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Emerging investigator series: bacterial opportunistic pathogen gene markers in municipal drinking water are associated with distribution system and household plumbing characteristics†

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Municipally-treated drinking water (DW) is a potential source of exposure to bacterial opportunistic pathogens (OPs), which can cause infection in susceptible individuals. In this study, we used quantitative PCR to determine concentrations of two bacterial species (*Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*) and three genera containing OPs (nontuberculous mycobacteria [NTM], *Achromobacter* spp., and *Burkholderia* spp.) in water and faucet aerator biofilm samples collected from 15 homes serviced by one DW treatment plant and distribution system. DNA from all five groups was detected in all samples. In addition to quantifying OP gene markers, we measured 15 water quality parameters and generated linear mixed-effect models, demonstrating that dissolved iron concentration was positively associated with the concentration of each bacterial group. Distribution system associated characteristics such as disinfectant concentration, water age, and pressure zone also had a significant association (individually and collectively) with OP abundance. Home specific factors influenced the abundance of some OPs. Specifically, water in newer homes and in homes with plastic plumbing contained higher concentrations of *S. maltophilia* and *Achromobacter* spp., respectively. Collectively, these findings provide insights into factors that may impact the abundance of OPs throughout the distribution system and building plumbing transect and will aid in the development of mitigation strategies to reduce waterborne opportunistic infections.

Water impact

Municipally-treated drinking water can be a source of bacterial opportunistic pathogens (OPs), which can cause infection in susceptible individuals; however, our understanding of which factors impact OP abundance is lacking. We observed that building plumbing material, water age, and dissolved iron concentration are associated with OP abundance in treated drinking water, information that may be used to inform future OP mitigation efforts.

Introduction

Infectious diseases account for millions of deaths annually worldwide.[1] In the US and other high-income countries, the majority of such deaths result from respiratory tract infections,[2,3] many of which are caused by bacterial opportunistic pathogens (OPs). Although most bacteria have the potential to cause human infection,[4] OPs can be described as organisms that typically pose little threat to healthy individuals but can cause infection in vulnerable populations. Due to increasing numbers of elderly and immunocompromised individuals, health-care associated and community acquired OP infections are important drivers of overall morbidity and mortality rates in the U.S.[5] Given the increase in reports of infectious disease outbreaks attributed

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† Electronic supplementary information (ESI) available: ESI for this study is provided and includes four figures (Fig. S1–S4), five tables (Tables S1–S5), a description of the sampling campaign procedures (Procedure S1) and a description of the analytical method developed to measure and quantify NDMA (Procedure S2). See DOI: 10.1039/d0ew00723d

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to waterborne OPs,[6–10] as well as the presence of OPs in municipally treated drinking water (DW), it is important to gain a better understanding of how household DW may contribute to opportunistic infections.[11,12] Moreover, a greater appreciation of the factors associated with OP abundance in DW is necessary to develop strategies to mitigate the risk of infection.

Approximately 85% of the US population receives DW from centralized treatment plants,[13] delivered through underground distribution systems followed by passage through building plumbing. Building plumbing consists of water meters, pipes, water heaters, faucets, shower hoses, and shower heads. Although utilities in most countries around the world provide a disinfectant residual to water at the end of the treatment process to control the growth of microorganisms in distribution systems and building plumbing, disinfected DW sampled from taps still contains a diverse community of microbes at levels ranging from 10^6–10^8 bacterial cells per L.[14] Surfaces in building plumbing favor the formation of biofilms, allowing microbes to persist and grow,[11,15–17] and overnight stagnation in plumbing can increase bacterial cell concentrations two- to three-fold.[18] A small fraction of the microbes found in DW are OPs, such as *Legionella* spp., *Achromobacter* spp., *Burkholderia* spp., nontuberculous mycobacteria (NTM), *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*.[6,7,12,18–29] With the exception of *Legionella* spp., none of the OPs are regulated by the U.S. Environmental Protection Agency or the majority of regulatory bodies in other countries.

Limited information is available about the factors that regulate concentrations of diverse OPs in real DW systems. Most previous studies have either focused on one OP group,[13] collected samples in a single site within a distribution system[16,22,24,29–32] (not capturing the importance of connections between treatment plant, distribution system, and building plumbing), or performed analyses in simulated systems,[33,34] thereby limiting the applicability of their findings to real systems. Furthermore, although prior work has compared microbial community structure temporally or spatially at various locations in treatment plants and distribution systems,[14,35,36] as well as in building plumbing,[15,16,18] and have linked community structure to water quality parameters, few studies have simultaneously evaluated the roles of water quality and distribution system and building plumbing characteristics in influencing OP presence and abundance in real DW systems. The paucity of such studies limits the development and implementation of mitigation strategies aimed at reducing OP exposure through DW.

In this study, we addressed these shortcomings, surveying the abundance of five of the most common OP groups found in DW (*Achromobacter* spp., *Burkholderia* spp., NTM, *P. aeruginosa*, and *S. maltophilia*) by sampling 15 homes served by one DW treatment plant that uses chloramine as its residual disinfectant; within each home, we sampled at five locations and we sampled the distribution system water after flushing. We did not include *Legionella* spp. in this study since *Legionella* spp. tend to be less abundant in chloraminated systems than in chlorinated systems.[37–39] Linear mixed-effect models were used to assess relationships between OP abundance and water quality parameters and other DW system features to identify potential targets for infection risk mitigation.

