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Distribution System Water Quality Affects Responses of Opportunistic Pathogen Gene Markers in Household Water Heaters

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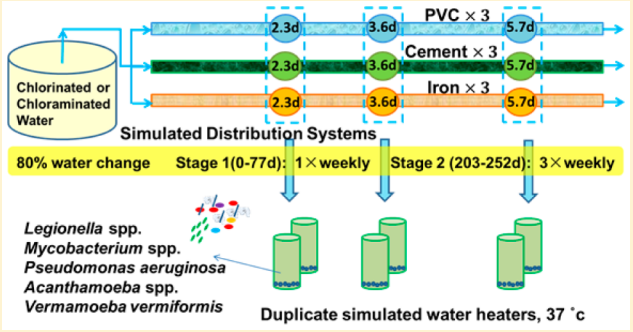
Supporting Information

ABSTRACT: Illustrative distribution system operation and management practices shaped the occurrence and persistence of *Legionella* spp., nontuberculous mycobacteria (NTM), *Pseudomonas aeruginosa*, and two amoebae host (*Acanthamoeba* spp., *Vermamoeba vermiformis*) gene markers in the effluent of standardized simulated household water heaters (SWHs). The interplay between disinfectant type (chlorine or chloramine), water age (2.3−5.7 days) and materials (polyvinyl chloride (PVC), cement or iron) in upstream simulated distribution systems (SDSs) profoundly influenced levels of pathogen gene markers in corresponding SWH bulk waters. For example, *Legionella* spp. were 3−4 log higher in SWHs receiving water from chloraminated vs chlorinated SDSs, because of disinfectant decay from nitrification. By contrast, SWHs fed with chlorinated PVC SDS water not only harbored the lowest levels of all pathogen markers, but effluent from the chlorinated SWHs were even lower than influent levels in several instances (e.g., 2 log less *Legionella* spp. and NTM for PVC and 3−5 log less *P. aeruginosa* for cement). However, pathogen gene marker influent levels correlated positively to effluent levels in the SWHs ($P < 0.05$). Likewise, microbial community structures were similar between SWHs and the corresponding SDS feed waters. This study highlights the importance and challenges of distribution system management/operation to help control opportunistic pathogens.



INTRODUCTION

Opportunistic pathogens residing in premise (i.e., building) plumbing systems, including bacteria (e.g., *Legionella pneumophila*, nontuberculous mycobacteria (NTM), *Pseudomonas aeruginosa*), and protozoa (e.g., *Acanthamoeba* spp.), have become an emerging public health threat, especially for immunocompromised populations.[1−5] Unlike traditional fecal pathogens, opportunistic pathogens naturally inhabit and proliferate within water distribution systems and household plumbing, posing a unique challenge for their mitigation.[6] Opportunistic pathogens are typified by various features, such as being oligotrophic,[7] heat-tolerant (e.g., can survive in >60 °C water heaters),[7,8] and disinfectant-resistant,[9,10] which make them strong competitors in drinking water environments, especially in premise plumbing. In addition, *Legionella*, NTM, and *P. aeruginosa* are capable of using amoebae as vehicles for protection and replication,[11−13] which enhances their survival in drinking water systems.

Premise plumbing is characterized by high surface-area-to-volume ratios, long retention times, and warm temperatures[14] and is recognized as an important reservoir and source of exposure (e.g., aerosol inhalation during showering, direct skin contact) of opportunistic pathogens.[6,15] Premise plumbing receives water from the main distribution system with varying physiochemical and biological characteristics, which can be ascribed to different distribution system properties, such as treatment and disinfection processes,[16,17] pipe materials and integrity,[18−21] and water age.[22,23] Occurrence and proliferation of opportunistic pathogens have been linked to some physiochemical parameters such as disinfectant type and concentrations,[24] trace metals,[25,26] assimilable organic carbon,[27] and temperature.[24] However, a controlled demonstration of how drinking water from upstream mains affects downstream opportunistic pathogen regrowth in premise plumbing is lacking. In particular, it is unclear to what extent the changes in water quality that occur during transport to homes located different distances from the treatment plant and through different pipe materials, might affect downstream opportunistic pathogen regrowth. Household water heaters are a particularly critical component of the premise plumbing because they are known reservoirs of several opportunistic pathogens,[16,28,29]

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especially when operated at lower temperatures that may save energy, but are conducive to pathogen growth. Establishing an understanding of the interplay between upstream distribution system water quality and regrowth of pathogens in premise plumbing can inform mitigation strategies, such as selective manipulation of engineering factors (disinfectant, pipe materials, etc.) to deliver water from the distribution system that might reduce opportunistic pathogen numbers in premise plumbing and protect consumers from exposure.[6]

The objective of this study was to investigate the influence of water quality from the main distribution system on the occurrence and persistence of *Legionella* spp., NTM, *P. aeruginosa* and two genera of host amoebae (*Acanthamoeba* spp., *Vermamoeba vermiformis*) in household water heaters. Simulated distribution systems (SDSs) with different disinfectants (chlorine, chloramines) and pipe materials (cement, iron, PVC) were operated to create influent with water age of 2.3, 3.6, and 5.7 days[23,30,31] to simulated household water heaters (SWHs). The SWHs were held at a constant temperature (37 °C) with infrequent water changes (1−3× per week), to track microbial changes during "worst case" stagnation events typical of weekends/holidays for premise plumbing and isolate the variables represented by the SDS water. Numbers of gene copies corresponding to opportunistic pathogens and ecologically relevant microorganisms were determined by quantitative polymerase chain reaction (q-PCR) as an indicator of their tendency to regrow or attenuate in the SWH effluent, relative to the influent. Differences in SWH response to the array of SDS influents illustrates the effect of utility management, water main distribution system material selection and distance from the treatment plant on opportunistic pathogen occurrence in premise plumbing and potential for consumer exposure.

