Published in final edited form as:
Sci Total Environ. 2021 June 20; 774: 145142. doi:10.1016/j.scitotenv.2021.145142.

Quantification of *Legionella pneumophila* by qPCR and culture in tap water with different concentrations of residual disinfectants and heterotrophic bacteria

Maura J. Donohue
United States Environmental Protection Agency, Office Research and Development, Center for Environmental Solutions and Emergency Response, Cincinnati, OH 45268, United States of America

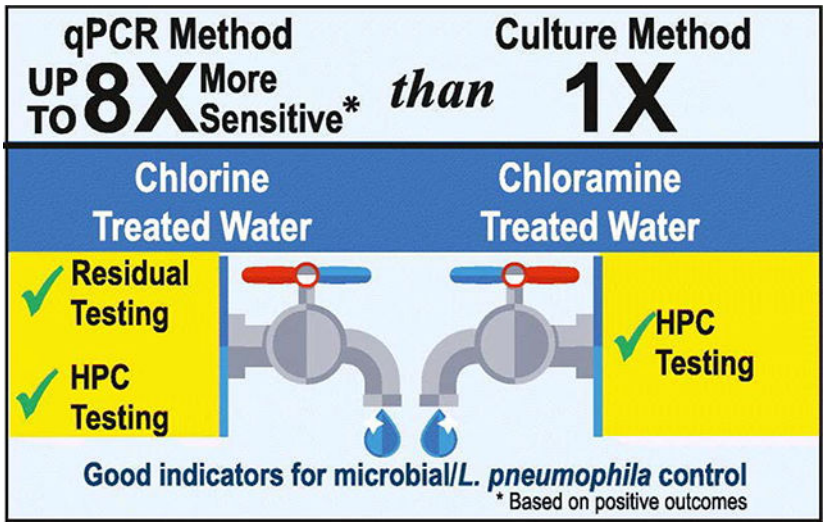
Abstract

Legionellosis prevalence is increasing in the United States. This disease is caused primarily by the bacterium *Legionella pneumophila* found in water and transmitted by aerosol inhalation. This pathogen has a slow growth rate and can "hide" in amoeba, making it difficult to monitor by the traditional culture method on selective media. Tap water samples (n = 358) collected across the United States were tested for *L. pneumophila by* both culture and quantitative Polymerase Chain Reaction (qPCR). The presence of other bacteria was quantified by heterotrophic plate counts (HPC). Residual disinfectant concentrations (free chlorine or monochloramine) were measured in all samples. *Legionella pneumophila* had the highest prevalence and concentration in the chlorinated water samples that had a free-chlorine value of less than 0.2 mg Cl_2/L. In total, 24% (87/358) of the samples were positive for *L. pneumophila* either by qPCR or 3% (11/358) were positive by culture. In chloramine-treated samples, *L. pneumophila* was detected by qPCR in 21% (31/148) and 1% (2/148) by culture, despite a high monochloramine residual >1 mg Cl_2/L. Despite the presence of a high disinfectant residual (>1 mg Cl_2/L), HPC counts were substantial. This study indicates that both culture and qPCR methods have limitations when predicting a potential risk for disease associated with *L. pneumophila* in tap water. Measuring disinfectant residuals and quantifying HPC in water samples may be useful adjunct parameters for reducing Legionellosis' risk from public water supplies at high-risk locations.

Graphical abstract

CRediT authorship contribution statement
The author met all criteria for authorship. Conceptualization, M.J.D; Sampling and data curation, M.J.D; Formal analysis, M.J.D; Investigation, M.J.D; Methodology, M.J.D.; Resources, M.J.D; Supervision, M.J.D; Visualization, M.J.D, Writing—original draft, M.J.D.; and Writing—review and editing, M.J.D.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Keywords

Legionella pneumophila ; Culture; Heterotrophic bacteria; qPCR; Monitoring; Residual

1. Introduction

Over 37,000 legionellosis cases were reported to the Centers for Disease Control and Prevention's (CDC) National Notifiable Disease and Condition List between 2011 and 2017. Legionnaires Disease and Pontiac Fever are two manifestations of a *L. pneumophila* infection. Legionnaires Disease is the most commonly reported form of legionellosis, usually caused by *Legionella pneumophila* (Hicks et al., 2012). Transmission of the *Legionella* bacterium primarily occurs through the inhalation of aerosols (Fraser et al., 1977). Although *L. pneumophila* is an environmental, water-borne bacterium (Bergey, 2012), it can survive drinking water treatment and be present in public water supplies (Stout et al., 1992). Therefore, it is critical to accurately and efficiently monitor these bacteria in water.

Traditionally, monitoring of *Legionella* populations in water samples relied on colony growth on selective media (APHA, 2012; ISO, 2017). The limitations of the culture methods to detect and quantify *Legionella* are well documented (Bonetta et al., 2010; Bopp et al., 1981; Whiley and Taylor, 2016). For example, the culture method was reported to recover only 10 to 40% of the *Legionella* population in a study reported by Lee et al. (2002). The culture method's limitations are due to *Legionella*'s slow growth rate (Bergey, 2012) and the fact that it can harbor inside amoeba, a single cell eukaryotic organism (Rowbotham, 1980) commonly found in water. Therefore, *Legionella* is difficult to monitor in water or other environmental media (Donohue et al., 2019a; Maze et al., 2014; Murdoch et al., 2013). Legionellosis can be a serious and expensive illness (Collier et al., 2012); thus, improved methods for monitoring *Legionella* in water are of high interest. Some molecular (Buse et al., 2019; Collins et al., 2015; Donohue et al., 2014; Joly et al., 2006; Wellinghausen et al.,

2001) and immunological (Berdal et al., 1979; Tilton, 1979) techniques have potential value as monitoring tools but need improvement.

Quantitative polymerase chain reaction (qPCR) is a DNA-based method that is widely used to demonstrate food safety (Allwood et al., 2020) for medical diagnostics (Kwok et al., 2018; Siracusano et al., 2020) and by forensic scientists. However, linking qPCR results to other measures of water quality is a new endeavor.

Heterotrophic plate counts (HPC) testing and measuring the disinfectant residuals are two tests traditionally applied to evaluate the microbiological quality of water. For example, a chlorine residual greater than 0.2 mg Cl_2/L, but not exceeding 4 mg Cl_2/L water (USEPA, 1994), signifies that microbial growth on an HPC plate will be low. On the other hand, water with a free-chlorine residual of less than 0.2 mg Cl_2/L is likely to have many colonies on an HPC plate (McCabe et al., 1970).

