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Water Impact Statement

Maintaining sustainable drinking water distribution systems will require enhanced monitoring methods that can quickly reveal when water quality is compromised. Methods for quantifying total microbial cells, like FCM and ATP, have tremendous potential to transform the way drinking water quality is monitored in drinking water distribution systems. Yet, research applying these novel tools to disinfected systems is scarce. This work demonstrates the unique insights gained from monitoring total microbial cells in several full-scale disinfected distribution systems and lays a foundation for statistical approaches that could provide a basis for applying these tools through water quality monitoring.

Effect of disinfectant residual, pH, and temperature on microbial abundance in disinfected drinking water distribution systems

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Abstract

In piped drinking water distribution systems, microbial water quality depends on the quantities and types of microorganisms present as well as the physicochemical conditions that influence them. Accurately assessing microbial water quality in these systems is important to maintain water quality throughout distribution. Microbial water quality can be assessed directly, using measures of microbial abundance, and indirectly, by measuring pH, temperature, and disinfectant residual. In the United States, total coliform bacteria measurement is the only regulated parameter for microbial abundance, but because levels are required to be maintained below the quantification limit, this parameter provides little insight into the total microbial abundance. In this study, alternate measures of microbial abundance were assessed in six drinking water distribution systems with a wide range of free chlorine (<0.02 to 2.14 mg/L as Cl_2) and total chlorine residuals (<0.02 to 2.9 mg/L as Cl_2). Five measures of microbial abundance were compared for quantifiability and variability throughout distribution: total and intact cell counts, total and intracellular ATP concentrations, and heterotrophic plate counts. We found that: (1) intracellular ATP and intact cell counts had significant and strong correlations with disinfectant concentration; (2) these correlations were stronger in chlorinated systems compared to chloraminated systems; (3) 97.6% of samples had a quantifiable intact cell counts, with only four samples below the intact cell count quantifiaction limit at the highest residual concentration in chlorinated drinking water distribution systems (1.5 - 2.0 mg/L as Cl_2); and (4) variance between technical replicates was lowest for total ATP followed by total and intact cell counts as compared to heterotrophic plate counts and intracellular ATP. We also demonstrated that a generalized linear mixed model could be used to estimate the combined effect of common water quality parameters on intact cell counts in a chloraminated distribution system: total chlorine had the greatest inverse effect on intact cells with a greater positive effect of temperature at lower levels of total chlorine. We discuss the purpose and interpretation of typical microbial water quality parameters, such as heterotrophic plate count

and disinfectant residual, and consider the future role of ATP and flow cytometry-based methods.

Key words: drinking water; distribution system; cell count; flow cytometry; ATP; chlorine; chloramine; generalized linear mixed model

1 Introduction

Microbial water quality in piped drinking water distribution systems depends on complex interactions between the microbial community (composition, abundance, and growth rates of microorganisms) and chemical and physical conditions. Over the last five years, researchers have made great progress to better understand these interactions with the common goal of guiding drinking water providers toward more efficient management of microbial water quality in piped drinking water systems with continuous or intermittent flow (1-11). Advances in meta-omics techniques allow researchers to characterize changes in the microbial community composition throughout piped distribution (12,13), but these techniques often do not quantify absolute microbial abundance. Increases in microbial abundance in piped drinking water distribution systems can signal mobilization of loose deposits (14,15), loss of disinfectant residual (16-18), treatment breakthrough (19), nitrification (20,21), stagnation (22,23), and intrusion or backflow (1). It is important to pair measures of microbial abundance with compositional data to better characterize microbial water quality in drinking water systems.

In the United States, total coliform bacteria are the only regulated parameter for microbial abundance, but because levels are required to be maintained below the quantifiaction limit, this parameter provides little insight into the total microbial abundance. Given this limitation, other measures of microbial abundance have been used that include heterotrophic plate counts (HPC), which are the most common (9,24), and newer methods that aim to capture the entire microbial community such as adenosine triphosphate (ATP) assays (25,26) and flow cytometry-based assays (27,28). Each assay has its limitations. The World Health Organization recommends HPC for monitoring the “general bacterial content” of water (24), but the HPC assay has been shown to quantify a varied fraction of total bacteria in drinking water (29) that can be several orders of

magnitude smaller than total cell counts and usually requires two days to complete (30). However, HPC may require less technical skill than ATP or cell counts if user-friendly proprietary HPC kits are used. As an alternative to HPC, the quantification of intracellular ATP has been used to estimate the viable biomass in water samples (31-33). However, ATP concentration depends on the types of microorganisms present (34) and local conditions (35,36), which hinders accurate quantification of microbial abundance. In addition, ATP assays require an extra filtration step during sample processing to measure total ATP (both intracellular and extracellular) as well as extracellular ATP (ATP in 0.1 μm filtered sample), which is subtracted from total ATP to obtain intracellular ATP (25). In contrast, flow cytometry-based methods can be used to quantify microbial cells (37,38) with high reproducibility (<5% error (39)), low limits of quantification (<25 cells/mL (40)), and rapid sample turnaround. Flow cytometry-based monitoring has been estimated to cost twice that of standard monitoring methods using HPC (41), and that cost does not include the cost of instruments needed, which for flow cytometry are currently more expensive than for HPC. For flow cytometry-based monitoring, an assessment of viability can be included by distinguishing between total cells and intact cells through staining procedures.

Drinking water distribution systems are dynamic, and changes in physical and chemical conditions in full-scale systems also influence the microbial abundance. For example, seasonal variations in drinking water quality have been linked to changes in intact cell count in a full-scale system without disinfectant residual (28). In drinking water systems with residual disinfectants, characterizing these impacts can be difficult because environmental factors that can impact microorganisms can also impact the efficacy of disinfection (e.g., temperature, pH) (42). In addition, high levels of residual disinfectant can make microbial abundance difficult to quantify because it might drive the quantity of microorganisms below the quantification limit of the assay. Flow cytometry-based methods have only been applied in full-scale systems with relatively low residual concentrations (<0.9 mg/L free chlorine and <1.8 mg/L combined chlorine) (8,16,18,43-45), while drinking water

systems in the United States have reported free chlorine concentrations of up to 4 mg/L as Cl_2 after primary disinfection (46). The understanding of disinfectant residual, and its interaction with other physical and chemical parameters, on total microbial abundance is still far from complete. Nonetheless, measures of microbial abundance that better reflect the entire microbial community, rather than a small fraction, and that are quantifiable throughout the range of conditions encountered in piped drinking water distribution systems, have the potential to provide more insight to guide the safe management of drinking water.