MATERIALS AND METHODS

Simulated Distribution Systems. The details of the SDSs have been previously described.[23] In brief, six triplicate SDSs comparing iron, cement, and PVC pipe materials were fed with either chlorinated (4.0 mg/L) or chloraminated (4.8 mg/L) tap water (Supporting Information Figure S1), considering the upper bound of allowed drinking water disinfectant residuals in U.S.[32] Water ages of 2.3, 3.6, and 5.7 days were achieved by maintaining the flow rate at 0.40 ± 0.005 mL/min in all SDSs, which were operated in parallel at 20 °C for about 6 months prior to SWH setup. Sixty-milliliters of water were collected from each of the triplicate pipes and combined aseptically into a glass bottle before feeding the SWHs, resulting in 18 distinct influent waters (i.e., 2 disinfectants ×3 pipe materials ×3 water ages).

Simulated Water Heaters. Thirty-six French square glass bottles (120 mL) were used to simulate 18 water heater conditions in duplicate by incubating at 37 °C, a temperature favorable to microbial growth and common at the bottom of water heaters[29] and in hot water recirculating systems.[28] Glass beads incubated in granular-activated-carbon (GAC)-filtered tap water for 10 days were transferred to each SWH to establish drinking water biofilm and indigenous microbes in water heaters. q-PCR analysis of glass bead biofilms revealed naturally occurring *Legionella* spp., *Mycobacterium* spp., and *V. vermiformis* from GAC-filtered water. In order to simulate low water usages representative of situations routinely occurring in schools and offices during the weekends and vacation homes during off-seasons, 80% water changes were performed, using 10 mL pipet, once per week during Stage 1 (days 0−77) (except for the first week during which two water changes occurred) and three times per week in Stage 2 (days 203−252). Weekly water changes were performed during days 77−203 for maintenance.

Water Quality Analysis. Free and total chlorine, dissolved oxygen (DO), total organic carbon and pH were measured every 2 weeks in the SDSs, as reported previously.[23] Disinfectant residuals were also measured in SWH effluents using the same method for SDS water.

Sample Collection and DNA Extraction. Sampling of SDS water was performed twice over the whole experiment as previously reported,[23] representative of each SWH operational stage. The first sampling occurred just prior to SWH setup (SWH day −1); while the second was performed in the middle of Stage 2 (SWH day 240). Freeze-drying was used to concentrate SDS water samples.

For SWHs, 11 main sampling events (i.e., samples were taken after 7-day stagnation for Stage 1 (6 samplings) and 2-day stagnation for Stage 2 (5 samplings)) were performed during the experiment. An additional four samplings occurred prior to the main samplings in order to assess initial microbial composition at the beginning of each stage: two in the first week after SWH setup and two immediately prior to switching to three water changes/week. Eighty-milliliters of water were filtered through a 0.22 μm-pore-size mixed cellulose ester filter (Millipore, Billerica, MA) and subject to DNA extraction. One biofilm sample was collected from glass beads (SWH day −1) in order to explore the microbiome introduced to SWHs. DNA extractions were performed using FastDNA SPIN kit according to manufacturer's instruction (MP Biomedicals, Solon, OH).

q-PCR. q-PCR methods (Supporting Information Table S1) and quality control for 16S rRNA genes (i.e., indicative of total bacteria), *Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba* spp., and *V. vermiformis* (referred as "target microorganisms" thereafter) were reported previously[29] and consistently applied in all samples in this study. In order to assess recovery efficiencies of different concentration methods for SDS and SWH water samples, a series of 10-fold diluted *Legionella pneumophila* cultures were spiked into 80-ml drinking water, which was subject to membrane filtration and freeze-drying in parallel prior to DNA extraction. Supporting Information Figure S2 demonstrated a linear relationship between log-transformed data from the two concentration methods, with a higher recovery rate noted for membrane filtration. Therefore, a conversion equation of "log-(Y(membrane filtration) + 1) = 1.07log(X(freeze-drying) + 1) + 0.12" was applied to previously published SDS q-PCR data,[23] to compensate for potential discrepancies resulting from different sample concentration methods prior to DNA extraction, and replotted in Figure 1.