This study was conducted to evaluate the relationship between the results from qPCR measurements of *L. pneumophila* in water samples compared to culture-based results, and HPC colony counts as they relate to disinfectant residuals. The results of this study should help inform the three separate monitoring approaches and identify their efficacy as monitoring tools for predicting the risk that a given sample could be carrying *L. pneumophila*.

2. Materials and methods

2.1. Study design

Between 2011 and 2017, potable water samples were collected from taps within forty-six states across the United States. Three hundred and fifty eight water samples were analyzed; 210 samples came from taps where the water was chlorinated, and 148 water samples came from taps where the water was chloraminated. The sample collection volunteers were sent all sampling supplies with instructions. They collected water at each tap in two-500 mL high-density polypropylene (HDPP) Nalgene bottles (Thermo Scientific, Waltham, MA) following 15 s flush time. One of the 500 mL HDPP bottle contained 0.015 g/500 mL sodium thiosulfate to preserve the sample for culture work. On the day the sample was collected, the bottles were packed in ice and sent by next day air to the US EPA; Cincinnati, Ohio.

2.2. Heterotrophic plate counts

Immediately upon a sample arrival, Standard Methods 9215B was used to enumerate the heterotrophic bacteria (APHA, 2012). The bottle containing sodium thiosulfate was briefly shaken. A 100 μL aliquot of water was spread-plated onto an R2A agar plate in duplicate (Becton, Dickinson and Company; Sparks, MD) and incubated at 25 °C for seven days. Colony forming units (CFU) were counted and recorded on day seven.

2.3. Legionella culture

Upon a sample arrival, the *Legionella* spp. was recovered from the sample following Standard Methods 9260Ja (APHA, 2012). Briefly, from the bottle containing sodium

thiosulfate, a 100 mL aliquot was vacuum-filtered through a 0.2 μm Supor membrane (Pall Life Sciences; Port Washington, NY). Once completed, using forceps, the membrane was aseptically transferred to a 5 mL macrocentrifuge tube (Celltreat Scientific Products, Pepperell, MA) containing 5 mL sterile water and vortexed with 3 × 30 s pulses. Two Buffer Charcoal Yeast Extract (BCYE) (Becton, Dickinson and Company; Sparks, MD) plates were spread plated with 100 μL of suspension. Plates were incubated at 35 °C for seven days. The theoretical limit of detection for culture is one CFU/2 mL. Presumptive *Legionella* colonies were identified to the species level using the MALDI Biotyper (Bruker Daltronics, Bellrinca, MA) (Dilger et al., 2016). Briefly, cells representing a single colony on a culture plate were spread into individual wells on the MALDI target plate. A microliter of Bacterial Test Mix Solution (Bruker Daltronics, Bellrinca, MA) was also plated and used to calibrate the instrument. Next, 1 μL of α-Cyano-4-hydroxycinnamic acid (CHCA) matrix was overlaid on top of the cells and bacterial text mix and left to air dry. Once the target plate was dry, it was inserted into the mass spectrometer for calibration and protein fingerprint analysis using Bruker's Real-time bacterial identification software.

2.4. Quantitative PCR

The qPCR method and data were previously reported by Donohue et al. (2019b). Below is a brief description of the process. The 500 mL of water was vacuum-filtered through a 47 mm, 0.4 μm Whatman® Nucleopore™ Track-Etched Membrane (Whatman Inc., Piscataway, NJ). The membrane was transferred aseptically with forceps to a sterile, 2 mL O-ring screw cap microcentrifuge tube (Sarstedt, Newton, NC) containing 0.30 ± 0.05 g 0.1 mm of sterile glass beads (BioSpec Products, Bartlesville, OK). Next, DNA was extracted by adding 500 μL of Tissue and Cell Lysis Solution (Lucigen Corporation, Middleton, WI) to the bead-beaded tube (2 mL O-ring screw cap microcentrifuge tube) and bead-beaten for 3 min. After a 5 min cooldown in ice, the tube's bottom was punctured with a 1/18 gage needle. The bead-beating tube was inserted into a 1.5 mL microcentrifuge tube (Eppendorf, Hauppauge, NY), and the lysate was collected into the 1.5 mL microcentrifuge tube by centrifugation for 5 min at 3500 rpm. Next, 2 μL of Proteinase K (50 μg/μL) (Lucigen Corporation, Middleton, WI) was added to the lysate and incubated at 65 °C in a water bath for 15 min. Then, 2 μL of RNase A (5 μg/μL) (Lucigen Corporation, Middleton, WI) was added to the lysate and incubated at 37 °C for 30 min. Subsequently, 350 μL of MPC Protein Precipitation Reagent (Lucigen Corporation, Middleton, WI) was added to precipitate the cellular proteins. The resulting supernatant was transferred to a microcentrifuge tube with an equal volume of ice-cold isopropanol (~−4 °C) and centrifuged at 10,000 ×*g* for 10 min. The isopropanol was poured off, and the resulting DNA pellet washed with 500 μL of ice cold (~−4 °C) 70% ethanol. Samples were centrifuged, and the ethanol was removed by pipet. The DNA pellet was air dry for 15 min to remove any remaining ethanol residual. Then, the DNA pellet was re-suspended in 25 μL of TE buffer (Integrated DNA Technologies, Coralville, IA) and stored at −80 °C until analyzed.

The preparation of standards (positive control) and other controls (method control and non-template control) for the qPCR analysis are previously published (Donohue et al., 2019b). The primer-probe set used to detect and quantify *L. pneumophila* (Donohue et al., 2014) in water was published previously. The primer-probe set

used to detect *L. pneumophila*, targets the 16S rRNA gene; it includes the forward primers (L pneum F1): GGAATTACTGGGCGTAAAGG, reverse primer (Lpneum R1): GAGTCAACCAGTATTATCTGACCG, and probe (Lpneum P1): 6FAM-AAGCCCAGGAATTTCACAGAT–BHQ. Quantitative PCR conditions and instrument used for the L. *pneumophila* assay is as follows. The qPCR total reaction volume was 25 μL and consisted of 17 μL of TaqMan® Universal PCR Master Mix plus 2 μL of a mixture of forward and reverse primers (500 nM), a 100 nM IDT Probe, 1 μL of 0.1% Bovine Serum Albumin (BSA), and 5 μL of DNA purified. Five microliters of DNA extract were analyzed per qPCR reaction equating to 100 mL of the original sample volume. Reactions were performed in triplicate for the *L. pneumophila* assay using a Roche LightCycler® 480 II Real-time PCR system (Roche Applied Science, Indianapolis, IN). The LightCycler® setting included monocolor-hydrolysis detection with a pre-incubation step of 10 min at 95 °C, with 40 cycles of the following thermocycling conditions 15 s at 95 °C, 60 s at 60 °C, and a cooling step of 5 min at 30 °C.