In this study, we compared five measures of microbial abundance (total and intact cell counts, total and intracellular ATP, and HPC) in six piped drinking water distribution systems. The drinking water systems had different treatment trains and used either free chlorine or chloramine as a residual disinfectant. We surveyed these systems to: (1) assess the impact of commonly measured parameters (disinfectant concentration, pH, and temperature) on microbial abundance, including statistical approaches to account for interactions between parameters; and (2) compare the quantifiability and variability of five measures of microbial abundance under the conditions of distribution. To our knowledge, this study is the first to apply flow cytometry-based total and intact cell counts in drinking water distribution systems with high disinfectant residual concentrations (>0.9 mg/L free chlorine and >1.8 mg/L total chlorine). These data will serve as points of comparison for future studies applying these methods in similar water systems.

2 Methods

2.1 Sampling Locations

Piped drinking water distribution systems in California and Texas were sampled as indicated in Table S1. Treatment processes and other metadata for these systems are shown in Table 1. Sampling efforts were coordinated with drinking water providers, and samples were collected from a subset of their routine monitoring locations. Systems A and B were sampled one time each in both 2016 and 2018. Systems C, D, and E were sampled one time in 2016. System F was sampled six times in 2018 (Table S1). Prior to bulk water grab sampling, drinking water distribution system site taps were flushed for 10 minutes and 500-mL grab samples of bulk water were aseptically collected in autoclaved-sterilized glass bottles. pH (Electrode Sealed SJ F; Fisher Scientific) was determined within eight hours of sampling. Temperature (Electrode Sealed SJ F; Fisher Scientific) and free and total chlorine measurements (HACH pocket colorimeter II) were determined onsite at the time of sampling. Samples for quantification of microbial abundance were treated with sodium thiosulfate in excess to quench disinfectant residual and kept at 4^{o}C until processing within 24 hours of sampling. For DWDS F, water ages for each site were provided by the utility based on an internal model of the full distribution system developed using SynerGEE Water (v4.7.0). Consumables, including filtered pipette tips (RAININ TerraRack or Finntip Flex) and 2-mL microcentrifuge tubes (Thermo Scientific) used for microbial analyses were purchased presterilized and free of DNA, DNase, and RNase as well as of ATP when available.

Table 1: Treatment processes for each drinking water distribution system sampled in this study, presented in their sequential order at the treatment plant, where source water type is either surface water (S) and/or ground water (G).

System	Source water type	Maximum capacity (million gallons per day)	Treatment Process											Secondary disinfectant
			free chlorine	coagulation, flocculation, sedimentation	ozone	free chlorine	filtration (anthracite & sand)	filtration (granular activated carbon & sand)	filtration (granular activated carbon)	membrane filtration (ultrafiltration)	chlorine dioxide	free chlorine	ammonia	
A	S	12		x				x				x		free chlorine
B1[+]	S	5.5		x			x					x		free chlorine
B2[+]	S	5.5		x						x		x		free chlorine
C	S	200	x	x			x					x	x	chloramine
D	S/G	40		x					x		x‡	x‡		free chlorine*
E	G	30		x					x			x		free chlorine*
F	S	144		x	x	x	x					x	x	chloramine

*These systems also have free chlorine addition at several locations in the distribution system
‡ both chlorine dioxide and free chlorine are used as primary disinfectants
[+]System B had two parallel trains fed with the same source water that are combined before distribution with about 40% of flow from B1and 60% of flow from B2

2.2 Cell counts by fluorescent staining and flow cytometry

Total and intact cell concentrations were measured following the methods of Miller et al. (40). Briefly, cell concentrations were measured using flow cytometry with SYBR® Green I (S9430; Sigma-Aldrich, St. Louis, MO) and propidium iodide (30 mM P1304MP; Life Technologies, Carlsbad, CA) to distinguish cells with intact membranes. From each bulk water grab sample, a 1000-µL aliquot of each triplicate was processed and the geometric mean and geometric standard deviation were calculated. Measurements were performed on two separate flow cytometers, an Accuri C6 flow cytometer (Accuri; BD Biosciences, San Jose, CA) and a BD FACSCanto cell analyzer (Canto; BD Biosciences, San Jose, CA). The Accuri was used to sample all locations but had to be sent in for repair during field sampling at DWDS F. While the Accuri was not available the Canto was used, which was during sampling of DWDS F (data files in the supplemental information include which cytometer was used to generate each data point). The Accuri was

equipped with a 50 mW laser emitting a fixed wavelength of 488 nm, and measurements were performed at the "fast" flow rate of 66 µL minute^{-1} on sample volumes of 50 µL. Microbial cell signals were distinguished and enumerated from background and instrument noise on density plots of green (FL1; 533 ± 30 nm) and red (FL3; >670 nm) fluorescence using FlowJo gating software (v 10.5.3). Gate positions were modified slightly from a template publicly available for the BD Accuri C6 (38) to adapt for FlowJo software. The Canto was equipped with a 20 mW laser emitting a fixed wavelength of 488 nm, and measurements were performed at a flow rate of 1 µL s^{-1} for 50 seconds. Microbial cell signals were distinguished and enumerated from background and instrument noise on density plots of green (FTIC; 530 ± 30 nm) and red (PerCP; 695 ± 40 nm) fluorescence using FlowJo gating software. Gate positions were modified slightly compared to BD Accuri C6 gating based on calibration beads (Spherotech, Catalog #NFPPS-52-4K, Lake Forest, IL). For the Accuri, the lower quantification limits were determined for intact cell count (22 cells per mL) and total cell count (12 cells per mL) by Miller et al. using the same instrument used in this study (40). All data from the Canto were deemed detectable based on the recommended lower quantification limit (>10^2 cells per mL; 38) after gate adjustment (more information can be found in the Supplemental Information). All of our flow cytometric measurements were at least an order of magnitude lower than the upper recommended upper quantification limit (<10^7 cells per mL; 38). For a negative control, 0.22 µm filtered, Millipore Milli-Q water was used.

2.3 Adenosine tri-phosphate concentrations

Total and intracellular ATP concentrations were measured following the methods of Miller et al. (40). Briefly, ATP concentrations were measured using the BacTiter-Glo™ Microbial Cell Viability Assay (G8231, Promega Corporation, Madison, WI) and GloMax® 20/20 Luminometer (Turner BioSystems, Sunnyvale, CA). From each bulk water grab sample, a 500-µL aliquot of each triplicate was processed and the geometric mean and geometric

standard deviation were calculated. Relative light units from the luminometer were converted to ATP concentrations using calibration curves made with a pure ATP standard (P1132; Promega Corporation, Madison, WI). Extracellular ATP was separated from total ATP prior to sample incubation through removal of microbial cells by filtration (0.1 µm, Millex-VV Syringe Filter Unit; Millipore, Billerica, MA). For total and extracellular ATP, the quantification limits were set by the standard curve, which ranged from $1x10^{-4}$ nM to 10 nM. No total or extracellular ATP measurement was higher than the upper quantification limit. The lower quantification limit for intracellular ATP was determined by Miller et al. (40) as $1.83x10^{-5}$ nM. Empty tube measurements and reagent-only measurements were used as negative controls and reagent controls respectfully.