Materials & methods

Sample information and distribution system residence times

Between October 2015 and February 2016, samples were collected from 15 single family homes in Ann Arbor, MI, USA (ESI† Fig. S1), all of which received water from the Ann Arbor Water Treatment Plant. This plant treats a mixture of source waters obtained from the Huron River (80–85%) and from groundwater wells (15–20%) using lime softening, coagulation, flocculation, sedimentation, ozonation, filtration, and chloramination.[14,25]

Homes were located in five distribution system pressure zones: gravity (homes 5, 7, 8, 10, 11), Geddes (homes 2, 3, 4, 13), northeast high (homes 9, 12, 14, 15), southeast high (home 6), and west high (home 1). Each of these zones has different typical operating pressures, and each has at least one storage tank for managing pressure and water demand. Seasonal (fall/winter) water residence times in the distribution system were calculated for each home by performing 168 h (one week) hydraulic simulations in EPANET[40] using a modified version of the hydraulic model used by the City of Ann Arbor.[41] Modification to the hydraulic model entailed applying reduction factors based on winter water demand patterns to each demand node based on the respective pressure zone. By comparing flow data between summer and winter 2015 in each pressure zone, reduction factors of 11.4% for the west high district, 19.0% for the Geddes and northeast high districts, and 29.2% for the gravity and southeast high districts were used. Based on distribution system residence times, which ranged from 13.9 h to 126.2 h, homes were divided into two areas: (i) shorter residence times ($\leq$24 h; homes 8–12, 14, 15), and (ii) longer residence times ($>$27 h; homes 1–7, 13). We previously reported residence times of the same homes based on seasonally uncorrected values.[26] Although the residence time values changed when applying reduction factors, the two residence time groups discussed in our previous study[26] were the same as those presented here. Information on distribution system pipe ages and materials in the areas of each of the homes sampled were provided by the City of Ann Arbor.[42] During sample collection at each home, a questionnaire was administered to the residents to collect information on home age and plumbing material. This study was deemed exempt by the University of Michigan Institutional Review Board (HUM00087246).

Sample collection

Water and biofilm samples were collected from kitchen faucets after a period of stagnation ($\geq$6 h). A swab of biomass was first collected from the faucet aerator (or the area around the aerator when the inside of the aerator could not be

accessed, *e.g.*, on extendible faucet heads) and placed in 30 mL of sterile phosphate buffer saline. Three sequential 1 L samples of building plumbing cold water were then collected followed by 4 L of water representing the distribution system. Finally, 4 L of hot water was collected. All water samples were collected with aerators removed. Both distribution system and hot water samples were collected after flushing the faucet for at least 5 min and waiting for the temperature to stabilize. Distribution system water is typically several degrees colder than water in building plumbing. Once a stabilized temperature difference was observed by continuously monitoring DW temperature during flushing, collection of water from the distribution system began. Additional details regarding the sampling campaign can be found in Procedure S1.†

Distribution system and hot water samples were filtered on site using a portable Masterflex peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) and 0.2 μm Sterivex filters (Fisher Scientific, Hampton, NH, USA). Filters with captured material were placed on dry-ice for up to 1 h before storage at −80 °C in the lab. Building plumbing samples and the 30 mL aerator biomass samples were stored on wet ice after collection, filtered within 1 h through 0.2 μm polycarbonate filters (Isopore Membrane Filters, EMD Millipore, Billerica, MA, USA) on top of 0.45 μm polycarbonate backing filters, and resulting 0.2 μm filters were stored at −80 °C. Prior to onsite or laboratory filtration, two 50 mL aliquots of water samples were removed and stored for water quality analyses.

Water quality measurements

Fifteen water quality parameters (Table S1†) were measured according to standard methods[43] in the first liter of stagnated building water, the distribution system water, and the hot water samples. Dissolved oxygen (DO), free chlorine, total chlorine, nitrate, nitrite, ammonium, and orthophosphate concentrations were determined at the time of collection using a portable DR900 spectrophotometer (Hach, Loveland, CO, USA). Temperature and pH also were monitored on site using a portable pH and temperature meter (Hanna Instruments, Ann Arbor, MI, USA) immediately after sample collection. Dissolved lead, copper, and iron concentrations were determined using 10 mL of filtered (0.45 μm polyvinylidene fluoride membrane syringe filter, Thermo Fisher Scientific, Waltham, MA) sample with inductively coupled plasma mass spectrometry (Agilent 7900, Agilent, Santa Clara, CA). Dissolved organic carbon (DOC) was measured using a Total Organic Carbon Analyzer (Shimadzu, Columbia, MD). Prior to analysis, samples were filtered through 0.45 μm nylon filters (Fisherbrand, Waltham, MA) previously washed with 40 mL MilliQ water and 10 mL of sample. All analyses, except DO, pH, and temperature, were performed in triplicate and the coefficient of variation was ≤12%. Monochloramine concentrations were estimated by subtracting the free chlorine concentrations from the total chlorine concentrations.[44] The Ct values (monochloramine concentration × contact time) of each home's cold and hot water samples were calculated using eqn (1), where C is the monochloramine concentration in a sample, DSt is the residence time in the distribution system and PPt is the stagnation time in building plumbing.