Illumina Sequencing. A subset of representative SWH samples (day 4 (prior to main samplings), 35 (during Stage 1), and 236 (during Stage 2) after SWH setup, n = 108), SDS water samples (n = 36), and one glass bead biofilm sample (day −1) were subject to Illumina sequencing of 16S rRNA gene amplicons. DNA extracted from triplicate SDSs[23] were combined by equal volume for each condition prior to PCR amplification in order to represent the overall microbiomes entering SWHs. 16S rRNA genes were amplified with barcoded primers 515/806R,[33] the PCR products of which were quantified, combined, and purified according to the Earth Microbiome Project 16S Illumina Amplification Protocol

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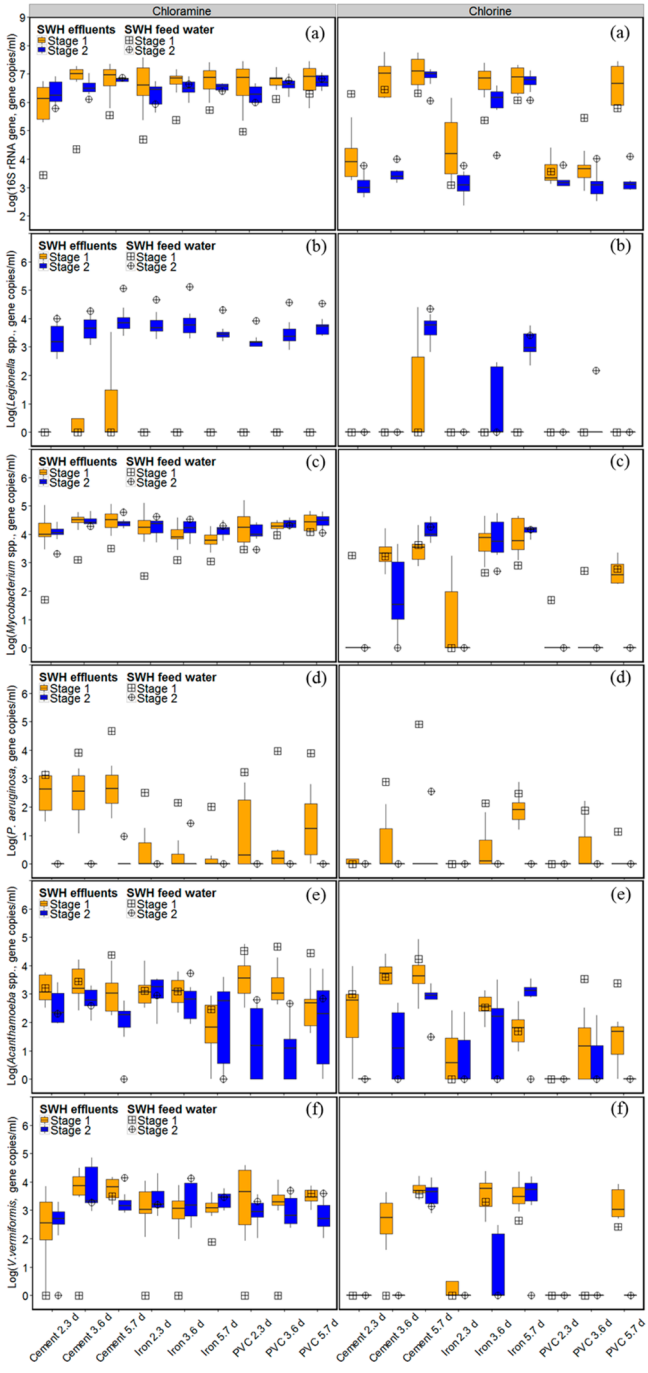

Figure 1. Enumeration of 16S rRNA gene, *Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba* spp., and *V. vermiformis* in effluents of simulated water heaters (SWHs) subject to 80% water changes at the following frequencies: Stage 1 = 1 × weekly, Stage 2 = 3 × weekly. ⊞ and ⊕ indicate gene copy numbers of target organisms in SWH feedwater determined in two previously reported[23] sampling events representative of Stage 1 and Stage 2, respectively. Data are $\log_{10}(x + 1)$ transformed. SDS data were adjusted to compensate for different sample concentration methods using the conversion equation in Supporting Information Figure S2. (a) 16S rRNA gene, (b) *Legionella* spp., (c) *Mycobacterium* spp., (d) *P. aeruginosa*, (e) *Acanthamoeba* spp., (f) *V. vermiformis*.

(http://www.earthmicrobiome.org/emp-standard-protocols/16s/) prior to sequencing on an Illumina Miseq benchtop sequencer using pair-end 250 bp kits at the Virginia Bioinformatics Institute. DNA sequences have been deposited into the European Nucleotide Archive (Accession Number: PRJEB8874).

Read pairs were assembled and processed by following the MiSeq protocol (http://www.mothur.org/wiki/MiSeq_SOP, accessed June, 2014) using Mothur 1.32.1. In order to ensure equivalent sequencing depth, subsampling 40 185 sequences from each sample was performed in consideration of sequencing completeness and reservation of the majority of samples for downstream analysis. Sequences were assigned to operational taxonomic units (OTUs) at a 3% dissimilarity level, which was used to calculate Bray−Curtis similarities using Primer-E 6.0 (Plymouth, United Kingdom). Multidimensional scaling analysis (MDS), cluster analysis, and analysis of similarity (ANOSIM) derived from Bray−Curtis similarity matrices were used to examine the degree of microbial community structure discrimination among different sample groups. The RELATE command of Primer-E was used to explore the correlations between similarity matrices from different SWH sampling days. Each sequence was classified against the Ribosomal Database Project 16S rRNA training set, with an 80% pseudobootstrap confidence score.