2.4 Heterotrophic plate counts

Heterotrophic plate counts (HPC) were determined using Quanti-Tray 2000 (IDEXX US; Westbrook, Maine) with HPC for Quanti-Tray media (IDEXX US; Westbrook, Maine) following the manufacturer's instructions with the trays incubated at 37°C for 44-72 hours. 100 mL of bulk water grab sample was transferred to autoclave sterilized bottles for each replicate and the geometric mean and geometric standard deviation were calculated. Technical duplicates of all samples were completed except samples from distribution system B in 2016, for which there were no replicates. The lower limit of quantification was set using the IDEXX Quanti-Tray format at a most probable number of one cell per 100 mL. The upper limit of quantification was set at a most probable number of 2419.6 cells per 100 mL (a fully positive IDEXX tray).

2.5 Statistical Analyses

Our dataset had inherent dependencies for which we needed to account in our analysis, including dependent variables that may be correlated with each other (e.g., pH, temperature, chlorine residual), samples collected from the same drinking water distribution system on the same day, or at the same location within a distribution system over time. Thus, relationships between microbial abundance and water quality parameters were assessed via correlation analyses and generalized linear mixed models using R (3.6.2) (47). To investigate potential multicollinearity, Spearman's Correlation values of all chemical and microbial water quality parameters were determined using Hmisc (4.3-0) (48) and GGally (1.4.0) (49). Data exploration was completed following Zuur et. al (50) using Cleveland dot plots to detect outliers, GGgally to assess colinearity, and scatter plots of all covariates to visualize relationships (50,51). Outliers and collinearity between covariates were not detected. Generalized linear mixed model (GLMM) analysis and validation was completed following the methods of Zuur et. al (51,52). Prior to analysis, microbial abundance metrics were tested for goodness of fit to a normal distribution, log-normal distribution, and gamma distribution (53) using goft (1.3.4) (54). The GLMM was fitted to raw intact cell counts from distribution system F with centered and scaled predictors (to improve the parameter optimization process) using lme4 (1.1-23) (55) with site as a random variable. The most optimal model was selected based on minimizing conditional Akaike information criterion with MuMIn (1.43.15) (56) through backward stepwise model selection. Wald confidence intervals for fixed effects were calculated using lme4. For correlation, GLMM, and summary statistic calculations, values below the quantification limit of intracellular ATP, total ATP, intact cell counts, total cell counts, HPC, free chlorine and total chlorine were replaced with the respective lower quantification limit for the assay to be conservative. However, for calculations of the coefficient of variation, only the quantifiable samples were used (Table 4). In figures, these data were plotted at a value below the quantification limit for visualization. Four HPC samples were above the quantification limit and were removed from all statistical analyses and figures. Plotting was

completed using ggplot2 (3.2.1) (57), tables were generated using stargazer (5.2.2) (58), plot fonts were set using extrafont (0.17) (59), figures with multiple plots were generated using ggpubr (0.4.0) (60), and color palettes were chosen from viridis (0.5.1) (61). The full reproducible code and csv files that have all data used in this paper is available in the Supplementary Materials as well as through GitHub (https://zenodo.org/record/3993877#.X5n0Qy9h1TZ).

3 Results

3.1 Impacts of physicochemical parameters on microbial abundance

We expected disinfectant residual concentration to be a master variable affecting microbial abundance across the various the drinking water distribution systems that were sampled. Thus, we plotted all data for each measure of microbial abundance as a function of disinfectant residual concentration. Of the five measurements of microbial abundance we applied, we observed inverse trends for two of them: intact cell counts (Figure 1A & 1D) and intracellular ATP (Figure 1B & 1E). The trends for HPC (Figure 1C and 1F), total cell counts in chloraminated systems (Figure S1A), and total ATP (Figure S1C and S1D) were less clear. In free chlorinated systems, a similar trend was observed for total cell count (Figure S1B) as for intact cell count (Figure 1D) likely because chlorine is a stronger disinfectant than chloramine (42). Thus, signal from non-viable cells and free DNA likely decreases more rapidly than in chlorinated systems. We also did not observe a trend for proportion of potentially viable cells (intact:total cells) (Figure S2).

In addition to disinfectant residual concentration, we recognized that temperature and pH might influence microbial abundance. To explore these relationships statistically, we used Spearman's correlation coefficients (r_s) to assess the strength of correlation between the various microbial abundance metrics, disinfectant concentration, temperature, and pH (Figures 2A & 2B). The correlation between microbial abundance metrics and residual disinfectant concentration is discussed first. In both chloraminated and chlorinated systems, total chlorine concentration was significantly and inversely correlated with both intracellular ATP and intact cell counts (r_s values between -0.65 and -0.85 ; p-values <0.001; Figures 2A & 2B), consistent with the visual trends in Figure 1. HPC were only significantly correlated with disinfectant residual in chloraminated systems (r_s= -0.46; p <0.001; Figure 2A). In contrast, we found that intracellular ATP was more strongly correlated with disinfectant concentration in chlorinated systems (r_s value -0.77 ; p

<0.001; Figures 2B) compared to chloraminated systems (r_s value -0.67 ; p <0.001; Figures 2A), but intact cell count was similar in both chlorinated (r_s values -0.63; p <0.01; Figures 2B) and chloraminated (r_s value -0.65; p <0.001; Figures 2A) systems. In chlorinated systems, the majority of the total chlorine concentration consisted of free chlorine except for in two cases for which total chlorine concentrations were <0.3 mg/L as Cl_2. Thus free and total chlorine concentrations were strongly correlated and appear to have similar impacts on measures of microbial abundance (Figure 2B). However, in chloraminated systems free chlorine concentration varied and was not significantly correlated with any microbial abundance parameters (Figure 2A).

In terms of the other two commonly monitored water quality variables, we observed significant correlations in chloraminated systems of temperature with intact cell counts (r_s = 0.44; p < 0.001) and with intracellular ATP (r_s = 0.48; p <0.001), but temperature was not correlated with any measures of microbial abundance in chlorinated systems (Figure 2A & 2B). pH was not significantly correlated with any measure of microbial abundance. We note that our dataset included ranges for disinfectant residual, temperature, and pH that are typical of drinking water distribution systems located in the western/ southwestern United States (Table S2).