$$\mathrm{Ct} = C \times (\mathrm{DSt} + \mathrm{PPt}) \quad (1)$$

Distribution system Ct values were calculated in a similar manner but included only the distribution system residence time. Additionally, the disinfection byproduct *N*-nitrosodimethylamine (NDMA) was measured using a high-resolution LC-MS (EQuan MAX system with Exactive Plus Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA) with a heated electrospray interface following the procedure described in Procedure S2.†

Total cell counts

To quantify the total number of cells in each water sample, 15 mL were filtered through a 0.2 μm polycarbonate filter (Isopore Membrane Filters) on top of a 0.45 μm polycarbonate backing filter. The resulting filter was cut into eight pieces using a sterile scalpel and the pieces were submerged for 5 min in a 1 μg mL^{-1} solution of 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) in sterile distilled water. Filter sections were rinsed using sterile water followed by 80% ethanol and allowed to air dry before mounting on glass slides. For each sample, eight microscopy fields were counted. The coefficient of variation was <20% for all samples. Cell counts were performed using a Zeiss Axio Observer D1 fluorescence microscope (Carl Zeiss Microscopy, LLC, Peabody, MA, USA).

Quantitative PCR (qPCR)

DNA was extracted from aerator biofilm and water biomass samples using a procedure[26] to enhance recovery of DNA from NTM, which are difficult to lyse. The total concentration of bacteria was determined using qPCR targeting the bacterial 16S rRNA gene. Quantitative PCR assays were used to target either species-specific (*P. aeruginosa* and *S. maltophilia*) or genus-specific (*Achromobacter*, *Burkholderia*, NTM) genes (Table S2†). Of note, the genus-specific assays quantify human OP species as well as species not known to cause human infection in each group. All DNA samples (n = 90) were analyzed in triplicate along with negative controls and standards. Assays were conducted in polypropylene 96-well plates on a CFX96 real-time quantitative thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each 10 μL reaction contained 5 μL of Fast EvaGreen qPCR Master mix (Biotium, Fremont, CA, USA), 0.8 μL of each primer (0.4 μM; Integrated DNA Technologies, Inc., Coralville, IA, USA), 2.4 μL water, and 1 μL of template DNA (approx. 0.1 ng μL^{-1}). PCR conditions for all assays comprised 40 cycles with the exception of 35 cycles for the *P. aeruginosa* assay. To avoid concerns of inhibition during qPCR, 0.625 mg mL^{-1} bovine serum albumin (Life Technologies, Carlsbad, CA,

USA) was used and DNA extracts were diluted at least ten times using molecular grade water. Melt curve analyses of the PCR products were conducted following each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer dimers or other artefacts. All qPCR assays demonstrated linear relationships between the log of the standards' DNA copy numbers and the calculated threshold cycle values across the specified concentration range ($R^2 > 0.99$ in all cases). Amplification efficiencies[45] varied from 1.8 to 2.0 across all assays consistent with those reported in other studies.[46,47] The limit of detection (LOD) and limit of quantification (LOQ) were determined for each assay (Table S2†) following the procedure outlined in Forootan *et al.*[48] Absolute concentrations of each OP were determined by normalizing qPCR results by individual sample weights. For water samples, this normalization resulted in units of gene copies per L (assuming 1 mL equals 1 g) and for aerator biofilm samples in gene copies per g wet weight.

Statistics

Significant differences in OP absolute abundances between sample types (building plumbing, distribution system, and hot water), pressure zones, and homes were determined by nonparametric multivariate analysis of variance (MANOVA). The functional relationships between abiotic (chemical and physical characteristics) and biotic parameters and individual OPs were analyzed using Kendall's correlation analysis (chosen due to it being a nonparametric test that did not violate any of the underlying assumptions of the test, unlike Pearson's (used for normally distributed data) and Spearman's (requires at least one ordinal variable) correlation analyses), with significance adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate.[49]

Linear mixed-effect models were generated using a stepwise Akaike information criterion (AIC) regression approach to determine the best combination of variables to explain the OP abundances. The data were analyzed as a single dataset across the different sample types. The dependent variable in each model was the OP group or total bacteria concentration and the explanatory variables explored included water quality and home specific factors (Table 1). The rationale for using linear mixed-effect models lies in the power of this modelling framework to deal with a wide variety of data types (*e.g.*, spatial, clustered). Prior to model generation, all OP abundances were transformed using the Box–Cox method to ensure normally distributed datasets, and all collinear variables were assessed and removed using the variance inflation factor (VIF) approach, with values <10 indicating an acceptable level of multicollinearity. In addition, power calculations revealed than no more than five explanatory variables should be included in the models. Furthermore, to determine if OP abundance was influenced by the concentration of dissolved iron, samples were evenly divided into two bins: ≤10 μg L^{-1} or >10 μg L^{-1} of dissolved iron, with significance tested using a Wilcoxon test. These two bins were chosen to be in line with previous studies.[50,51] All statistical analyses were performed in *R*,[52] with significance set at a p-value < 0.05. The exact p-values were noted where appropriate.