The qPCR results were interpreted as follows: a sample was considered positive for *L. pneumophila* if two or more of the triplicates had a quantification cycle (C_q) value <39. Quantitative PCR Cq values were transformed to cell equivalence per mL (CE/mL) using a master standard curve linear equation. Quantitative PCR limit of detection can theoretically detect one genomic target per 100 mL. However, the limit of detection definition applied in this study is the lowest number of gene targets detected 100% of the time. For the Lp16S assay, this was 10 cell equivalence (CE)/100 mL or 10 genomic target/100 mL (Donohue et al., 2014).

2.5. Chlorine residual

These analyses were performed in the first 24 h after sample collection. The chlorine residual and monochloramine (chloramine) levels were measured colorimetrically using Hach's DPD-Free Chlorine and Monochlor F reagents (Loveland, CO). The manufacturer's instructions were followed.

2.6. Statistical analysis

Percentage calculations were performed in Excel (Microsoft Corporation, Redmond, WA). Statistical significance was determined using Mann-Whitney Rank Sum *t*-test Sigma Plot 14.0 (Systat, San Jose, CA) a significance level of 0.05.

3. Results

Legionella pneumophila was detected in 25% of the water samples (88/358) by either qPCR or culture. By qPCR, *L. pneumophila* was detected in 24% of the samples (87/356): i.e., 27% (56/210) from chlorinated water sources and 21% (31/148) from chloraminated water sources. By culture, *L. pneumophila* was recovered from far fewer samples 3% (11/358) of all samples, 4% (9/210) from chlorinated water, and 2% (2/148) from chloraminated water sources. Four colonies of *L. anisa* were the only other *Legionella* spp. recovered. The *L. anisa* colonies were all recovered from one water sample. There was one chloramine sample (1/2) that was culture positive but qPCR negative. Overall, *L. pneumophila* concentrations

measured by qPCR were about one to three logs greater than those estimated by culture (Fig. 1A and B).

Fig. 2 shows *L. pneumophila* prevalence and concentrations from the culture and qPCR analyses, as modulated by residual disinfectant concentrations. Culture detection of *L. pneumophila* (Fig. 2A) occurred in cases where there was no measurable free chlorine or when monochloramine concentrations were >1.0 mg Cl_2/L (see two samples designated with blue open diamond). Samples with different disinfectant residuals, *L. pneumophila* detections by qPCR (orange circles) are shown in Fig. 2B and C. *Legionella pneumophila* was detected in 73% of samples (41/56) that had no measurable free-chlorine residual, i.e., less than 0.1 mg Cl_2/L (Fig. 2B). Ninety percent (28/31) of the *L. pneumophila* detections in chloraminated waters had a monochloramine residual >1 mg Cl_2/L (Fig. 2C). Samples positive by both culture and qPCR are also shown in Fig. 2B and C (blue diamond, culture positive). In chloraminated water samples, *L. pneumophila* was detected by qPCR and by culture in water that had a wide range of monochloramine concentrations, 0.1 to 8 mg Cl_2/L water.

Sample's HPC results and residuals (free-chlorine or monochloramine) are graphed in Fig. 3. Additional designations were added to a sample's HPC result; if that sample was positive for *L. pneumophila* by both culture and qPCR (blue diamond) or qPCR (only) (red circle). The majority of *L. pneumophila* positive water samples had an HPC count exceeding 101 CFU/mL. The HPC data demonstrated a quarter log reduction in the bacterial count for every mg of free-chlorine present in the water (Fig. 3A). For chloraminated samples, the HPC data (Fig. 3B) demonstrated that the monochloramine residual did not impact the heterotrophic bacterial counts. A slight bacterial increase was observed for every mg of monochloramine present.

Fig. 4 shows *L. pneumophila* detection frequency and depicts concentration (box and whisker plot) in relationship to the heterotrophic bacterial count (shaded square). For each HPC segmentation, *L. pneumophila* detection frequency ranged from 18 to 34% by qPCR or 3–6% by culture.

Results indicate that heterotrophic bacteria at the 1–10 CFU/mL were not suppressing *L. pneumophila* regrowth. As the HPC concentrations increase, *L. pneumophila* concentrations (box and whisker plot) start shifting to the left of the HPC zone, signifying that heterotrophic bacteria are suppressing *L. pneumophila* regrowth. The farther the box and whisker plots are from the HPC zone could indicate that the impact of HPC bacterial utilization of nutrients acts to suppress the replication of the *L. pneumophila* microbes. The area (from the top of the HPC zone to the box and whisker plot 75th percentile) increases as the heterotrophic bacterial counts increase. The culture results showed a 2 to 3 log suppression and the qPCR results indicate a 1.5 to 4 log suppression of *L. pneumophila*.

In Fig. 4A and C, the qPCR data provides a finer resolution, revealing other trends. In Fig. 4A, the median concentrations of *L. pneumophila* decreased as the HPC counts increased (the differences between HPC >10 CFU/mL and >1000+ CFU/mL are statistically significant, $P = 0.015$). The box and whisker plot area that overlaps with the HPC zone

indicates that these samples were also culture positive. Further investigation into samples that were both culture and qPCR positive is illustrated in Fig. 5. Another apparent trend is the fact that the *L. pneumophila* box whisker plots are right-skewed. This distribution type suggest that several water quality factors could be impacting *L. pneumophila* survival and replication relative to those influencing the heterotrophic bacteria population. However, the occurrence of these circumstances is not frequent, as indicated by the long tailing of the distribution curve.

Fig. 5A compares the eleven *L. pneumophila* culture-positive samples, from both chlorine- and chloramine-treated water, to the samples' heterotrophic bacteria counts. It is clear from this figure that the culture confirmed *L. pneumophila* concentrations are small relative to the heterotrophic bacteria count from those same samples. The *L. pneumophila* concentration was higher than the heterotrophic bacteria count for only 1 of the 11 samples. The heterotrophic bacteria were one to three log units higher than the *L. pneumophila* concentrations for the other ten samples. There is no consistent relationship between the total HPC value and the probability that any given sample will harbor viable *L. pneumophila* as identified by culture (see samples 2, 5, 7, and 11).

