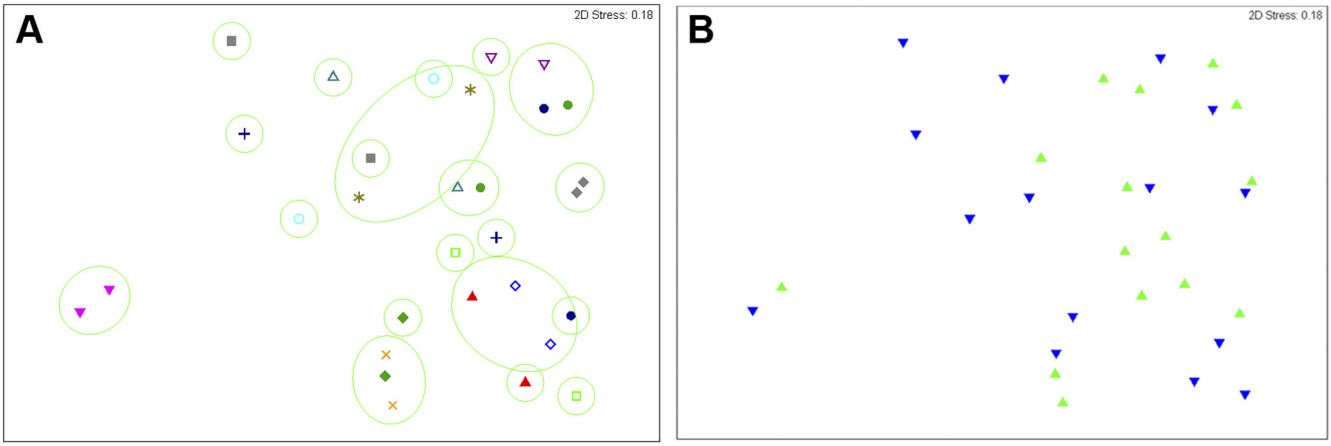

FIG 3 Multidimensional scaling analysis of bacterial community composition (T-RFLP profiles) for first-draw and postflushing samples from PCF. (A) Each symbol pair represents paired first-draw and postflushing samples from the same location ($n = 15$). The green circles represent a similarity level of 50% as determined by cluster analysis. (B) Green symbols represent first-draw samples and blue symbols postflushing samples.

that each bacterium has a single copy of the 16S rRNA gene. *Mycobacterium* spp. in postflushing samples ranged from 15 to 3.0×10^3 gene copies/ml (average = 495 gene copies/ml), which is comparable to qPCR estimation of levels of *Mycobacterium* spp. in hospital tap water (1.0×10^3 to 2.0×10^7 CFU/500 ml) (36) but higher than reported levels of *Mycobacterium* spp. in drinking water systems (e.g., 10×10^5 to 7×10^5 CFU/liter [33], 15 to 140 CFU/liter [72], and 1 to 1,000 CFU/500 ml [77]) determined by culturing methods according to the assumption that each mycobacterium has one genome copy of the 16S rRNA gene. Note that all qPCR assays applied in this study yielded slightly higher estimates than CFU counts (slope of 1.2 to 1.6), except the *Mycobacterium* and *M. avium* qPCR assays, which yielded lower estimates (slope of 0.8 to 0.9) (see Fig. S1 and Table S9 in the supplemental material). This is likely due to the greater losses of mycobacteria during upstream processing, such as filtration and DNA extraction, accounted for in the qPCR estimate. Mycobacteria are notorious for such analytical challenges imposed by the unique hydrophobic and impermeable nature of their cell envelope (3). Nonetheless, the results reported here support the conclusion of the previous CDC study that switching to chloramines favors the growth of *Mycobacterium* spp. (54). The prevalence of *Mycobacterium* spp. is likely to further increase the incidence of disease, particularly through aerosol inhalation by susceptible individuals. Identical DNA fingerprints found for samples from patients with mycobacterial pulmonary disease and household plumbing isolates has indicated that *Mycobacterium* species inhabiting premise plumbing are a probable source of pulmonary infection (30, 32).

A recent literature review examining drinking water systems across 14 different countries provides evidence of ubiquitous free-living amoebas in treated drinking water, especially in reservoirs and in premise-plumbing tanks. In particular, *Acanthamoeba* and *Hartmanella* were the most frequently identified genera in tap water (70). In the present study, the FOD and abundance of *Acanthamoeba* spp. were significantly lower than those of *H. vermiformis.* It has been suggested that *Hartmannella* spp. are more ecologically relevant than *Acanthamoeba* spp. in premise plumbing. Thomas and colleagues (71) reported that the dominance of *Acanthamoeba* spp. over *Hartmanella* spp. was reversed in a drinking water treatment plant after sand filtration. In a biofilm batch test examining protozoan hosts for *L. pneumophila* under a range of controlled water conditions, indigenous *H. vermiformis* grew whereas *Acanthamoeba* spp. did not (75).