In the chloraminated distribution system, microbial abundance measures were strongly correlated with both disinfect residual and temperature, and pH was strongly correlated with disinfectant residual. We wanted to assess relationships between these variables using a model. However, we needed to account for interactions between variables and for measurements from the same location that were not independent. For this approach, we focused on intact cell counts and developed a mixed model using data from distribution system F (n=80). Raw intact cell counts were not normally or log normally distributed, but the fit to a gamma distribution was not rejected (53). Thus, scaled and centered predictor variables (pH, temperature, free and total chlorine) and raw intact cell counts were used in a generalized linear mixed model with log link function

(Equation 1). The log link function was chosen because it requires positive fitted values. Sampling location within distribution system F ("site") was used as a random intercept to account for dependency associated with samples taken from the same site.

(Equation 1)

$$ICC_{ij} \sim Gamma(\mu_{ij},\tau)$$

$$\log(\mu_{ij}) = total\ chlorine_{ij} + free\ chlorine_{ij} + pH_{ij} + temperature_{ij} + total\ chlorine_{ij}\ X\ pH_{ij} + free\ chlorine_{ij}\ X\ pH_{ij} + free\ chlorine_{ij}\ X\ temperature_{ij} + total\ chlorine_{ij}\ X\ temperature_{ij} + site_i$$

$$site_i \sim N(0,\sigma^2_{site})$$

In Equation 1, ICC_{ij} is the intact cell count (with mean μ_{ij}) for the jth observation of $site_i$. ICC_{ij} is assumed to follow a gamma distribution with scale parameter, μ_{ij}, and shape parameter, τ. The random intercept, $site_i$, is assumed to be normally distributed with mean of 0 and variance of σ^2_{site}. Fixed effects include total chlorine, free chlorine, pH, temperature, and their interactions (included as interaction terms). We applied stepwise model selection (Table S3) to determine the most optimal model (Equation 2) with parameter estimates in Table 2.

(Equation 2)

$$ICC_{ij} \sim Gamma(\mu_{ij},\tau)$$

$$\log(\mu_{ij}) = total\ chlorine_{ij} + pH_{ij} + temperature_{ij} + free\ chlorine_{ij}\ X\ pH_{ij} + total\ chlorine_{ij}\ X\ temperature_{ij} + site_i$$

$$site_i \sim N(0,\sigma^2_{site})$$

The most optimal model shows that lower total chlorine concentrations resulted in higher intact cell counts; as expected, there was also an interaction with temperature that could result in higher intact cell counts at lower total chlorine values and higher temperatures (Figure 3). In Figure 3,

quantiles of temperature, from lowest (purple line) to highest (yellow line), are used in Equation 2 at a range of total chlorine concentrations. The total chlorine term was the largest parameter estimate for a fixed effect in this model (Table 2), which indicates that total chlorine had a large inverse effect on intact cell counts. In addition, higher pH and temperature values resulted in higher intact cell counts (Figure S3 & Table 2). However, the effect of temperature and pH on intact cell counts was smaller than that of total chlorine (Table 2). In addition, the interaction between pH and free chlorine in the optimized model was indistinguishable from 0 (0 falls within the confidence intervals shown in Table 2). It is known that free chlorine disinfection is more effective at pH values below 7.5 (42), but the minimum pH value in system F was 7.4 (Table S4). Thus, the pH in this system likely did not vary enough to produce an accurate estimate for this interaction term (Table S4).

Table 2: Estimated parameters, standard errors, and confidence intervals for each covariate of the most optimal model of intact cell counts in distribution system F (Equation 2). Generalized linear mixed model for intact cell counts with sampling location ("site") as a random variable, where $\sigma^2_{site} = 0.26$ and $\tau = 1.72$.

parameter	estimate	standard error	lower confidence interval (5%)	upper confidence interval (95%)
intercept	8.6	0.19	8.3	9.0
total chlorine	-1.3	0.13	-1.6	-1.1
pH	0.40	0.17	0.062	0.73
temperature	0.35	0.097	0.16	0.54
pH * free chlorine	0.39	0.23	-0.066	0.84
total chlorine * temperature	-0.24	0.12	-0.47	-0.0087

*Indicates interaction terms

We hypothesized that another variable in drinking water distribution systems that may correlate with microbial abundance is water age, given that the concentration of chlorine residual is known to diminish with water age, which could have substantial impacts on microbial abundance (62).

To investigate the impacts of water age on water quality, we compared water age with intact cell counts, intracellular ATP, HPC, and total chlorine concentration in distribution system F (Figure 4). Surprisingly, the measures of microbial abundance generally did not trend with water age (Figure 4 A-C). However, total chlorine generally decreased with water age during each specific sampling event (Figure S4). To investigate the variability in chlorine residual at individual sampling sites, we aggregated data from a year of sampling at 21 sites in distribution system F (Figure S5). Total chlorine at each sited varied over the course of a year depending on the location sampled and was not directly correlated with the water age at that site (Figure S5). These results suggest that total chlorine had a large impact on microbial abundance that was independent of water age in distribution system F.

3.2 Quantifiability and variability of five measures of microbial abundance

To evaluate the utility of the microbial abundance assays, we determined which measures of microbial abundance were most frequently quantifiable in disinfected drinking water systems. Intact cell counts yielded the highest percentage of results that were above lower quantification limits (97.6% of samples, n= 166; Table 3). In contrast, intracellular ATP was quantifiable in only 68.9% of samples (n= 113), and HPC were quantifiable in only 81.4% of samples (n= 102; 18.6% of samples either above or below limits of quantification). Total ATP and total cell counts were quantifiable in 100% of samples, as no samples were below the limit of quantification (Table 3). Interestingly, quantifiability of intracellular ATP was dependent on the system sampled (Tables S5 & S6). In particular, we saw a greater fraction of samples with concentrations of intracellular ATP above lower quantification limits from distribution system A (90.9% of samples with n= 11; Table S5) and distribution system B (90% of samples with n= 10; Table S5) and lower quantifiability in samples from distribution system F (64.1% of samples with n= 92; Table S5).

To evaluate the variability of the microbial abundance assays, we determined which measures of microbial abundance had the lowest average coefficients of variation. The coefficient of variation is commonly used to assess variability in quantitative bioassays and is reported as a percentage with a higher percentage indicating more variation among replicates (63). To summarize the variability across all samples taken in this study, we calculated an average coefficient of variation for each of the measures of microbial abundance by taking the arithmetic mean of all sample coefficients of variation (Table 4). Notably, variability was lower for total ATP (9.29%; Table 4), total cell counts (17.0%; Table 4), and intact cell counts (16.9%; Table 4), compared to intracellular ATP (56.0%; Table 4) and HPC (49.4%; Table 4).

Table 3: Percent of samples above quantification limit, below quantification limit, and quantifiable in all drinking water distribution systems sampled for this study for each microbial water quality assessment method. "n" is the number of samples per assay.