Fig. 5B and C respectively reflect the findings from the chlorine and chloramine treated water samples. The individual sample numbers are identified on the X-axis. The black Y-axis background identifies the HPC counts for the individual samples arrayed from the lowest to highest HPC concentration for the chlorine (Fig. 5B) systems and chloramine (Fig. 5C). The red portion of the figure provides the qPCR results for each sample. The green stars are the culture positive samples aligned in accordance with the sample number for those that were both qPCR and culture positive. In chlorine-treated water (Fig. 5B), 17% (10/56) of samples had *L. pneumophila* concentration (CE/mL) that neared or exceeded the heterotrophic bacteria counts. Interestingly, these samples also had the highest likelihood of being culture positive. In chloramine-treated water, the HPC counts were 4 to 6 logs greater in concentration compared to the *L. pneumophila* concentrations in many water samples, 73% (24/33).

4. Discussion

Legionella pneumophila contamination in tap water can be determined by both culture and qPCR methods. However, each approach has its own limitations in estimating risk. The culture method lacked the ability to detect all live *L. pneumophila* microorganisms, including those that were viable but nonculturable cells (VBNC) or amoeba encysted. The culture method often underestimates the levels of *L. pneumophila* in a water sample. On the other hand, the qPCR method can detect both alive and damaged/dead cells by detecting decaying DNA. Therefore, the method can overestimate the *L. pneumophila* concentrations (Joly et al., 2006). These limitation are clearly recognized in the published literature (Behets et al., 2007; Collins et al., 2017; Joly et al., 2006; Merault et al., 2011; Wullings and van der Kooij, 2006; Yanez et al., 2005). Thus, additional research is needed to further refine monitoring approaches (culture, qPCR, or some combination of the two) to provide a stronger link between the presence of *L. pneumophila* and the risk for disease.

In tap water, disinfectant residuals and heterotrophic bacteria counts are water quality parameters that provide important context relative to *L. pneumophila* occurrence risk. As expected, in free-chlorine treated water, most of the *L. pneumophila* detections (culture and qPCR) were in water with free chlorine residuals below 0.2 mg Cl_2/L water, suggesting that the distribution system's guidelines require residuals to be greater than 0.2 mg Cl_2/L are health protective (USEPA, 2006). However, in cases where the water was treated with chloramine, this was not the case. *L. pneumophila* detections occurred across the monochloramine concentration span. The lack of a correlation between monochloramine residual and the detection of *L. pneumophila* by both qPCR and culture is apparent. When viable bacteria counts were examined, 75% (24/32) of the *L. pneumophila* qPCR detections were from samples that had high HPC (>101 CFU/mL water) viable bacterial counts; despite a high monochloramine residual concentration (Fig. 3B). These results suggest that the free-chlorine residual has a stronger antimicrobial potency than monochloramine.

Independent of a sample's heterotrophic bacterial count, the culture method only achieved a 3–6% recovery of *L. pneumophila* (Fig. 4). Compared to the qPCR quantification, the culture detection threshold for *Legionella* indicates that it has fundamental limitations. Either not enough water was analyzed, or the *L. pneumophila* concentration was well below the culture method's recovery limit (APHA, 2012). Another possible explanation for the poor performance of the culture method is low method robustness, due to other water quality characteristics (e.g., hardness, total organic carbon, pH) that affect *Legionella's* recovery (Kuchta et al., 1983). Method selectivity may also be limiting culture performance. Although most *Legionella* spp. can grow on the selective BCYE media, a heavy inoculum is needed (Joly et al., 2006; Lee et al., 1993; Wang et al., 2012; Wullings and van der Kooij, 2006).

The culture recovery rates of the other *Legionella* spp. are even lower than *L. pneumophila* recovery rates. In this study, 0.3% (1/358) samples yielded a *Legionella* non-*pneumophila* species compared to the 3% of *L. pneumophila* positive samples. The Kyritsi et al. (2018) study had similar results. In this case, 9% (46/505) of samples were *Legionella* non-*pneumophila* positive, and 38% (192/505) were *L. pneumophila* positive (Kyritsi et al., 2018). However, studies that used a *Legionella* spp. (genus) qPCR methods reveal that ~83% of samples were *Legionella* spp. positive (genus), and about 28% of the samples were *L. pneumophila* positive (Schwake et al., 2016; Wang et al., 2012). With the use of molecular tools, it becomes evident that *Legionella* spp. are more prevalent in the water than what culture methods are indicating. Therefore, it cannot be assumed that the culture method is suitable for recovering all environmental *Legionella* spp., and perhaps not suited for all environmental *L. pneumophila* strains.

HPC testing is a good measure of microbial control. For in the presence or absence of treatment, it does quantify the number of heterotrophic microbes present in the water. However, HPC results are only a fair predictor of *L. pneumophila* occurrence in drinking water (Bargellini et al., 2011; Duda et al., 2015). This is due to *L. pneumophila* not being able to compete with the heterotrophic bacteria for nutrients. Evidence of this is observed in Fig. 4A and C. The *L. pneumophila* highest detection frequencies of 33 and 36% were in the 101–1000 CFU/mL range. As the heterotrophic bacteria exceeded 1001 CFU/mL the

L. pneumophila detection frequency dropped to 20 to 23% of samples being positive. It is only under the strict conditions of temperature, nutrients, and pH that *L. pneumophila* can grow under laboratory conditions, requiring 3 to 10 days for a visible colony to appear (Warren and Miller, 1979). *L. pneumophila* has a generation time (the amount of time needed to duplicate) of 4 to 6 h (Ristroph et al., 1981; Warren and Miller, 1979). The heterotrophic bacteria generation time is minutes (LaBauve and Wargo, 2012; Mahdi et al., 2014). This evolutionary strategy of a longer generation time allows *L. pneumophila* to persist, not overpopulate in most water conditions. This persistence strategy was observed in the qPCR data (Fig. 4A and C). Regardless of the heterotrophic bacteria concentrations, approximately 30% of the samples were positive for *L. pneumophila* in both chlorine and chloramine treated water (Fig. 4). Additionally, in King et al. source and drinking water study demonstrates that even in ambient waters *L. pneumophila* median concentration is low (2.4 CE/mL) relative to HPC results of 3 to 4 logs (CFU/mL) and comparable to the results observed in treated water (unpublished HPC results) (King et al., 2016). This paper emphasizes *L. pneumophila* persistence strategy in untreated water.

In chloramine treated water, the monochloramine residual was unexpectantly not a reliable indicator of microbial control. Without performing the HPC test, the loss of microbial control would not be detected. Therefore, the positive qPCR results from the chloramine treated water samples could not be fully explained without realizing that a large viable heterotrophic bacterial population thrived.