Results & discussion

Occurrence and abundance of OPs

DNA from all of the five OP groups (*P. aeruginosa*, *S. maltophilia*, *Achromobacter* spp., *Burkholderia* spp., and NTM) was detectable in all water and biofilm samples and was quantifiable in 90% of samples (Table S3†). The concentration of each OP group was different in each water type (Fig. 1). Total bacteria and OPs were present at high levels in aerator biofilm samples (Table S3†) (median of total bacteria: 2.19×10^9 16S rRNA gene copies per g wet biomass). Among the building plumbing water samples, total bacteria and OPs were typically most abundant in the first liter of stagnated water with lower levels in the second- and third-liter samples. The lowest concentrations of OPs were generally found in hot water samples (Fig. 1). The single copy *atpE* gene of NTM ranged from <200 to 7.76×10^6 gene copies per L in all water samples with 1.5 to 3 orders of magnitude higher concentrations in the first liter of stagnated water compared with other water samples. Likewise, the target gene for *P. aeruginosa*, *oprL*, was present

Table 1 Linear mixed-effect models for each OP and total bacteria showing the variables that explain the OP concentrations across all water samples

			Overall model	
Taxa	Data transformation	Model components[a]	Explained[b] (%)	Most influential abiotic component after water type[c]
Achromobacter	$\log_{10}(x)$	Building plumbing material$^{17\%}$ + iron$^{13\%}$ + *S. maltophilia*$^{41\%}$ + orthophosphate$^{2\%}$ + water age$^{3\%}$	76	Building plumbing material
Burkholderia	$\log_{10}(x)$	Iron$^{18\%}$ ± water type$^{63\%}$ + *P. aeruginosa*$^{9\%}$ − nitrite$^{1\%}$	91	Iron
NTM	$x^{0.1}$	Iron$^{5\%}$ − water age$^{1\%}$ ± pressure zone$^{2\%}$ ± water type$^{88\%}$	95	Iron
P. aeruginosa	$\log_{10}(x)$	Ct$^{5\%}$ + iron$^{9\%}$ + *Burkholderia*$^{37\%}$ ± water type$^{24\%}$ − pH$^{2\%}$	74	Iron
S. maltophilia	$x^{-0.1}$	Iron$^{14\%}$ − home age$^{6\%}$ + *Achromobacter*$^{47\%}$ + *Burkholderia*$^{12\%}$ + copper$^{3\%}$	64	Iron
Total bacteria	$x^{1.6}$	Monochloramine$^{3\%}$ ± water type$^{95\%}$ + *Burkholderia*$^{0.7\%}$ − pressure zone$^{0.5\%}$	98	Monochloramine

[a] Superscript numbers proceeding each component in the models show their relative percentage contribution to the overall model. [b] Percentage explained pertains to the adjusted R^2. All models were significant at p-values < 0.001 (Table S5†). [c] Water type was not a significant parameter in *Achromobacter* and *S. maltophilia* linear mixed-effect models.