Data Analysis and Statistics. R[34] was used to perform statistical comparisons. Gene copy numbers were log transformed (log(x+1)) prior to analysis. Parametric one-way analysis of variance (ANOVA) or nonparametric Kruskal−Wallis ANOVA was performed to compare target microbes in various SWH effluents depending on the normality and variance of the data, which was followed by corresponding multiple comparison analysis. The nonparametric Wilcoxon rank sum test was used to compare the target microorganisms between two groups. The correlations between target microorganisms in SDSs (average numbers from triplicate pipes) and SWHs, and between target microorganisms in SWHs and disinfectant residuals in SDSs were conducted using Spearman rank correlation analysis. Statistical significance was set at $P < 0.05$.

RESULTS

Properties of SWH Influents. As previously reported,[23,31] the SDSs successfully generated a matrix of waters with a wide range of physiochemical properties (Supporting Informaiton Table S2), reflecting trends known to occur in practice as water is transported through distribution systems and before it enters buildings. Briefly, disinfectant concentration and DO decreased with increasing water age in all SDSs, while TOC increased ($P < 0.05$). pH increased ∼1 unit in cement SDSs due to lime leaching. The chlorine residuals were highest in the PVC SDSs, particularly on day 2.3 and 3.6 ($P < 0.05$), followed by cement and iron SDSs. One atypical trend compared to practical field experience was faster chloramine decay relative to chlorine (Supporting Information Table S2), which was a result of nitrification evidenced by increased nitrite level and presence of nitrifiers in chloraminated SDSs.[23,31]

Variation of Microbial Numbers in SWH Influent and Effluent. Although not a direct measure of viability, numbers of gene markers in and out of SWHs with time were considered as indicators of regrowth or attenuation in the SWHs throughout the study.

Higher levels of virtually all target microorganisms were present in the influent to SWHs receiving water from the chloraminated SDSs relative to chlorinated SDSs (Figure 1), consistent with much lower levels of disinfectant residuals (0.02−0.36 mg/L) in the chloraminated SDSs[23] versus the

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chlorinated systems (0.06−2.57 mg/L) due to nitrification. Higher influent chlorine residuals were correlated with lower gene copy numbers of target microorganisms in chlorinated SWHs (ρ = −0.7759 ∼ -0.3349, P < 0.05) (Supporting Information Table S3). In contrast, only weak correlations were occasionally observed between total chlorine and 16S rRNA genes, *Legionella* and *Mycobacterium* in chloraminated SWHs (ρ = −0.4715 ∼ −0.2731, P < 0.05) probably due to the much lower levels of residuals.

In terms of effluents from the SWHs, the dominant trend was higher levels of virtually all microbial DNA in effluent of reactors fed chloraminated water. The SDS sampling events provided two snapshots for comparing overall SDS and SWH trends. Correlation analysis was performed between target microbial numbers in SWH effluents and the most recent SDS sampling days (i.e., SWH day 14 for Stage 1, day 245 for Stage 2), assuming negligible temporal variation of SDS water quality during this short time period (1−2 weeks). Positive correlations were noted for all four target microorganisms (ρ = 0.4736−0.8277, P < 0.05) (Supporting Information Table S4), suggesting an overarching influence of upstream microbial composition on prevalence of opportunistic pathogens in downstream household water heaters, even after long stagnation (i.e., 2−7 days) at the warm temperature of 37 °C.

Total Bacteria in SWHs. Total bacterial numbers (16S rRNA genes) had a tendency to be higher in SWHs receiving chloraminated SDS water, relative to chlorinated SDS water (Figure 1(a)). In chloraminated SWH effluent, the average number of 16S rRNA genes varied less than 1 log for all conditions studied ($2.0 \times 10^6 - 1.1 \times 10^7$ gene copies/ml). These numbers were comparable to the upper range of 16S rRNA gene levels found in real-word distribution systems[35] and premise plumbing.[29] A wider range of 16S rRNA gene numbers were noted in SWHs receiving chlorinated SDS water ($6.1 \times 10^3 - 1.9 \times 10^7$ gene copies/ml in Stage 1; $1.7 \times 10^3 - 6.4 \times 10^6$ gene copies/ml in Stage 2), which also had higher and more variable levels of disinfectant residuals in the influent (Supporting Information Table S2). Importantly, disinfectant residual was never detected in the effluent of any SWH reactors and thus in and of itself could not serve as the driving force of trends observed throughout the study. Water age had noticeable influence on the levels of total bacteria in the SWHs receiving chlorinated SDS water, for example, lower levels of 16S rRNA genes were found in SWHs receiving chlorinated water aged 2.3 days relative to water aged 5.7 days (P < 0.05), regardless of SDS pipe materials.