Quantitative PCR assays can detect DNA from damaged and dead cells. The positive samples do not necessarily pose a risk to human health. If residuals were greater than 0.1 mg Cl_2/L water and the heterotrophic plate counts were less than 101 CFU/mL, then qPCR only detected *L. pneumophila* in 16% (14/88) of the water samples. This microbial subpopulation is unlikely to be of risk to human health. Most qPCR positive water samples (84%) were in water that had no chlorine residual. Nevertheless, the thresholds of 0.1 mg Cl_2/L free-chlorine and >101 CFU/mL in water were arbitrary in this analysis and need to be validated or modified based on water samples associated with actual legionellosis cases or outbreaks.

Measurement of disinfectant residuals and HPC testing in water samples are parameters that can support the risk analysis when paired with results from culture or qPCR applications. Chloramine-treated water demonstrated a lack of microbial control despite a high residual. Therefore, the positive *L. pneumophila* detection in chloramine-treated water cannot be dismissed without further assessment. The results indicate that bacterial control measures were not performing at the point-of-use locations. Therefore, the frameworks for monitoring *L. pneumophila* at public water systems may need refinement. Similar monitoring adjustments may also be needed for cooling towers and HVAC systems because these sources are prone to *Legionella* spp. and *L. pneumophila* contamination (WHO, 2007).

Acknowledgments

Disclaimer

The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here. It has been subjected to the Agency's administrative review and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Abbreviations

qPCR	quantitative Polymerase Chain Reaction
HPC	heterotrophic plate counts
CFU	colony forming units
US EPA	United States Environmental Protection Agency
CDC	Centre for Disease Control and Prevention
CE	cell equivalence

References

Allwoodet al., 2020Allwood JS, Fierer N, Dunn RRThe future of environmental DNA in forensic scienceAppl. Environ. Microbiol, 86 (2020), 10.1128/AEM.01504-19, APHA 2012 APHA

Standard Methods for the Examination of Water and Wastewater: American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF), Legionella Method9215b (2012)

Bargelliniet al., 2011Bargellini A, Marchesi I, Righi E, Ferrari A, Cencetti S, Borella P, et al.Parameters predictive of Legionella contamination in hot water systems: association with trace elements and heterotrophic plate countsWater Res., 45 (2011), pp. 2315–2321 [PubMed: 21316728]

Behetset al., 2007Behets J, Declerck P, Delaedt Y, Creemers B, Ollevier FDevelopment and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samplesJ. Microbiol. Methods, 68 (2007), pp. 137–144, 10.1016/j.mimet.2006.07.002 [PubMed: 16914218]

Berdalet al., 1979Berdal BP, Farshy CE, Feeley JCDetection of *Legionella pneumophila* antigen in urine by enzyme-linked immunospecific assayJ. Clin. Microbiol, 9 (1979), pp. 575–578 [PubMed: 383744]

Bergey, 2012Bergey DHBergey's Manual of Systematic Bacteriology (2nd ed.), vol. 5, Springer-Verlag, New York, NY (2012)

Bonettaet al., 2010Bonetta S, Bonetta S, Ferretti E, Balocco F, Carraro EEvaluation of *Legionella pneumophila* contamination in Italian hotel water systems by quantitative real-time PCR and culture methodsJ. Appl. Microbiol, 108 (2010), pp. 1576–1583, 10.1111/j.1365-2672.2009.04553.x [PubMed: 19796090]

Boppet al., 1981Bopp CA, Sumner JW, Morris GK, Wells JGIsolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective mediumJ. Clin. Microbiol, 13 (1981), pp. 714–719 [PubMed: 7229015]

Buseet al., 2019Buse HY, JM B, Struewing IT, Szabo JGChlorine and monochloramine disinfection of *Legionella pneumophila* colonizing copper and polyvinyl chloride drinking water biofilmsAppl. Environ. Microbiol, 85 (2019), 10.1128/AEM.02956-18

Collier, 2012Collier SA, Stockman LJ, Hicks LA, Garrison LE, Zhou FJ, Beach MJDirect healthcare costs of selected diseases primarily or partially transmitted by waterEpidemiol. Infect, 140 (2012), pp. 2003–20013, 10.1017/S0950268811002858 [PubMed: 22233584]

Collinset al., 2015Collins S, Jorgensen F, Willis C, Walker JReal-time PCR to supplement gold-standard culture-based detection of *Legionella* in environmental samplesJ. Appl. Microbiol, 119 (2015), pp. 1158–1169, 10.1111/jam.12911 [PubMed: 26218315]

Collinset al., 2017Collins S, Stevenson D, Walker J, Bennett AEvaluation of *Legionella* real-time PCR against traditional culture for routine and public health testing of water samplesJ. Appl. Microbiol, 122 (2017), pp. 1692–1703, 10.1111/jam.13461 [PubMed: 28376265]

Dilgeret al., 2016Dilger T, Melzl H, Gessner ARapid and reliable identification of waterborne Legionella species by MALDI-TOF mass spectrometryJ. Microbiol. Methods, 127 (2016), pp. 154–159, 10.1016/j.mimet.2016.05.028 [PubMed: 27260989]

Donohueet al., 2014Donohue MJ, O'Connell K, Vesper SJ, Mistry JH, King D, Kostich M, et al.Widespread molecular detection of *Legionella pneumophila* Serogroup 1 in cold water taps across the United StatesEnviron. Sci. Technol, 48 (2014), pp. 3145–3152, 10.1021/es4055115 [PubMed: 24548208]

Donohueet al., 2019aDonohue MJ, King D, Pfaller S, Mistry JHThe sporadic nature of *Legionella pneumophila*, *Legionella pneumophil*a Sg1 and *Mycobacterium avium* occurrence within residences and office buildings across 36 states in the United StatesJ. Appl. Microbiol, 126 (2019), pp. 1568–1579, 10.1111/jam.14196 [PubMed: 30891905]

Donohueet al., 2019bDonohue MJ, Vesper S, Mistry J, Donohue JMImpact of chlorine and chloramine on the detection and quantification of *Legionella pneumophila* and *Mycobacterium* speciesAppl. Environ. Microbiol, 85 (2019), 10.1128/AEM.01942-19

Dudaet al., 2015Duda S, Baron JL, Wagener MM, Vidic RD, Stout JELack of correlation between *Legionella* colonization and microbial population quantification using heterotrophic plate count and adenosine triphosphate bioluminescence measurementEnviron. Monit. Assess, 187 (2015), p. 393, 10.1007/s10661-015-4612-5 [PubMed: 26038316]