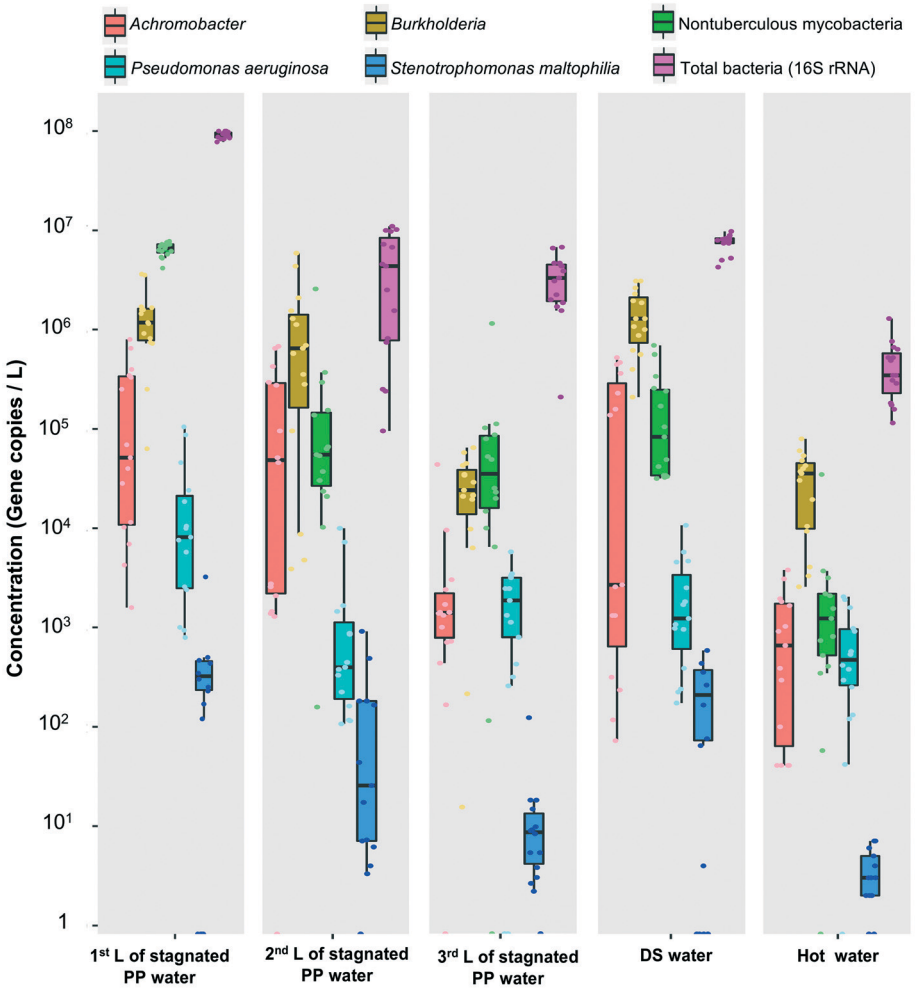

Fig. 1 Boxplots illustrating the concentration of total bacteria and five OPs in various water samples collected in 15 homes. Black lines inside the boxplot represent the median and top and bottom lines represent the upper and lower quartiles of the dataset. The whiskers represent the upper and lower quartiles plus or minus 1.5 times the interquartile range. PP refers to building plumbing water and DS indicates distribution system. Values < 1 indicate samples that were below the LOQ (see Table S2† for LOQ values). Note that the NTM levels in the first liter of stagnated building plumbing water samples were included in a previous publication.[26]

at significantly higher concentrations in the first liter samples (p-value: 0.016) than in other water samples.

The OP abundances in distribution system samples were generally most similar to those in the third liter of building plumbing water samples, although *Burkholderia* and *S. maltophilia* concentrations were significantly higher in distribution system samples (p-value: 0.001, and *Achromobacter* showed a greater variance (Fig. 1)). Although previous studies have detected OPs in treatment plants, distribution systems, and building plumbing in varying abundances,[6,7,18,19,21–23,25–29] few[18,22,32] have sampled multiple sites along the DW transect through the distribution system and within homes. Our results suggest and are consistent with other studies in that concentrations of OPs vary greatly across the DW transect and that OP concentrations in distribution system samples do not necessarily provide a good indication of OP concentrations in building plumbing water.

Within the distribution system, no significant difference was found in the concentrations of total bacteria or OPs between homes with short or long residence times (p-value: 0.463), except for NTM, which were present at significantly (p-value: <0.001) higher abundances in homes with longer residence times, consistent with our previous study[27] (Fig. 2). It should however be noted that although the difference in distribution system water temperatures between the residence time groups was not statistically significant (p-value 0.051), shorter residence time homes were generally sampled in colder months, which may have resulted in a seasonal effect as temperature is known to impact the abundance of many OPs.[22] *Burkholderia* and *P. aeruginosa* displayed strong positive correlations in all but the aerator biofilm samples; otherwise, there were no significant associations between any two OPs within the different water types (Fig. S3†). Collectively, these observations imply that future mitigation approaches may need to be tailored for distribution systems and building plumbing systems separately.

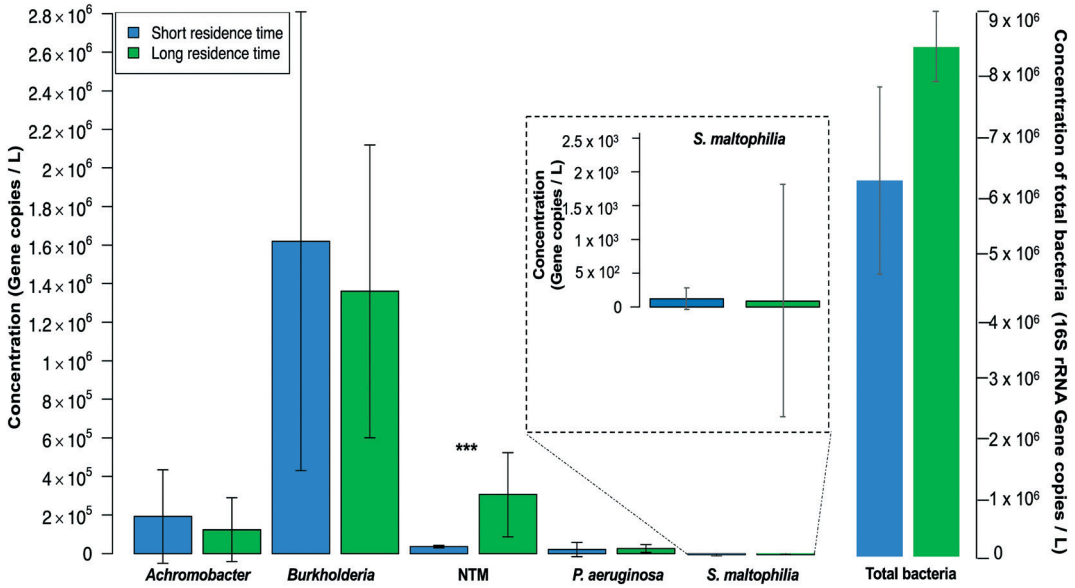

Fig. 2 Average concentrations of total bacteria and OPs in distribution system samples with short (≤24 h, blue) and long (>27 h, green) residence times. Error bars represent standard deviations. ***Designates statistically significant (p-value: <0.001) differences in the abundance between the two groups as determined by paired Wilcoxon tests.