Assay	n	percent quantifiable	percent below quantification limit	percent above quantification limit
intact cell counts	166	97.6	2.4	0
total cell counts	166	100	0	0
intracellular ATP	113	69.9	30.1	0
total ATP	113	100	0	0
HPC	102	81.4	14.7	3.9

Table 4: Ranges in coefficient of variation determined by geometric mean (%; min, median, and max) and average coefficient of variation (%) for replicates taken in all drinking water distribution systems sampled for this study for each microbial water quality assessment method. “n” is the number of samples per assay.

Assay	n	Min coefficient of variation	Median coefficient of variation	Max coefficient of variation	Average coefficient of variation
intact cell counts	162	0.0266	9.78	148	16.9
total cell counts	166	0.318	6.15	255	17.0
intracellular ATP	79	43.0	48.6	328	56.0
total ATP	113	0.389	4.81	66.0	9.29
HPC	73	0	27.1	293	49.4

4 Discussion

We compared five measures of microbial abundance by surveying drinking water systems that apply residual disinfectants in California and Texas. In one chloraminated system, we used a generalized linear mixed model to estimate the effect of commonly measured water quality parameters on intact cell counts. In the following sections, we discuss the purpose and interpretation of typical microbial water quality factors and consider the future role of enhanced measures of microbial water quality for three applications: routine monitoring, diagnostics, and research.

4.1 Considerations for routine monitoring of drinking water systems

A key finding from this study is that disinfectant concentration in drinking water distribution systems provided an indirect measure of microbial abundance, which has useful implications for routine monitoring of distribution systems. Disinfectant residual had the largest inverse correlation coefficient regardless of residual type (Figure 2A & 2B). Furthermore, total chlorine had the greatest inverse effect on intact cell counts in a chloraminated drinking water distribution system

365 (distribution system F). Gillespie et al. (16) and Nescerecka et al. (18) also surveyed disinfected
366 distribution systems, but did not report trends between intact cell counts and disinfectant residual.
367 Gillespie et al. (16) sampled in chlorinated systems with free chlorine <0.8 mg/L as Cl_2 and
368 recommend maintaining free chlorine above 0.5 mg/L as Cl_2 to keep the fraction of potentially
369 viable cells below 0.2. In contrast, we did not observe a similar trend with the fraction of viable
370 cells (Figure S2), but we did find that intact cell count decreased to <100 cells/mL at free chlorine
371 concentrations above 1.5 mg/L as Cl_2. We assessed intact cell counts at a wider range of
372 disinfectant concentrations and observed a clear trend between disinfectant residual and intact
373 cell counts.
374 We presented a proof of concept that development of mixed models could help relate routinely
375 monitored physicochemical data to intact cell counts in drinking water distribution systems.
376 Drinking water providers in the United States commonly monitor pH, temperature, free chlorine,
377 and total chorine, and we incorporated these data into a model to estimate intact cell counts using
378 data from a chloraminated distribution system. The most optimal model (Equation 2) suggests
379 that total chlorine had the largest effect on intact cell counts and that this effect depended on
380 temperature. Zhang et al. (64) also found that disinfectant concentration had an inverse effect on
381 log-transformed HPC and visually observed higher values of log transformed HPC in the summer
382 than in the winter, but statistical results were inconsistent, likely due to variability in HPC results.
383 Using intact cell counts, instead of just the small fraction of total coliform bacteria or HPC, holds
384 promise to model a commonly observed phenomenon: in summer, a higher residual disinfectant
385 is necessary to maintain microbial water quality (65). This study focused on routinely measured
386 parameters in drinking water distribution systems in the southwestern United States, but more
387 research is needed to expand the dataset and modeling approach. This approach could include
388 a dataset that accounts for seasonal variability and source water quality changes as well as
389 includes additional biological (e.g., assimilable organic carbon) and physicochemical parameters
390 (e.g., total organic carbon concentration). However, including more parameters would require a

larger sample size than we collected for our model (n=80). In addition, modeling completed using data from multiple distribution systems will introduce a nested dependency structure in which both samples from the same system will be correlated as well as samples from the same site within a distribution system over time. With a more complete dataset, it might be possible to generate a model for which consistent deviations from model predictions at specific sites may be indicative of water quality problems, such as pipe corrosion or nitrification.

4.2 Intact cell counts and intracellular ATP assays as diagnostic tools

To better understand observed or expected changes in water quality, such the impact of nitrification, upgrading treatment processes, or incorporating a new treated water source (e.g., potable reuse), diagnostic monitoring can be necessary. However, the culturing methods commonly employed in routine monitoring, such as for total coliforms and HPC, often produce unquantifiable or unrepresentative results. For example, in a survey of U.S. drinking water providers, 57% of respondents reported never detecting total coliforms while the other 43% reported having fewer than 12 positive samples per year (n= 256 respondents; (46)). Similarly, our results support previous claims that HPC vastly underestimates drinking water microbial abundance as compared with intact cell counts (30). HPC only quantifies bacteria that can utilize organic nutrients for growth (29,41) and they have been shown to comprise <1% of bacteria in some drinking water samples (31,66). Prest et al. (28) reported a very high fraction of treated drinking water samples with HPC results below 5 CFU/mL while total cell counts ranged from 9.0 x 10^4 to 4.5 x 10^5 cells/mL.

For diagnostic purposes, use of intact cell counts would allow drinking water providers to detect changes in microbial water quality that are not observable using traditional microbial monitoring methods like HPC or total coliform quantification (30,41,43,67-69). In this study, 97.6% of samples

had quantifiable intact cell counts. Only four samples were below the intact cell count quantification limit, which occurred at the highest residual concentration observed in chlorinated drinking water distribution systems (1.5 - 2.0 mg/L as Cl_2; Figure 1). Intact cell counts spanned four orders of magnitude in chloraminated systems (from <22 cell/mL to 1.09 x 10^5 cells/mL) and more than two orders of magnitude in the chlorinated systems (<22 cells/mL to 2.12 x 10^3 cells/mL). As might be expected, these cell counts were lower than those reported in other studies with lower maximum residual disinfectant values or in systems without disinfectant residuals. For chlorinated distribution systems, the maximum cell counts from this study are about 1000 times less than those reported in Gillespie et. al (16). In addition, the geometric mean of intact cell counts of all distribution system samples in our study (3 x 10^3 cells/mL) was about 100 times lower than that of total cell counts reported for a system that does not apply a residual disinfectant (1 x 10^5 cells/ mL) (70).