Fraseret al., 1977Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, et al.Legionnaires' disease: description of an epidemic of pneumoniaN. Engl. J. Med, 297 (1977), pp. 1189–1197, 10.1056/NEJM197712012972201 [PubMed: 335244]

Hickset al., 2012Hicks LA, Garrison LE, Nelson GE, Hampton LMLegionellosis—United States, 2000–2009Am. J. Transplant, 12 (2012), pp. 250–253, 10.1111/j.1600-6143.2011.03938.x [PubMed: 22244124]

ISO, 2017 ISO ISO 11731: Water Quality-Detection and Enumeration of *Legionella*International Organization for Standardization (ISO), Geneva, Switerland (2017)

Jolyet al., 2006Joly P, Falconnet PA, Andre J, Weill N, Reyrolle M, Vandenesch F, et al.Quantitative real-time *Legionella* PCR for environmental water samples: data interpretationAppl. Environ. Microbiol, 72 (2006), pp. 2801–2808, 10.1128/AEM.72.4.2801-2808.2006 [PubMed: 16597985]

Kinget al., 2016King DN, Donohue MJ, Vesper SJ, Villegas EN, Ware MW, Vogel ME, et al.Microbial pathogens in source and treated waters from drinking water treatment plants in the United States and implications for human healthSci. Total Environ, 562 (2016), pp. 987–995, 10.1016/j.scitotenv.2016.03.214 [PubMed: 27260619]

Kuchtaet al., 1983Kuchta JM, States SJ, McNamara AM, Wadowsky RM, Yee RBSusceptibility of *Legionella pneumophila* to chlorine in tap waterAppl. Environ. Microbiol, 46 (1983), pp. 1134–1139 [PubMed: 6360045]

Kwoket al., 2018Kwok MK, Lin SL, Schooling CMRe-thinking Alzheimer's disease therapeutic targets using gene-based testsEBioMedicine, 37 (2018), pp. 461–470, 10.1016/j.ebiom.2018.10.001 [PubMed: 30314892]

Kyritsiet al., 2018Kyritsi MA, Mouchtouri VA, Katsioulis A, Kostara E, Nakoulas V, Hatzinikou M, et al.*Legionella* colonization of hotel water systems in touristic Places of Greece: association with system characteristics and physicochemical parametersInt. J. Environ. Res. Public Health, 15 (2018), 10.3390/ijerph15122707

LaBauve and Wargo, 2012 LaBauve AE, Wargo MJ Growth and laboratory maintenance of Pseudomonas aeruginosa Curr. Protoc. Microbiol (2012), 10.1002/9780471729259.mc06e01s25 (Chapter 6: Unit 6E 1)

Leeet al., 1993Lee TC, Vickers RM, Yu VL, Wagener MMGrowth of 28 Legionella species on selective culture media: a comparative studyJ. Clin. Microbiol, 31 (1993), pp. 2764–2768 [PubMed: 8253978]

Leeet al., 2002Lee JV, Surman SB, Hall M, Cuthbert LDevelopment of an international EQA scheme for the isolation of Legionella spp. from environmental specimens

Marre R, Abu Kwaik Y, Bartlett CL, Cianciotto NP, Fields S, Frosch M, et al. (Eds.), Legionella, ASM Press, Washington DC (2002), pp. 271–274

Mahdiet al., 2014Mahdi O, Eklund B, Fisher NLaboratory culture and maintenance of Stenotrophomonas *maltophilia*Curr. Protoc. Microbiol, 32 (2014), 10.1002/9780471729259.mc06f01s32 (Unit 6F 1)

Mazeet al., 2014Maze MJ, Slow S, Cumins AM, Boon K, Goulter P, Podmore RG, et al.Enhanced detection of Legionnaires' disease by PCR testing of induced sputum and throat swabsEur. Respir. J, 43 (2014), pp. 644–646, 10.1183/09031936.00191913 [PubMed: 24311761]

McCabeet al., 1970McCabe LJ, Symons JM, Lee RD, Robeck GGSurvey of community water supply systemsJ. AWWA, 62 (1970), pp. 670–687

Meraultet al., 2011Merault N, Rusniok C, Jarraud S, Gomez-Valero L, Cazalet C, Marin M, et al.Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samplesAppl. Environ. Microbiol, 77 (2011), pp. 1708–1717, 10.1128/AEM.02261-10 [PubMed: 21193672]

Murdochet al., 2013Murdoch DR, Podmore RG, Anderson TP, Barratt K, Maze MJ, French KE, et al.Impact of routine systematic polymerase chain reaction testing on case finding for Legionnaires' disease: a pre-post comparison studyClin. Infect. Dis, 57 (2013), pp. 1275–1281, 10.1093/cid/cit504 [PubMed: 23899682]

Ristrophet al., 1981Ristroph JD, Hedlund KW, Gowda SChemically defined medium for *Legionella pneumophila* growth J. Clin. Microbiol, 13 (1981), pp. 115–119

Rowbotham, 1980Rowbotham TJPreliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebaeJ. Clin. Pathol, 33 (1980), pp. 1179–1183 [PubMed: 7451664]

Schwakeet al., 2016Schwake DO, Garner E, Storm RO, Pruden A, Edwards MA*Legionella* DNA markers in tap water coincident with a spike in legionnaires disease in Flint, MIEnviron. Sci. Technol. Lett, 3 (2016), pp. 311–315, 10.1021/acs.estlett.6b00192

Siracusanoet al., 2020Siracusano S, Rizzetto R, Porcaro ABBladder cancer genomicsUrologia, 87 (2) (2020), pp. 49–56, 10.1177/0391560319899011 [PubMed: 31942831]

Stoutet al., 1992Stout JE, Yu VL, Muraca P, Joly J, Troup N, Tompkins LSPotable water as a cause of sporadic cases of community-acquired legionnaires' disease N. Engl. J. Med, 326 (1992), pp. 151–155, 10.1056/NEJM199201163260302

Tilton, 1979Tilton RCLegionnaires' disease antigen detected by enzyme-linked immunosorbent assayAnn. Intern. Med, 90 (1979), pp. 697–698, 10.7326/0003-4819-90-4-697 [PubMed: 434657]

US EPA, 1994US EPA National Primary Drinking Water Regulations: Disinfectants and Disinfection ByproductsUnited States Environmental Protection Agency, Washington DC (1994), pp. 38668–38829

US EPA, 2006US EPA Long term 2 enhanced surface water treatment rule. 71 Fed. Regist. (2006), pp. 654–786