In this study, we targeted OP groups using three genus-specific qPCR assays (*Achromobacter* spp., *Burkholderia* spp., and NTM) that target known human OP species as well as non-pathogenic species. We previously reported that clinically important NTM, such as *Mycobacterium avium*, *Mycobacterium abscessus*, and *Mycobacterium chelonae*, were present in the first-liter samples from building plumbing.[26] Despite referring to the five target groups in this study as OPs, we recognize that the genus-specific assays target both human OP species as well as species not currently known to cause human infection.

The higher abundance of most OPs in building plumbing compared to distribution system samples is likely due to building plumbing characteristics, which provide a favorable niche for OP growth and survival. Building plumbing typically has a high surface-to-volume ratio, intermittent stagnation, lower disinfectant residual relative to the distribution system, and warming cycles.[12] These factors, in addition to characteristics of many OPs previously found in DW, including their high resistance to disinfectants, tendency to form biofilms, and capability to survive at low DO and nutrient concentrations,[53] indicate that their growth and survival in building plumbing is difficult to prevent. For example, compared to *Escherichia coli*, the $Ct_{99.9\%}$ for inactivation with chlorine for environmentally recovered *M. avium*, *P. aeruginosa*, and *Burkholderia pseudomallei* were 2000-fold, 40-fold, and 28-fold higher, respectively.[53,54] Another important consideration when assessing the inactivation of OPs is their ability to enter into a viable but nonculturable state,[55] indicating that culture-based methods typically used in inactivation studies overestimate the efficacy of disinfection.

While NTM have been detected in building plumbing and distribution system samples in several other studies,[22,30,31] their concentrations (Fig. 1) and frequencies of detection (Table S3†) were higher in the current study. Reasons for higher NTM abundance and frequency of detection in the drinking water system we sampled may be related to its source water (primarily surface water), the use of chloramine as a residual disinfectant (increased frequency of detection of NTM has been reported for chloraminated systems compared with chlorinated systems),[56] or our improved NTM DNA extraction method, which recovered between three to eight times more DNA from NTM than two widely used commercial kits.[26]

Burkholderia spp., unlike the other OPs, were quantifiable in all samples (Table S3†). This result may not be surprising given the genetic and ecological breadth of this genus.[57] Furthermore, although *P. aeruginosa* and *S. maltophilia* were quantifiable at a slightly lower frequency than the other OPs, possibly due to the narrower target range (species *vs.* genus), they were detected at higher frequencies than in other studies.[22,31,32] Such differences in detection frequency are likely due to a combination of differences in water quality, disinfection practices (chlorine residual switched to chloramine,[22] no residual disinfectant,[32] and chlorine residual[31] compared to chloramine in this study), and methods. Specifically, our procedure for extracting DNA from hard-to-lyse cells,[26] alongside the lower LOD and LOQ for our qPCR assays likely resulted in higher rates of detection of OPs in our study.

Abiotic sample characteristics

Abiotic sample characteristics, including physical–chemical water quality parameters, as well as distribution system, building plumbing, and home features for the 15 homes sampled are presented in Table S4 and Fig. S3.† Dissolved iron concentrations in distribution system water samples

varied based on distribution system pipe materials. Homes serviced by cast iron pipes installed before 1920 (n = 2), between 1920–1949 (n = 4), after 1949 (n = 3), and homes serviced by ductile iron pipes (n = 6) showed average (±SD) dissolved iron concentrations of 14.1 ± 12.6, 6.4 ± 5.2, 31.6 ± 13.7, and 20.0 ± 14 μg L^{-1}, respectively. By combining results for samples from cast iron pipes installed before 1949 (n = 6), dissolved iron concentrations in these water samples were found to be significantly different from those collected from cast iron pipes installed after 1949 (n = 3; p-value: 0.047). Specifically, the former water samples contained, on average, 70% lower dissolved iron concentrations than the latter. Although these results suggest that dissolved iron concentrations fluctuate with the age of cast iron pipes, due to the small sample size, additional data would be required to verify such a relationship. Note that we measured dissolved iron concentrations in this study, rather than total iron concentrations, because dissolved iron concentrations were more likely to be associated with OP concentrations than total iron concentrations, as discussed below.

Associations between OP concentrations and abiotic characteristics

Univariate analyses identified both positive and negative linear correlations between the abundance of total bacteria and each OP with abiotic parameters (Fig. 3). We developed linear mixed-effect models for concentrations of total bacteria and each OP by combining all water types (cold and hot building plumbing and distribution system water samples but not aerator biofilm samples) to increase the power of the statistical method (Tables 1 and S5†). We found that between 64% and 98% of the OP concentrations could be explained by different components of the DW transect. More specifically, features associated with disinfection (*e.g.*, monochloramine concentration, Ct, and water age), dissolved iron concentrations, and characteristics related to homes (home age and plumbing material) are associated with OP abundance, as discussed below.