Intracellular ATP may also be useful for diagnostic purposes because the values measured in this study correlated strongly with intact cell counts and ATP assays are less expensive. Drinking water providers monitoring microbial abundance for diagnostic purposes will need to choose measures of microbial abundance that maximize information gained and minimize expense. For this reason, it is important to consider how much each technique overlaps with other measures of microbial abundance and with chemical or physical water quality parameters. Intact cell counts and intracellular ATP results were strongly correlated (Figure 2A & 4B), and other studies have found similar correlations between ATP and intact cell counts among both chloraminated and chlorinated systems (18,28,30,66). Our results support the likelihood that most microbial abundance information will be obtained if either intact cell counts or intracellular ATP is measured. However, intact cell count was still more quantifiable and consistent compared to intracellular ATP. Intracellular ATP was quantifiable in only 69% of samples (Table 3) and technical replicates

varied considerably (average coefficient of variation = 55%; Table 4). Thus, intracellular ATP may only be preferable when expense is a primary concern.

4.3 Assessment of biostability and risk in disinfected drinking water systems

While there is no evidence that the safety of drinking water is compromised simply due to variations in microbial abundance, microbial growth in distribution systems is generally considered to be a risk (27,71,72). Choosing universal guidelines to maintain microbial water quality is not straightforward because microbial abundance is not directly linked to specific risks to infrastructure or public health. For example, setting a numerical operational limit for cell counts (e.g., 100 cells/mL) is not logical because microbial abundance varies considerably by water source and even within the same distribution system (17). In lieu of numerical operational limits, researchers have proposed maintaining biologically stable water, in which microbial abundance and composition does not significantly change throughout a distribution system (70,73). However, biologically stable drinking water is difficult to maintain in disinfected drinking water distribution systems (17,18) because disinfectant residual concentration has been shown to degrade in drinking water distribution systems as it reacts with pipe walls and organic matter (Figure S4) (62). In this study, disinfectant residual varied over a large range within chloraminated and chlorinated distribution systems, and there was a strong inverse correlation between the residual concentration and the microbial abundance.

Instead of maintaining biologically stable water, setting more subjective operational limits might be necessary in disinfected drinking water systems. Subjective operational limits have been set for HPC in the United Kingdom, France, the Netherlands, and Belgium where the upper limit is “no abnormal change” in HPC (30). While it is difficult to define “normal” in drinking water systems, normal can be operationally defined by measuring microbial water quality under a range of

conditions encountered in the system to establish a baseline and to discern contamination events from natural fluctuations (74,75) that have been well documented in drinking water distribution systems (76). To establish a baseline microbial abundance in drinking water systems, water providers could monitor intact cell counts or intracellular ATP data throughout the range of chemical and physical water quality conditions encountered in their systems under routine operations. The generalized linear mixed model presented in this paper represents one way to establish such a baseline and the methodology could be applied in other systems.

To more thoroughly assess health risk in drinking water systems, more research is needed to pair absolute microbial abundance measures with assessments of microbial community composition and the concentration of specific pathogens of concern. Significant research is underway to characterize microbial communities in drinking water using high-throughput sequencing technologies (e.g., 16S rRNA gene amplicon and metagenomic sequencing). Some researchers have paired microbial abundance data with sequencing data using quantitative polymerase chain reaction (qPCR) methods to provide a deeper characterization of microbial water quality (77,78,79). Combining qPCR with viability dyes brings a similar benefit as cell counts and ATP assays in that cell membrane damage can be used as a viability metric (80). However, these methods have limitations discussed previously (81), including limited resolution (twofold changes in gene copies; 82), bias introduced from assay design (82,83), and bias introduced with PCR (84). Others have paired flow cytometry with sequencing data to provide a similar characterization of microbial water quality without bias introduced from PCR (39,81,85). Ultimately, these studies may provide a sophisticated understanding of the complex interactions and factors that govern microbial ecology in drinking water systems. However, not all microbial ecology studies report absolute microbial abundance data. Pairing measures of microbial abundance with sequencing results has the potential to characterize microbial water quality in greater resolution than using any single method. This approach can provide more insight into risk in drinking water distribution

systems including potential exposure to opportunistic pathogens and other microbially induced issues, such as pipe corrosion (86,87), nitrification (88,89), and aesthetic deterioration of finished water (90).

For meta-omics research, we believe that the microbial abundance measures we studied that will be most useful to include are intact and total cell counts. Our flow cytometry results indicate that a varied fraction of cells in the sites we sampled were viable (Figure S2). Intact and total cell counts are quantified by a fluorescent dye that intercalates with DNA (91) and are a more direct measure of microbial abundance compared to ATP assays. Though cell count data were correlated with ATP data, ATP results were varied and often unquantifiable in these systems. While total cell count is more reflective of the sequenced microbial community, intact cell count is more reflective of the risk imposed by the microbial community. Thus, both total and intact cell counts could be useful to pair with meta-omics data and provide a more informative assessment of microbial water quality in drinking water systems.

5 Conclusions

Applying measures of microbial abundance in piped drinking water systems can be useful for routine monitoring, diagnostics, and research. Our results support that disinfectant residual is an indirect measure of microbial abundance, and the necessity of pairing it with direct measures is questionable for routine monitoring. However, for diagnostic purposes, additional monitoring data in systems with large ranges in microbial and physicochemical water quality conditions could help drinking water providers diagnose issues early and move beyond the goal of ensuring total coliforms are not detectable (92,93). For research, pairing meta-omics data with measures of microbial abundance can help researchers better characterize microbial water quality. Our results support that HPC assays are uninformative in these systems because these results are variable and often unquantifiable. Microorganisms are present throughout drinking water systems, and by

limiting analyses to HPC, the true microbial water quality cannot be observed. Instead, we recommend using either intracellular ATP or intact cell counts for diagnostic purposes and both intact and total cell counts to pair with meta-omics data. Our main findings are summarized as follows:

- Intact cells were measured in all six piped drinking water distribution systems, including chloraminated sites with total chlorine > 2.5 mg/L as Cl_2
- Only 2.4% of sampling sites, with the highest free chlorine concentrations (i.e., 1.5-2 mg/L as Cl_2), had intact cell counts below quantification limits
- Residual disinfectant concentration was significantly and strongly correlated with intracellular ATP and intact cell counts in distribution systems
- Negative correlations between residual disinfectant concentration and intracellular ATP were stronger in chlorinated systems than in chloraminated systems
- The parameter that had the greatest impact on intact cell counts in a chloraminated drinking water distribution system was total chlorine concentration, which interacted with temperature
- Of the five measures of microbial abundance, only total cell counts and total ATP were quantifiable in all samples, but these assays do not assess viability of cells
- Total ATP had the least variability among technical replicates followed by intact cell counts and total cell counts