Wanget al., 2012Wang H, Edwards M, Falkinham JO 3rd, Pruden AMolecular survey of the occurrence of *Legionella* spp., *Mycobacterium* spp., P*seudomonas* aeruginosa, and amoeba hosts in two chloraminated drinking water distribution systemsAppl. Environ. Microbiol, 78 (2012), pp. 6285–6294, 10.1128/AEM.01492-12 [PubMed: 22752174]

Warren and Miller, 1979 Warren WJ, Miller RD Growth of Legionnaires disease bacterium (Legionella pneumophila) in chemically defined medium J. Clin. Microbiol, 10 (1979), pp. 50–55 [PubMed: 500795]

Wellinghausenet al., 2001Wellinghausen N, Frost C, Marre RDetection of legionellae in hospital water samples by quantitative real-time LightCycler PCRAppl. Environ. Microbiol, 67 (2001), pp. 3985–3993 [PubMed: 11525995]

Whiley and Taylor, 2016 Whiley H, Taylor M Legionella detection by culture and qPCR: comparing apples and oranges Crit. Rev. Microbiol, 42 (2016), pp. 65–74, 10.3109/1040841X.2014.885930 [PubMed: 24580080]

WHO, 2007WHO *Legionella* and the Prevention of LegionellosisWorld Health Organization (2007)

Wullings and van der Kooij, 2006 Wullings BA, van D der Kooij Occurrence and genetic diversity of uncultured *Legionella* spp. in drinking water treated at temperatures below 15 degrees C

Appl. Environ. Microbiol, 72 (2006), pp. 157–166, 10.1128/AEM.72.1.157-166.2006 [PubMed: 16391038]

Yanezet al., 2005Yanez MA, Carrasco-Serrano C, Barbera VM, Catalan VQuantitative detection of Legionella pneumophila in water samples by immunomagnetic purification and real-time PCR amplification of the dotA geneAppl. Environ. Microbiol, 71 (2005), pp. 3433–3441, 10.1128/AEM.71.7.3433-3441.2005 [PubMed: 16000746]

Highlights

- Culture underestimates *L. pneumophila* detection frequency and concentration.
- Quantitative PCR is more sensitive than culture for *L. pneumophila* detection.
- Free-chlorine residual is a good indicator of microbiological quality control.
- Monochloramine residual is not a good indicator of microbiological quality control.
- For chloramine water, HPC analysis is the better indicator of microbial control.

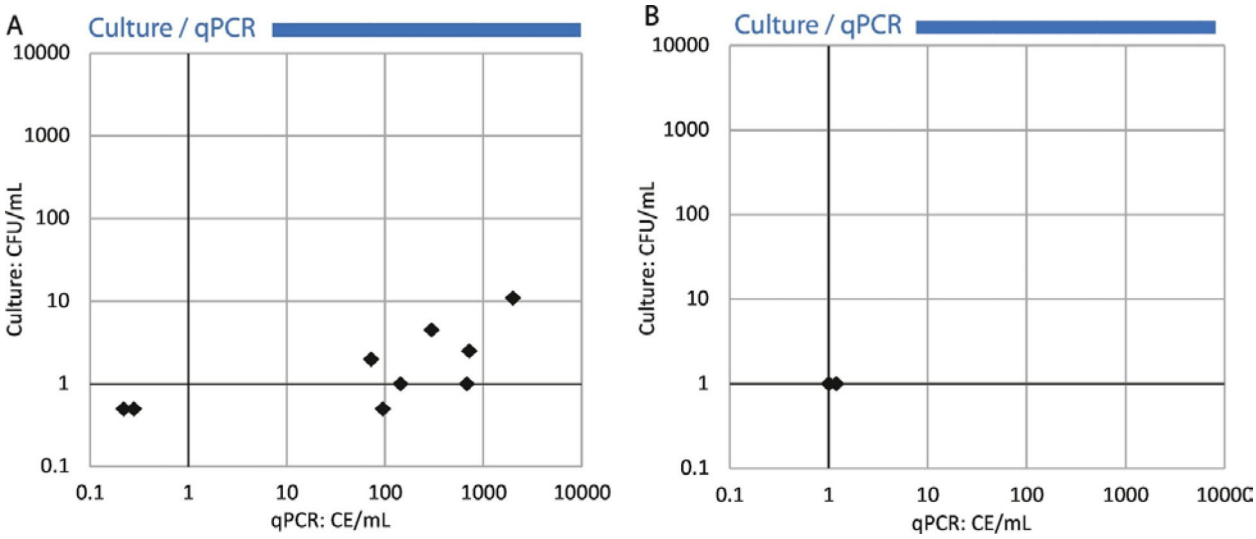


Fig. 1.

Legionella pneumophila positive samples by culture and qPCR results. A. Chlorine-treated water, B. Chloramine-treated water.

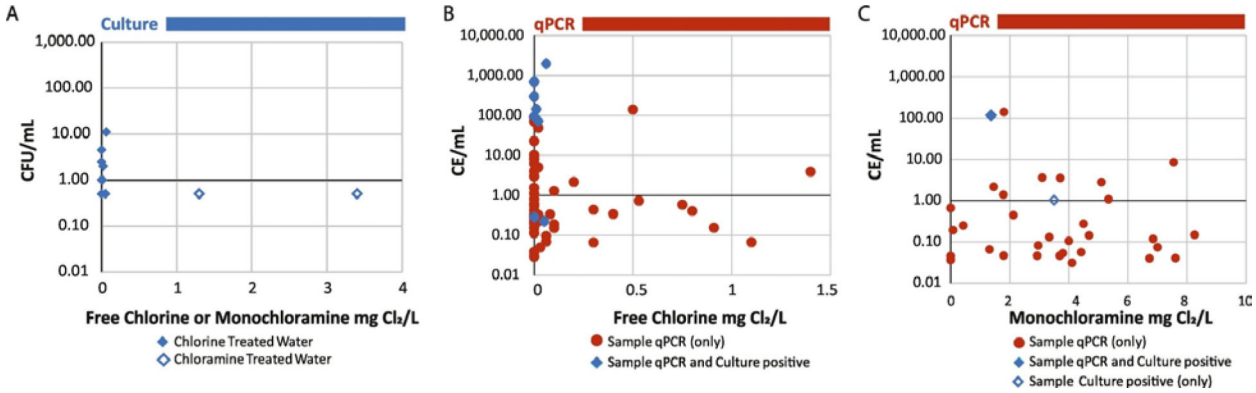


Fig. 2.