The importance of disinfection and its impact on DW microbial communities is well documented.[34,36,58–60] In particular, several reports[61–63] have suggested that chloramine leads to the selective enrichment of certain OPs, including NTM. Consistent with this, we observed significant associations with measures of disinfection (*i.e.*, Ct and monochloramine concentration) and total bacteria and *P. aeruginosa* abundances (Tables 1 and S5†). Although monochloramine concentration was not associated with OP abundances, a direct measure of disinfection (Ct) and parameters that indirectly impact disinfection (pressure zone and water age) are significant variables in four out of the five linear mixed-effect models for OPs (Table 1).

Our results demonstrate that the abundance of *Achromobacter* spp., NTM, and total bacteria levels vary by pressure zone and/or water age (Tables 1 and S5†). The influence of water age in our study agrees with previous studies that have shown it to be a key parameter in explaining microbial community composition.[18,26,34,64] Likewise, water storage towers used for pressure management have been shown by one study to be a source of OP contamination.[65] Furthermore, considering that temperature and source water composition fluctuate with season and have been previously shown to impact DW microbial communities[66,67] and DBP formation,[68] future research

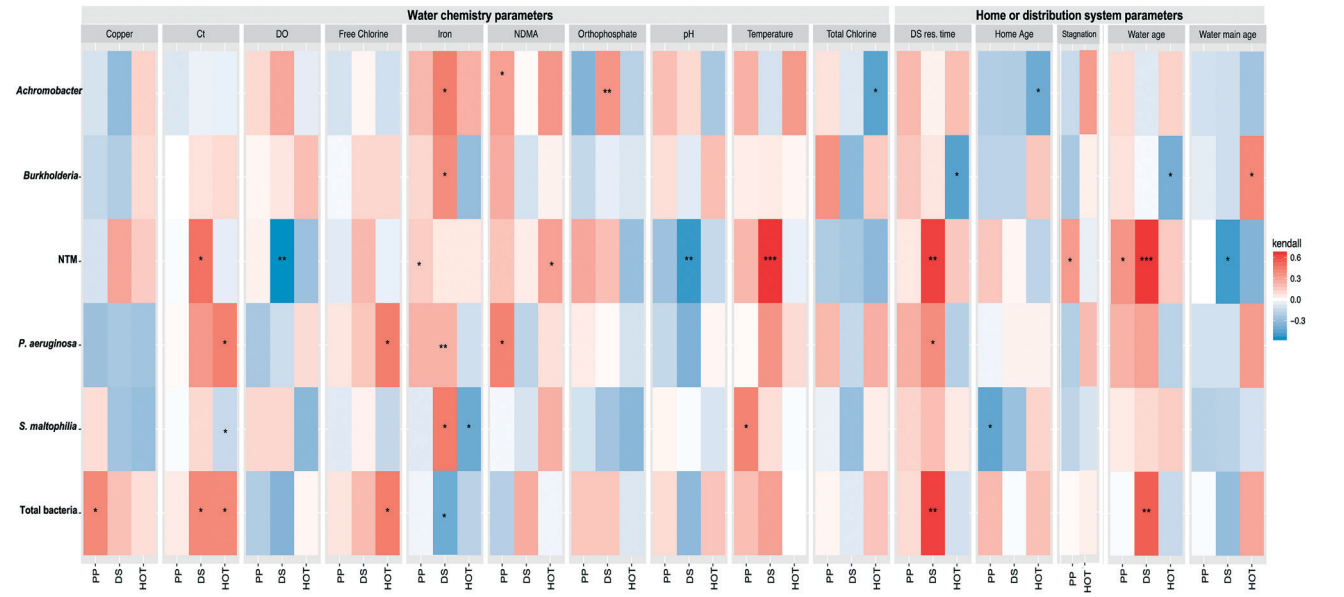

Fig. 3 Kendall correlation analysis showing the relationships between the various abiotic characteristics and the absolute abundance of the OPs. Only abiotic characteristics with at least one statistically significant correlation are shown. PP refers to building plumbing water, DS indicates distribution system and hot pertains to building plumbing hot water. Red boxes indicate positive correlations and blue boxes represent negative correlations, p-values are denoted by *: 0.05, **: 0.01, ***: 0.001.

should focus on gathering temporal data to capture any seasonal disinfection dynamics, which may influence OP abundance. Elucidating temperature effects is particularly relevant to confirm the findings of this study since short residence time homes generally were sampled in colder months as discussed above.

Following water type, dissolved iron concentration was the second most influential abiotic parameter in four of the six models, explaining 18%, 5%, 9%, and 14% of the abundances for *Burkholderia*, NTM, *P. aeruginosa*, and *S. maltophilia*, respectively (Tables 1 and S5†). Dissolved iron concentration always exerted a positive association with OP abundance, with samples with $\leq$10 μg L^{-1} dissolved iron containing, on average, ten times lower abundances of the five OPs than samples with >10 μg L^{-1} dissolved iron (*p*-value: 0.006). The presence and significance of the dissolved iron concentration in all OP models is consistent with the importance of this micronutrient, which is essential for bacterial cell function, including electron transport, amino acid and pyrimidine biosynthesis, DNA synthesis, quorum sensing, and biofilm formation.[50,69,70] Moreover, the OPs targeted in this study, especially those capable of intracellular growth (*P. aeruginosa*, *S. maltophilia*, *Burkholderia* spp., and NTM), possess siderophores capable of chelating very low concentrations of iron,[69–71] making these organisms particularly well adapted to survival in low iron environments such as in DW. Although human pathogens, including the aforementioned OPs, grow optimally with 0.3–1.8 μM (16–100 μg L^{-1}) of Fe(II) (24% of our samples fell within this range – Table S4†), they have been shown to grow and survive at much lower concentrations.[72]