***Legionella* spp.** *L. pneumophila* was not detected in this study (data not shown), but broader trends at the genus level, which contains other pathogenic members, were noted. The general pattern of *Legionella* occurrence in the SWHs tended to follow that of the numbers measured in the SDS influent for each corresponding Stage/condition (Figure 1(b)). During Stage 1, the detection frequencies of *Legionella* spp. decreased from 91.7% of all SWH samples on day 4 to 2.8% on day 21 (Supporting Information Figure S3), which likely resulted from influent water characteristics that were depleted in *Legionella* and inhibitory to growth of *Legionella* previously established on the glass bead biofilms. By far, the dominant trend was that *Legionella* were more prevalent in SWHs receiving water from chloraminated SDSs, than from chlorinated SDSs, during Stage 2 when they were consistently detected in the chloraminated SDS influents, although *Legionella* has a tendency to decrease relative to influents. Importantly, this was the case even though both disinfectants were absent in the SWHs themselves, indicating that broader impacts of the disinfectants on the water chemistry and/or microbial ecology can have persistent impact. SDS water age and pipe material also had some effect, with *Legionella* spp. gene numbers found in SWHs receiving chloraminated water from the cement and PVC SDSs aged 2.3 days lower relative to corresponding water aged 5.7 days in Stage 2 (P < 0.05). Chlorinated SDSs appeared to provide the optimal condition, with *Legionella* spp. completely non-detectable across all but four conditions (Figure 1(b)).

***Mycobacterium* spp.** While *Mycobacterium avium* was nondetectable (data not shown), the *Mycobacterium* genus was tracked as representative of NTM species. Mycobacteria were markedly more persistent in SWHs receiving water from chloraminated, relative to chlorinated SDSs, with average concentrations ranging from 7.9×10^3 to 5.8×10^4 gene copies/ml throughout both Stage 1 and Stage 2 (Figure 1(c)). Mycobacteria in SWHs receiving water from chlorinated iron or cement SDSs generally followed the trend of the influent measurements throughout Stages 1 and 2. However, except for one case, mycobacteria were strikingly absent in SWHs receiving water from chlorinated PVC SDSs. Remarkably, this was true even in Stage 1, when mycobacteria were prevalent in the influent (2.3 and 3.6 days), suggesting the water itself had a low regrowth potential in the warm, stagnant water heater environment. There was no apparent effect of water age from the chloraminated SDSs on mycobacterial numbers in the SWHs, but increased water age from chlorinated cement or iron SDSs did result in increased mycobacterial numbers (P < 0.05).

***P. aeruginosa*.** *P. aeruginosa* were also more prevalent in the SWHs receiving chloraminated SDS water (Figure 1(d)). *P. aeruginosa* was only detected in the SWH effluent ($0-2.8 \times 10^3$ gene copies/mL) and influent from SDSs[23] during Stage 1. However, *P. aeruginosa* had a tendency to decrease relative to influent in all SWHs, suggesting that the warm, stagnant SWH environment was not conducive to their regrowth. Higher numbers of *P. aeruginosa* were also noted in SWHs receiving water from the chloraminated cement SDSs relative to from the iron and PVC SDSs (2−3 log higher, P < 0.05), indicative of pipe material effect.

***Acanthamoeba* spp.** *Acanthamoeba* spp. in SWH effluents tended to track numbers measured in the influent, with the chlorinated PVC condition standing out as the best case scenario (Figure 1(e)). Further, *Acanthamoeba* spp. gene copy numbers were significantly reduced in SWH effluents in several cases when receiving PVC SDS influent, whether chlorinated or chloraminated (1−2 log). SDS water age did not have an effect on *Acanthamoeba* numbers in SWHs receiving chloraminated water (P > 0.05), except for a higher level of *Acanthamoeba* noted in PVC 2.3 d SWHs relative to 5.7 days in Stage 1 (P < 0.05). Water age appeared to be somewhat more influential in SWHs receiving chlorinated SDS water, with lower numbers of *Acanthamoeba* found in cement 2.3 days SWHs (<detection limit in Stage 2) compared to 5.7 days SWHs in both stages, and in iron 2.3 days SWHs compared to 3.6 days in Stage 1 and to 5.7 days in Stage 2 (P < 0.05), respectively.

***V. vermiformis*.** *V. vermiformis* is not itself a pathogen, but is viewed as an important host for opportunistic pathogen amplification in premise plumbing.[36] *V. vermiformis* was especially predominant in SWHs receiving chloraminated SDS water, with the average numbers ranging from 1.4×10^3 to 1.2×10^4 gene copies/ml during Stage 1 and from 7.6×10^2

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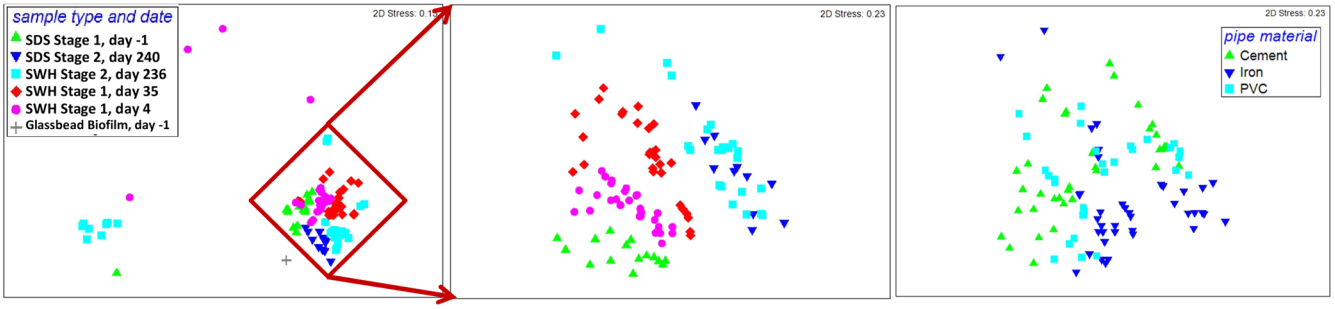