6 Conflicts of Interest

There are no conflicts to declare

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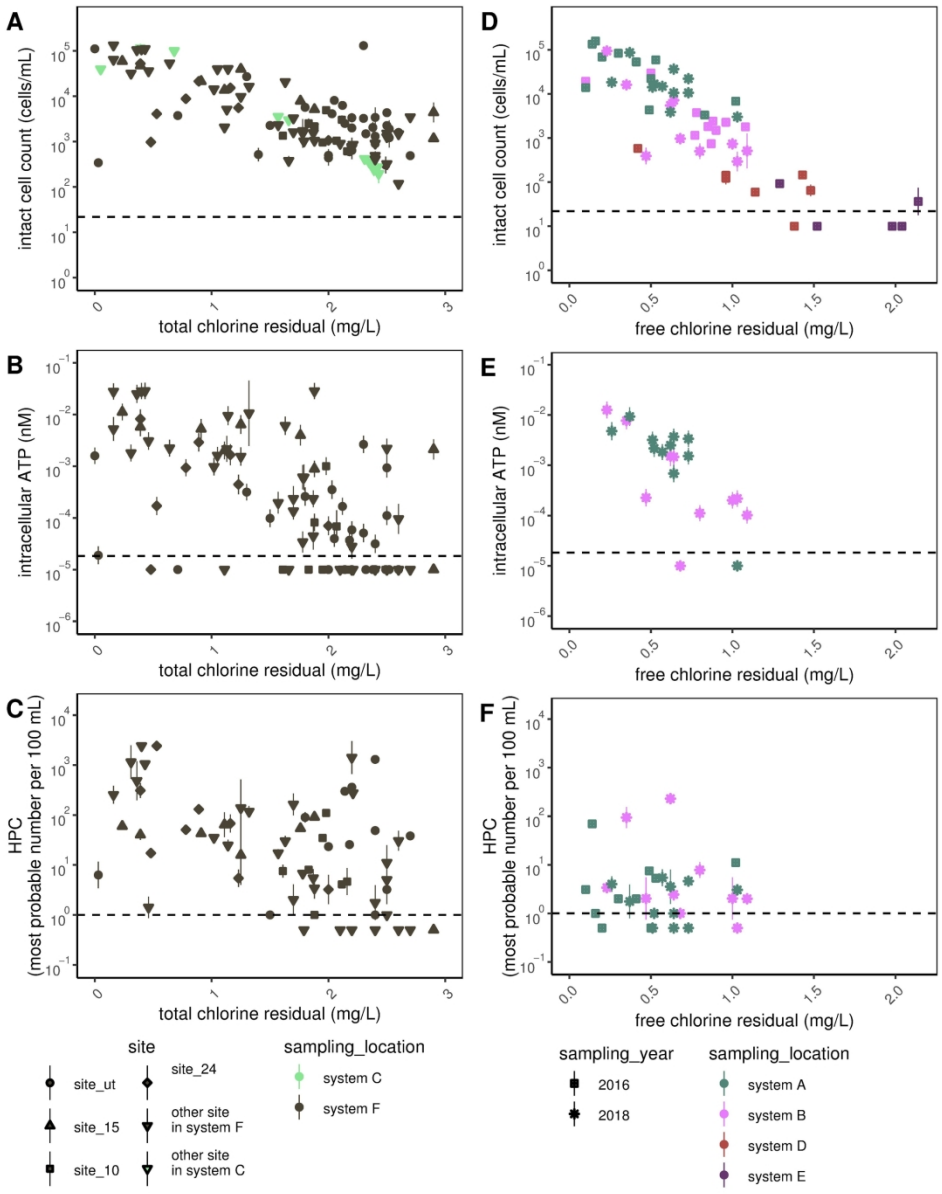
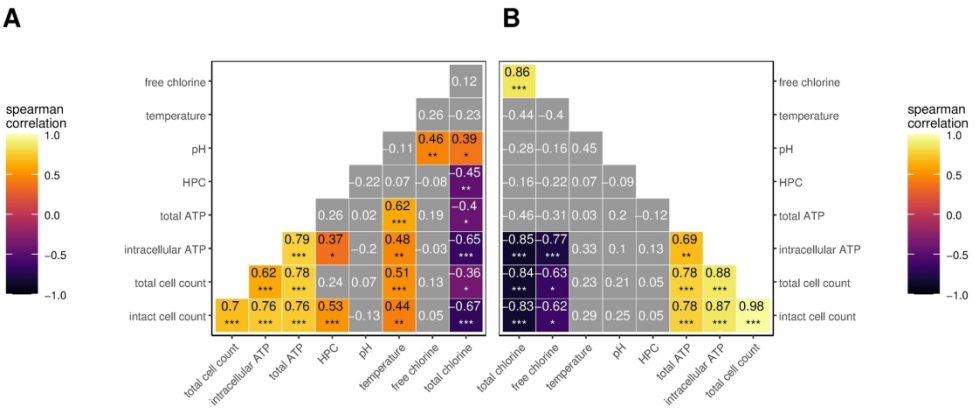


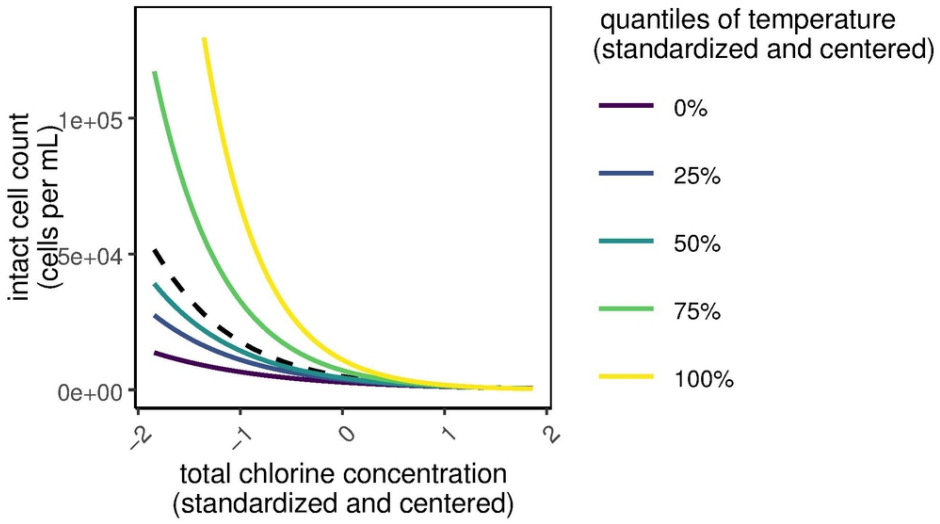
Microbial abundance measures in the drinking water distribution systems sampled in this study by disinfectant concentration. Left (chloraminated systems): intact cell counts (A), intracellular ATP (B), and HPC (C) in distribution system C (A) and distribution system F (A-C). Shapes denote sites in distribution system F that were sampled at least six times between August 2017 and April 2018. Right (chlorinated systems): intact cell counts (D), intracellular ATP (E), and HPC (F) in distribution system A (D-F), distribution system B (D-F), and distribution systems D and E (D). Shapes denote locations in distribution systems A and B that were sampled once in 2016 and repeated in 2018. Horizontal dashed lines denote quantification limits for each assay. Points are the geometric mean of the technical replicates and error bars represent the variation associated with technical replicates as quantified by the geometric standard deviation for technical replicates.