Overall, dissolved iron concentration <10 μg L^{-1} was associated with an order of magnitude fewer total OPs, likely due to the 1.6–10 times lower than optimal concentration of this critical micronutrient.[72] This finding is also in agreement with previous studies that observed significantly lower growth rates for many OPs grown at iron concentrations of $\leq$10 μg L^{-1} compared to those for cultures grown in iron-replete conditions.[50,51] The finding that distribution system samples exhibited higher median concentrations of dissolved iron (14.2 μg L^{-1}) compared to building plumbing (4.2 μg L^{-1}) and hot water samples (4.1 μg L^{-1}) suggests that the distribution system could be a site for OP mitigation efforts through reducing dissolved iron concentrations.

The linear mixed-effect models (Tables 1 and S5†) show that building plumbing material and home age explain 17% and 6% of the abundances of *Achromobacter* spp. and *S. maltophilia*, respectively. Specifically, newer homes and homes with plastic building plumbing material (predominately cross-linked polyethylene (PEX) although other types of plastic were also used) positively corresponded with the abundance of *Achromobacter* spp. and *S. maltophilia*, respectively (Table S5†). It is likely that building plumbing material and home age are interacting or confounding parameters, given that plumbing in newer homes is typically constructed using plastic plumbing materials.[73] However, no significant interaction between these two parameters was found, possibly due to the small sample size. While the effect of varying pipe materials on biofilm formation, microbial community composition, and the abundance of OPs has been studied in both real and simulated distribution systems,[34,74–76] the influence of building plumbing material on OP abundance is not well studied.[17,77] To our knowledge, no prior studies have observed an association between building plumbing material and the absolute abundance of specific OPs besides *L. pneumophila*.[78] In addition, *L. pneumophila* was also found to be significantly enriched in PEX plumbing compared to copper plumbing,[77] consistent with our observations for *Achromobacter* and *S. maltophilia* (Table 1, Fig. S4, and Table S5†).

Possible reasons for the higher abundance of these two OPs in newer homes and/or homes with plastic plumbing may lie in their surface charge and ability to metabolize or survive exposure to components leached from plastic.[78] Microbial adhesion to surfaces is a complex process, influenced by both properties of the microorganism and surface, the most significant of which are hydrophobicity and surface charge. Under most physiological conditions, the surface of bacteria is typically negatively charged, with a few exceptions.[79] Therefore, given that surfaces such as plastic are negatively charged,[79] bacteria are typically repulsed when approaching these surfaces. However, due to the positive and slightly negative charge (as indicated by measuring the whole-cell isoelectric point) of *S. maltophilia* and *Achromobacter*, respectively,[79,80] it is possible that these two OPs could outcompete other organisms when colonizing plastic plumbing, explaining their positive association with plastic. Likewise, the ability of *S. maltophilia* and *Achromobacter* to metabolize a wide range of compounds, including many of the volatile and semi-volatile organic compounds (*e.g.*, ethyl *tert*-butyl-ether, toluene, and xylene[73]) released into DW from various types of plastic plumbing materials, makes them more adapted to survival in building plumbing composed of plastic.[81–83] Therefore, it is possible that their ability to metabolize leached organic compounds from plastic, alongside their preferential surface charges, makes them pioneer organisms in early plastic building plumbing biofilms, a hypothesis supported by the exponential decay relationship seen between their abundance in first-liter building plumbing water samples and home age (Fig. S4†). A detailed building plumbing characterization was outside the scope of this study; it is likely that homes contain a multitude of plumbing materials, therefore more work is needed to investigate the observed relationship between plastic plumbing and *S. maltophilia*.

Recommendations

Our results add to the growing evidence that OPs are commonly present in municipal DW. Given that at least some of these organisms may become regulated in the future,[84] it is important to identify the abiotic factors associated with OP abundance. This study observed that abiotic characteristics, such as building plumbing material, water age, dissolved iron

concentration, and Ct, are associated with concentrations of five OPs in municipally treated DW collected from homes. Although the conclusions from this study were obtained based on a dataset representing grab samples collected at one time point from 15 homes serviced by the same utility, the statistical rigor used (power calculations based on an effect size of 0.3, power level of 0.8, and probability level of 0.05) ensured that conclusions were not biased by the small number of samples collected. Nevertheless, future studies are needed to determine the mechanisms behind the observed relationships and whether they are valid for other municipal drinking water systems, and assessments must be made to discern seasonal effects, water usage influences, and day-to-day variation within each home. Overall, the models generated within this study provide insight into possible OP mitigation strategies and areas for future study such as:

a) Determining whether the type of distribution system material or iron pipe topography explain the association between dissolved iron and OP abundance observed in this study.

b) Evaluating the impact of plastic building plumbing aging with respect to its enrichment or selection for OPs. This is of particular importance given the increased use of PEX building plumbing in new residential homes, commercial properties, and hospitals.[73]

Conflicts of interest

The authors declare no conflict of interest.

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