Detection of *Legionella* by cultivation. *Legionella* are of particular interest due to the severe pneumonia that they can cause and the relatively high number of reported outbreaks associated with drinking water systems (17). To provide some comparison to the previous CDC study (54), PCF water samples were cultured for recovery of *Legionella* spp. It is of interest that the only culture-positive sample was collected from a water heater where *L. pneumophila* water had been detected during both the chlorine and chloramine disinfection phases of a previous CDC study (54). Consistent with these observations, the culture-positive sample also yielded the highest gene copy numbers of *Legionella* spp. and *L. pneumophila* according to qPCR. However, the qPCR estimate (220 cells/ml based on one gene copy per cell [40]) was about 100-fold higher than the culture-based estimate. A similar difference in magnitude between qPCR and culturing of *L. pneumophila* was reported for cooling towers (44).

One possible explanation for the lower estimates of frequency and density of culture-positive *Legionella* samples is a higher detection limit of the cultivation method used here compared to the CDC method (54). A recent pilot study for the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) program demonstrated that interlaboratory differences in *Legionella* culture enumeration were as high as 1,660-fold (51). Importantly, the optimal method for isolation from environmental samples appears to be sample specific and *Legionella* concentration dependent (7, 25, 49) and can vary greatly as a result of sample shipping and processing (53). qPCR is also generally subject to a higher estimate of *Legionella* because of detection of DNA from dead cells (67, 82) but also because it can detect cells in a viable but nonculturable (VBNC) state, which has been widely reported in other drinking water studies (26, 80, 81). Another possible explanation for the low recovery of *Legionella* isolates could have been the reported fall in numbers of *Legionella* spp. following the switch to chloramine disinfection (54). Finally, additional sample pretreatment steps required for culturing could diminish recovery relative to qPCR.

Effect of 3-min flushing. Postflushing samples are representative of the water distribution system, whereas first-draw samples are indicative of the premise plumbing. The reduction of numbers of *Legionella* spp., *Mycobacterium* spp., and *H. vermiformis* and of 16S rRNA gene densities in postflushing samples suggests that even when the distribution system itself is highly chloraminated, conditions in premise plumbing may still permit the persistence and growth of bacteria and protozoa harbored in the biofilm. Greater differences were observed between first-draw samples and postflushing samples in BCV relative to PCF, which was likely due to longer water stagnation time for BCV. Residents in BCV were advised to abstain from water use for 8 h prior to the sampling. However, it was impractical to impose the same restriction in PCF due to the limits of sampling time and building complexity.

The impact of flushing on numbers of bacteria and amoebas is consistent with findings of a study investigating the influence of overnight stagnation on bacterial densities assayed by flow cytometry, adenosine triphosphate (ATP) concentration, and heterotrophic plate counts (HPC) (43). Samples taken after overnight stagnation (first draw) yielded 2-to-3-fold, 2-to-18-fold, and 4-to-580-fold-higher cell numbers, ATP concentrations, and HPC, respectively. The differences in water quality between the premise plumbing and main distribution system reinforce prior research indicating that routine monitoring as employed by water utilities does not reflect conditions (e.g., stagnant periods) encountered in premise plumbing (57). The differences also demonstrated regrowth of bacteria and protozoa in premise plumbing, indicating that qPCR is capable of capturing information about live cells in the system.

Associations with biotic and abiotic factors. The positive correlation between *H. vermiformis* and total bacterial numbers is likely a direct reflection of *H. vermiformis* grazing on bacteria for food (78). However, this relationship was observed only in water heaters and was not strong ($\rho < 0.7$), indicating that other environmental factors may account for *H. vermiformis* growth. Furthermore, *H. vermiformis* and other amoebas can serve as hosts for amoeba-resisting bacteria like *Legionella* spp. and *Mycobacterium* spp. (8, 14). Observed correlations between *Legionella* spp. and *H. verimformis* in BCV were possibly a reflection of pathogen-host effect. However, the absence of strong correlations between pathogens and hosts in either BCV or PCF suggests the impor-

tance of the broader microbial ecology of drinking water systems, representing complex interactions involving both abiotic and biotic factors. For example, cell-to-cell interaction between microbes can either stimulate or inhibit the growth of opportunistic pathogens in drinking water. Specifically, one isolate of *B. subtilis* was able to reduce *L. pneumophila* numbers in either the absence or presence of amoebae by lysing *Legionella* cells or inhibiting internal replication in amoebae, respectively (69). Among members of one collection of aquatic bacteria, 66% inhibited *L. pneumophila* growth (35).