190x239mm (300 x 300 DPI)



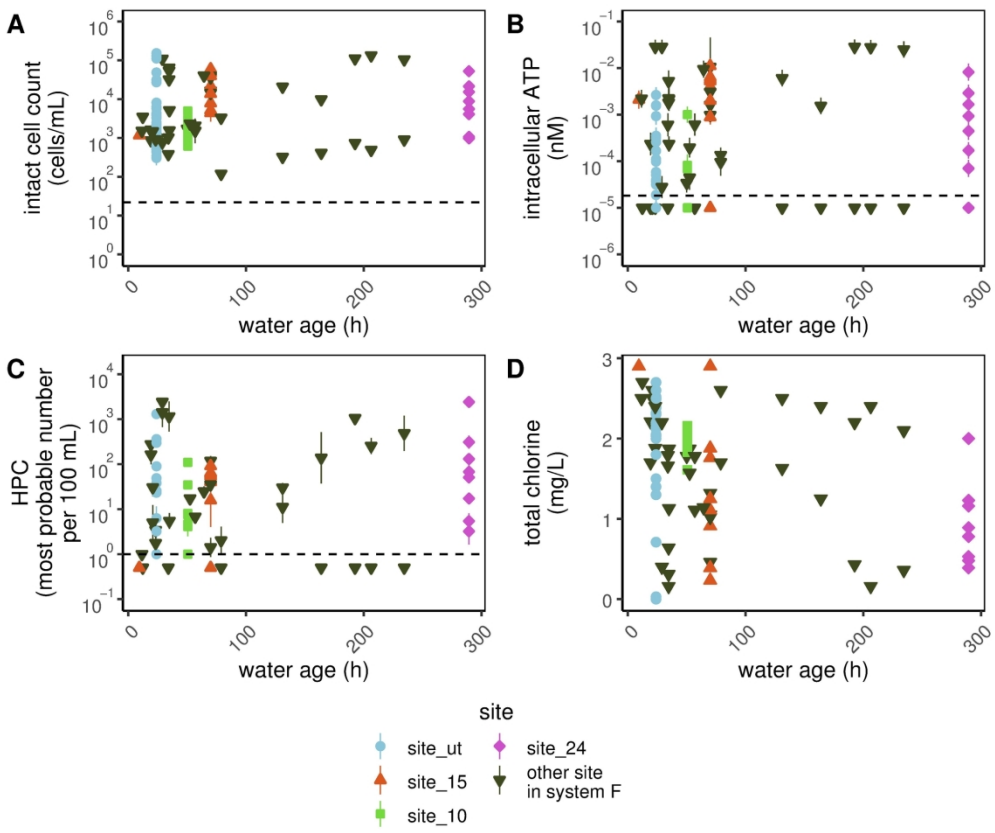
A) Spearman's correlation coefficient heat map for all samples with complete water quality data collected from a chloraminated drinking water distribution system (n= 61) (B) Spearman's correlation coefficient heat map for all samples with complete water quality data collected from chlorinated drinking water distribution systems (n= 21). Insignificant coefficients are shown in grey where significance is coded as * p< 0.01; ** p<0.001; *** p<0.0001.

190x95mm (300 x 300 DPI)



Visual representation of the most optimal model of intact cell counts in distribution system F (Equation 2). To generate dashed grey line, all fixed effects were held constant at their average value except for total chlorine (with bootstrapped 95% confidence intervals are shown in grey). To generate other lines, temperature was varied in the model at each quantile value (-1.9, -0.10, -0.53, 0.87, and 2.1). In Figure S3, other fixed effects are shown.

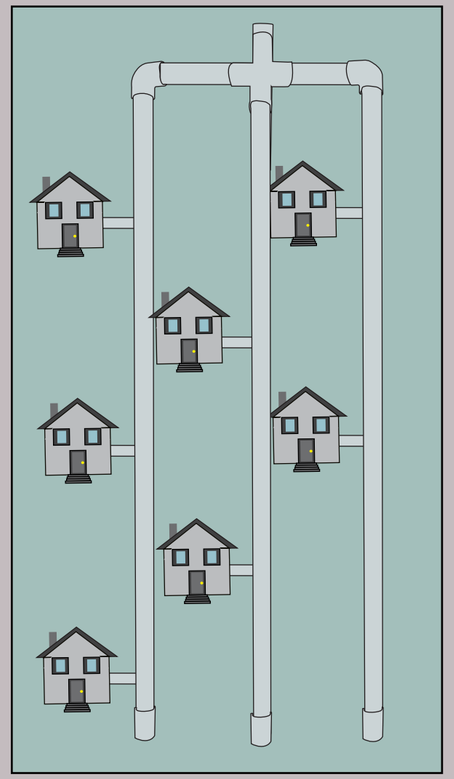
89x47mm (300 x 300 DPI)



Intact cell counts (A), intracellular ATP (B), HPC (C), and total chlorine concentration (D) by water age (hours) in distribution system F. Shapes denote locations in distribution system F that were sampled at least six times between August 2017 and April 2018. Horizontal dashed lines denote quantification limits for each assay. Points are the geometric mean of the technical replicates and error bars represent the variation associated with technical replicates as quantified by the geometric standard deviation for technical replicates.

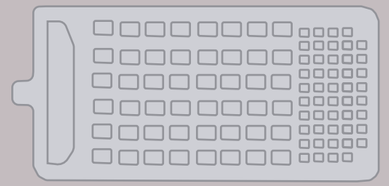
190x160mm (300 x 300 DPI)

1. Sample
drinking water distribution systems

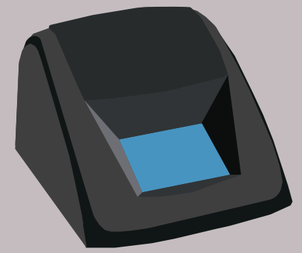


2. Compare
microbial abundance measures

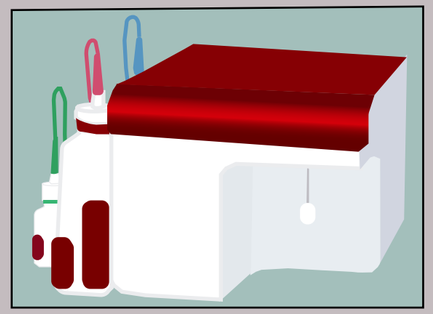
Heterotrophic plate counts



Intracellular ATP



Intact cell counts



3. Model
relationships