Figure 2. Multidimensional scaling analysis (MDS) of simulated distribution system (SDS) bulk water, simulated water heater (SWH) bulk water and glass-bead biofilm samples. SDS samples were collected 1 day before SWH set up to represent Stage 1 (day −1) and on day 240 of SWH operation to represent Stage 2. SWH samples were collected on day 4, day 35 (in the middle of Stage 1), and day 236 (in the middle of Stage 2). (b) and (c) are the same subsets of the MDS analysis presented in (a) but color coded by sample type (b) or pipe material (c), excluding 14 total samples from the SDS (n = 1), SWH(n = 12), and biofilm(n = 1) (Supporting Information Table S6). Legend in (a) also applies to (b).

to 1.8 × 10^4 gene copies/ml during Stage 2 (Figure 1(f)). Interestingly, *V. vermiformis* was nondetectable in the influent during Stage 1 for the majority of conditions, but regrew 2−4 log in six cases receiving chloraminated SDS water (Figure 1(f)). This suggests that the chloraminated SDS water had high *V. vermiformis* regrowth potential and that the warm, stagnant conditions of the SWH were particularly conducive to their amplification. The chlorinated PVC SDS condition again stood out as optimal, effectively suppressing downstream *V. vermiformis* growth. Water age was a significant factor in SWHs receiving water from chloraminated cement SDSs and from all chlorinated SDSs in both stages (P < 0.05).

Effect of SWH Water Change Frequency Relative to Shift in SDS Influent Composition. Frequent water changes (3× per week) contributed to reduced microbial levels in some SWHs (Supporting Information Table S5). For example, reduced *V. vermiformis* was observed in SWHs fed with chloraminated cement SDS water aged 5.7 days and PVC SDS water aged 5.7 days, while their corresponding influent levels were similar during Stage 1 and 2. However, in most cases, water change frequency had little effect on target microorganisms. For example, decrease of target microorganisms in SWHs occurred simultaneously with marked reductions also occurring in corresponding SDS influents (Supporting Information Table S5).[23] This implies that the microbial composition of the SDS influent water may be a more dominant factor in governing downstream microbial composition and opportunistic pathogen occurrence in SWHs than stagnation. Biofilms in the SWHs could also serve as a reservoir that buffers the effect of water change frequency.

Comparison of SDS Influent and SWH Microbial Communities. Subsampling of 40,185 16S rRNA gene sequences for each sample resulted in exclusion of 25 samples (of 145 total) from further analysis. Ninety-six percent of excluded samples were collected from chlorinated SDS water aged 2.3 and 3.6 d or corresponding SWHs (Supporting Information Table S6), likely a result of lower DNA biomass due to high chlorine residuals.

A total of 4554 OTUs were defined based on clustering 4 822 200 sequences using a dissimilarity cutoff of 3%, with 2283 singleton OTUs assigned. Sample coverage ranged from 99.55% ∼ 99.95%, suggesting microbial communities were well-represented by the obtained sequences. Sequences were classified among two archaeal (only 21 sequences) and 20 bacterial (>99.999% of sequence reads) phyla, with *Proteobacteria* (70.5%) and *Bacteroidetes* (24.7%) as the most dominant phyla. Unclassified bacteria accounted for 2.8% of total sequences (Supporting Information Figure S4(a)). At the class level, *α-Proteobacteria* (34.1%), *β-Proteobacteria* (26.2%), *Sphingobacteria* (22.3%), and *γ-Proteobacteria* (7.4%) represented the majority of sequences, with 42 other classes accounting for <10% of sequences (Supporting Information Figure S4(b)).

MDS plots of SDS, SWH, and glass bead biofilm samples (Figure 2(a)) demonstrated a tight cluster of the majority of samples and several scattered (i.e., dissimilar) points, with the glass bead biofilm sample immediately proximal to the major cluster. The points scattered outside the main cluster (see "Excluded from MDS subset" in Supporting Information Table S6) were generally associated with higher SDS chlorine residual (Supporting Information Table S2). Analysis including only samples in the major cluster suggested a gradual shift in the SWH microbial community structure with time in parallel with a shift that occurred between the Stage-1 and Stage-2 sampling of the SDSs (Figure 2(b)). SWH day 4 samples were most similar to SDS samples from Stage 1 whereas SWH day 236 samples most similar to the SDS samples from Stage 2. ANOSIM analysis confirmed the same trend, demonstrating clear separation among each group of samples (Global R = 0.535−0.869, P = 0.001), except between the Stage-2 SDS and 236 d SWH samples (P = 0.078). Less separation between SDS and SWH samples during Stage 2 relative to Stage 1 may be associated with succession of glass bead biofilm composition with time and higher water change frequency during Stage 2.