Limiting TOC has been proposed as a potential control measure for limiting pathogen regrowth in drinking water distribution systems. Previous studies linked heterotrophic plate counts (HPC) and mycobacterial abundance to AOC (29, 33, 72, 76). This study also identified a correlation of 16S rRNA genes, *Mycobacterium* spp., and *H. vermiformis* with TOC in BCV first-draw samples. However, low ρ values indicated that TOC is not the only factor contributing to their growth. Furthermore, the fact that the correlation existed only in first-draw samples and that 16S rRNA genes also correlated suggests that the true correlation may have been with biofilm constituents, which are detectable by TOC but do not represent actual TOC from the distribution system. The strong correlation between *Legionella* spp. and TOC in PCF water heater samples and the absence of such a relationship in other samples implies that TOC may be a factor favoring growth of *Legionella* spp. under certain conditions. Further laboratory control experiments are needed in order to better delineate potential relationships with TOC.

While temperature is known to be a critical factor for controlling opportunistic pathogen occurrence (30, 34, 42, 52, 55), no correlation between *Legionella* or mycobacteria and temperature was observed in this study. This was not unexpected, because temperature typically elicits a threshold response at extremes and the temperatures captured in this study were not necessarily representative of the narrow range corresponding to a linear growth rate response.

Characteristics of the broader bacterial community. ANOSIM demonstrated that the bacterial community compositions were significantly different between the BCV and PCF systems, which could be explained by the geographic separation and water source difference as well as the different physical characteristics of these two systems. In BCV, all of the transmission lines are cement-lined ductile iron pipe. In contrast, PCF pipes are mainly made of copper (70% to 75%) and PVC (25% to 30%). Several studies have previously demonstrated a significant influence of pipe materials on microbial community structure in simulated drinking water systems (37, 46, 83).

The low Shannon diversity index in samples with a water age of 3 to 6 days might be attributable to relatively higher concentrations of chloramine (average = 2.6 ± 0.2 mg/liter, $P < 0.05$). ANOSIM demonstrated that water age has a weak effect on the bacterial community structure (Table 5). Even within the same water age group, the similarity of the bacterial community profiles among the samples was as low as 0, indicating that water age is not the sole driving factor. Further, BEST analysis failed to identify any relationship between the microbial communities and environmental parameters measured in Table 1. It is likely that other characteristics of the main distribution systems and premise plumbing not investigated in the present study, such as pipe age and materials, are also significant drivers of the broader microbial community composition.

The 3-min flush caused visible microbial community change in postflushing water samples in this study. Likewise, a considerable change was noted in bacterial community composition by denaturing gradient gel electrophoresis after a 5-min flush in household water (43). Slight increases in evenness and richness indices were also noted in the present study after flushing. The visible microbial community changes between first-draw and postflushing samples further call into question the validity of routine monitoring methods, which may overlook important microbes that reside in premise plumbing (57) and that are the source of direct human exposure.

Conclusions. This report provides a comprehensive and quantitative snapshot of the prevalence of *L. pneumophila* and other *Legionella*, *M. avium* and other mycobacteria, *P. aeruginosa*, and two amoeba hosts in two representative chloraminated drinking water distribution systems. The results confirmed that drinking water systems, especially premise plumbing, are a reservoir for microorganisms and pathogens even in the presence of high chloramine residuals. Overall, this study demonstrated the value of qPCR for simultaneously monitoring genetic markers of multiple opportunistic pathogens and their protozoan hosts. These microorganisms are unique in comparison to traditional pathogens of concern (i.e., those leaving the drinking water treatment plant, typically of fecal origin) because they readily inhabit drinking water distribution systems and in-home plumbing. Thus, although qPCR does not provide a direct measure of viable organisms, it does provide a measure of organisms that were presumably viable at one point in time in the distribution system. Regrowth of organisms in first-draw samples representative of premise plumbing illustrates this principle and also highlights the fact that general water distribution system monitoring approaches likely overlook regrowth of opportunistic pathogens in premise plumbing, which is the front line of exposure to consumers. Nonetheless, future effort is merited to translate gene copy numbers into meaningful risk criteria, which currently are based on culture measurements. The relationships identified among targeted bacteria and amoebas, as well as associations between their numbers and abiotic environmental factors identified in the study, shed light on the importance of understanding drinking water pathogen ecology in order to better discern the mechanisms of opportunistic pathogen persistence in drinking water. Further, investigations of the influence of disinfectant type, pipe materials, and water age, in conjunction with microbial ecology, on opportunistic pathogen occurrence and persistence under controlled laboratory conditions would be beneficial in order to identify the most critical factors that could be modified to limit opportunistic pathogen proliferation in drinking water systems.

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