Cluster analysis of samples from the major cluster demonstrated that samples from duplicate SWHs harbored similar microbial communities (P > 0.05, SIMPROF test) and illustrated little divergence of microbiomes between duplicates with time. RELATE analysis of SWH samples collected on 4, 35, and 236 days indicated similar patterns of microbial community resemblances among the three sampling events (P = 0.001). All chlorinated 2.3 days, chlorinated cement 3.6 days, and chlorinated PVC 3.6 and 5.7 days samples were excluded from analysis, in order to ensure same sample numbers among different samplings for RELATE analysis. Overall this implies that influent SDS water was an overarching factor in shaping SWH microbial communities.

As revealed by MDS analysis, clustering of samples according to SDS pipe materials were observed within each individual SWH sampling event (Supporting Information Figure S5) and

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pooled samples (Figure 2(c)), especially for samples corresponding to iron SDS water. ANOSIM (Supporting Information Table S7) confirmed significant, yet reduced effect of SDS pipe materials on SWH microbial communities with time, noted by decreased global R values. Sharper separation by different SDS pipe materials was observed in chlorinated SWHs compared to chloraminated SWHs (Supporting Information Figure S5(a) and S5(b)), likely caused by interactions between pipe materials and higher chlorine residuals. Meanwhile, elevated effect of disinfectant type on SWH microbial communities was suggested by increased global R values (Supporting Information Table S8) with time. However, the influence of disinfectant might be underestimated due to exclusion of some chlorinated SWH samples.

DISCUSSION

This research demonstrates the sometimes dominant influence of disinfectant type and dose, water age and distribution system materials on downstream microbial levels under standardized household water heater conditions. Although methods employed in this study may overestimate target microorganisms by detecting DNA that is not necessarily present inside a viable cell (especially in the presence of disinfectant), the increase in numbers of target microorganisms in SDSs with water age, as well as their reduction in SWHs relative to influent water (e.g., *Legionella*), indicate that q-PCR successfully captured trends of target microorganisms. The microbial composition of the influent water was found to be an overarching influence, with significant correlations of all target microbes in influent SDS waters and SWH effluents, even after 7 day stagnation in the SWHs and long after any disinfectant had decayed. At the same time, beneficial interactions between SDS water and SWH conditions were observed, as indicated by relatively lower level of *Legionella*, *P. aeruginosa* and *Acanthamoeba* in several SWHs compared to their influent level (e.g., *Legionella*: chlorinated PVC 2 log reduction; *P. aeruginosa*: chlorinated cement 5.7 days SWHs 2−5 log reduction, 3.6 days chloraminated PVC up to 4 log reduction; *Mycobacterium*: chlorinated PVC 2−3 log reduction), suggesting that these waters had low regrowth potential and that beneficial microbiomes (i.e., "probiotics"[37]) may have been established in SWHs to exclude these opportunistic pathogens. Broadly speaking, the chlorinated PVC SDS condition was found to be optimal for comprehensive control of the target microorganisms in this study; thus, water main materials are also expected to exert an important role in practice even though specific results from this work are not necessarily generalizable to full-scale systems.

Although *L. pneumophila* was not found in SDS or SWHs in the present study, the capability of gradual acclimation in this study of other *Legionella* spp., which might contain other human pathogens such as *L. micdadei*, *L. longbeachase*, *L. bozemannii*,[38] highlighted the potential of premise plumbing as a reservoir and exposure source of *Legionella*, especially when favorable conditions are met.

Effect of Upstream Engineering Factors on Opportunistic Pathogens and Microbial Communities in Downstream Water Heaters. Employment of different disinfectants (i.e., chloramine and chlorine) can select for distinct microbial community structures[35,39] and, in particular, opportunistic pathogens[16,24,40] in drinking water. In the present study, nitrification in chloraminated SDS resulted in much lower chloramine residuals relative to chlorine in the upstream SDSs, supporting recent studies indicating that chloramine decay is sometimes faster than chlorine decay in at least some unusual circumstances.[41] Thus, expected benefits of more persistent chloramine residuals were not observable in these SWHs and nitrification was associated with conditions conducive to regrowth.

Maintaining a target residual level is a fundamental strategy for inhibiting microbial regrowth in premise plumbing. In the present study, *Legionella* was below the detection limit or only sporadically detected in SWHs when the average influent chlorine residual was >0.4 mg/L even with very long stagnation times and disinfectant absence in the SWHs. This conceptually supports the American Society of Heating, Refrigeration, and Air Conditioning Engineering's recommendation of >0.5 mg/L free chlorine oxidants (as Cl_2) for *Legionella* control at the point of entry to buildings.[42] However, this standard is not necessarily sufficient for inhibiting other groups of opportunistic pathogens, such as *Acanthamoeba* and *Mycobacterium* (Figure 1).

Water main pipe materials can alter water chemistry via complex physiochemical and microbiologic processes,[23,43−45] which may affect downstream microbial regrowth. Although influence of pipe materials on bacterial numbers and microbial communities in bulk water and biofilm have been extensively studied in real and simulated distribution systems,[20,46−49] few studies have investigated the effect of upstream pipe material on downstream microbial regrowth, especially with respect to opportunistic pathogens.[50,51] One recent study indicated that upstream copper pipes decreased overall microbial diversity in downstream biofilm, but promoted *L. pneumophila* colonization.[51] Another study found that replacing old pipes (likely metal-based pipes) with corrosion-resistant plastic pipes in a municipal distribution network reduced downstream colonization of mycobacteria,[50] supporting the notion that upstream pipe materials and their status (e.g., aging, corrosion) can be a major factor governing downstream opportunistic pathogen regrowth. In the present study, much higher (∼2 log) *P. aeruginosa* in SWHs receiving water from chloraminated cement SDSs relative to iron and PVC SDSs (during Stage 1) is likely due to favorable chemistry and microbial composition of cement SDS water toward *P. aeruginosa* persistence in SWHs.

Other pipe material effects on the broader microbial communities were well-apparent, with clear clustering of samples receiving water from the iron SDSs noted (Figure 2(c)). This is consistent with previous reports of the strong selection power of iron pipes on biofilm and bulk water microorganisms.[49,52] For example, water passage through iron rigs increased the proportion of Gram-positive bacterial isolates from 52% to 100%.[52] Iron pipes were also able to select for a variety of bacteria capable of utilizing different iron and manganese constituents, such as *Lysinibacillus* spp., *Geobacillus* spp., and *Magnetobacterium* spp., which, incidentally, were not identified in the present study.[49] Decreased pipe material effects with time might be associated with temporal variation of influent waters from SDSs. For example, development of distinct microbial communities in chlorinated iron 3.6 days SWH samples relative to other iron SWH samples occurred together with a ∼1-log reduction of 16S rRNA genes during Stage 2.[23]

Effect of Stagnation on Water Heater Microbiomes. Water stagnation in premise plumbing is often associated with water quality deterioration, such as low disinfectant residuals, higher metal concentrations (e.g., Cu^{2+}), and microbial

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accumulation.[53−55] Overnight stagnation can increase cell numbers 2−3 fold, as measured by flow cytometry, or 4−580 fold, as indicated by HPC counts, in premise plumbing water.[22] Our previous study also demonstrated increased *Legionella* spp., mycobacteria, and *V. vermiformis* in the first draw samples representing premise plumbing water compared to postflushing samples representing distribution water after overnight stagnation.[29] Although significant reduction of target organisms in Stage 2 relative to Stage 1 found in some SWHs may imply effectiveness of the frequency of water changes, temporal variation in SDS water (influent to the SWHs) during Stage 2 (e.g., reduction of 16S rRNA genes, *Acanthamoeba* spp., and *V. vermiformis* in some chlorinated SDS water) likely contributed more to the observed reduction during Stage 2, especially given the predominant influence of influent microbial composition. Moreover, biofilm succession in SWHs on the glass beads and walls may also account, in part, for the temporal variations in SWHs during two stages.

Effect of Temporal Variation of Distribution Water on Microbiomes in Premise Plumbing. While the focus of the present study was on how a range of water chemistries associated with disinfectant, pipe material, and water age influenced microbiology in standardized downstream household water heaters, the two snapshots of the influent chemistry to the SWHs revealed that the water chemistry also varied with time. Distinct temporal variations of drinking water physiochemical (e.g., nitrate/nitrite, ions) and microbial characteristics (e.g., HPC counts, fecal indictors) have been frequently reported in drinking water distribution systems, often associated with seasonal changes and nitrification events.[35,56−59] However, the effect of temporal variations in distribution water on the premise pluming microbiome has been subject to doubt, given that household plumbing itself can serve as the seeding source and essentially functions as a warm biological reactor (i.e., pipes are room temperature, water heater at set point) that may dominate main distribution system effects. In this study, we observed that the microbial composition of the influent water was a dominant factor, with significant correlations between influent SDS and effluent SWH numbers for all target organisms. Nonetheless, as noted above, there were potentially a few key "probiotic" instances in which influent pathogens did not regrow, which is of interest for further study.

Insights into Opportunistic Pathogens Control in Premise Plumbing. Biostability of drinking water is often represented by microbial regrowth potential assays which typically monitor the growth of one species or a microbial consortium within a certain period of time in filter-sterilized dechlorinated water.[60] This study evaluated the potentials of various SDS waters influenced by disinfectant, pipe materials, and water age to support or inhibit downstream growth of opportunistic pathogens by considering the effects of disinfectant residuals and indigenous microbes from both the influent water (i.e., SDS water) and affected environment (glass bead biofilm simulating indigenous water heater microbiome). This approach is suitable for capturing a variety of complex real-world scenarios and avoids using lab-adapted cells as seed, which are not necessarily representative of naturally occurring opportunistic pathogens in terms of their susceptibility to disinfection,[61] proliferation potential in drinking water,[62] and interactions with other microbes (e.g., resistance to infection).[12,63]

This study provides a controlled, replicated, head-to-head comparison of downstream response of opportunistic pathogens to distribution waters with various physiochemical and biological properties under the hypothetical worst-case scenario of household water heaters (i.e., long stagnation time, optimal microbial growth temperature). The governing influence of distribution water quality in shaping the downstream microbiome and opportunistic pathogen occurrence was illustrated and persisted even after long stagnation (i.e., 7 days) in the simulated household water heater environment. Therefore, integration of upstream water quality improvement, such as water main replacement, along with better downstream plumbing management is critical for successful achievement of opportunistic pathogens mitigation.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1−S5 and Table S1−S8 as noted in the text. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b01538.

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Notes

The authors declare no competing financial interest.

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