L. pneumophila concentration by disinfectant residual. A. L. *pneumophila* culture recovery, B. Chlorine treated water, *L. pneumophila* detection by qPCR C. Chloramine treated water, *L. pneumophila* positive qPCR samples.

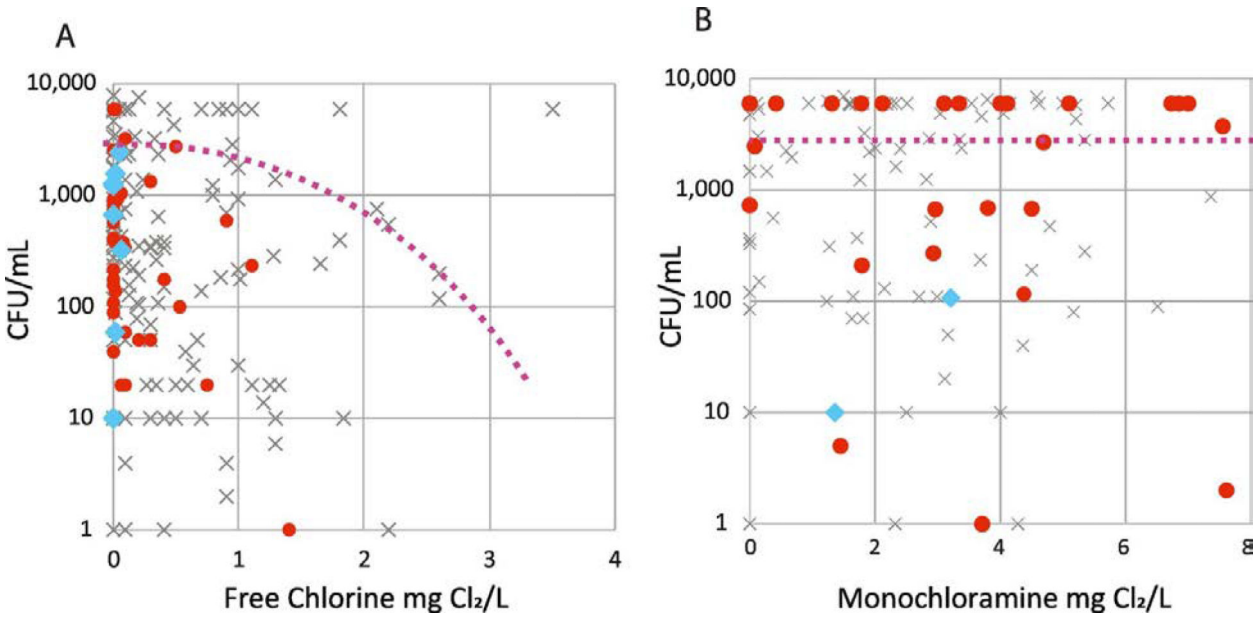


Fig. 3.

Heterotrophic plate count (CFU/mL) by disinfectant residual in (A) Chlorine treated water and (B) Chloramine treated water. *L. pneumophila* positive samples by culture (blue triangle) and qPCR (red dot), and *L. pneumophila* negative samples (grey x). The linear regression line is the dotted pink.

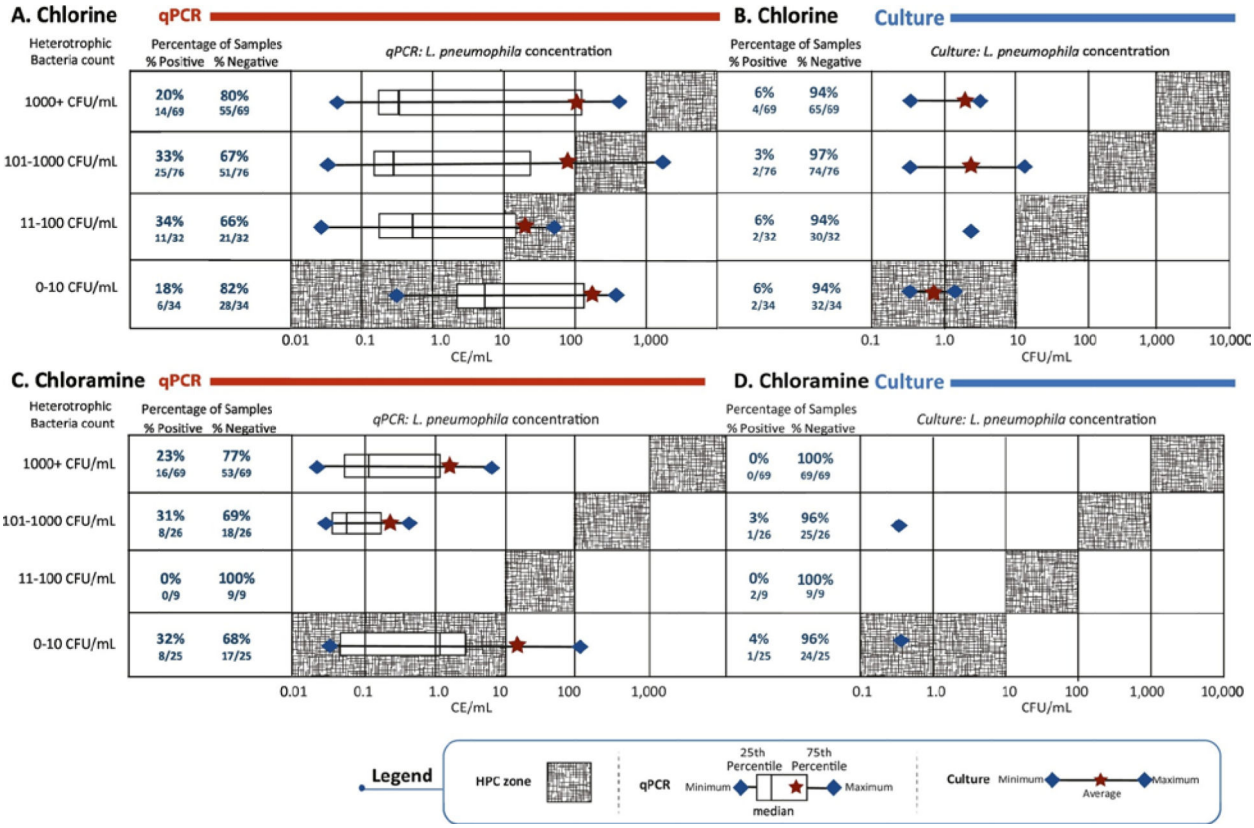


Fig. 4.

Legionella pneumophila qPCR and culture detection frequency and concentration relationship with heterotrophic bacteria counts.

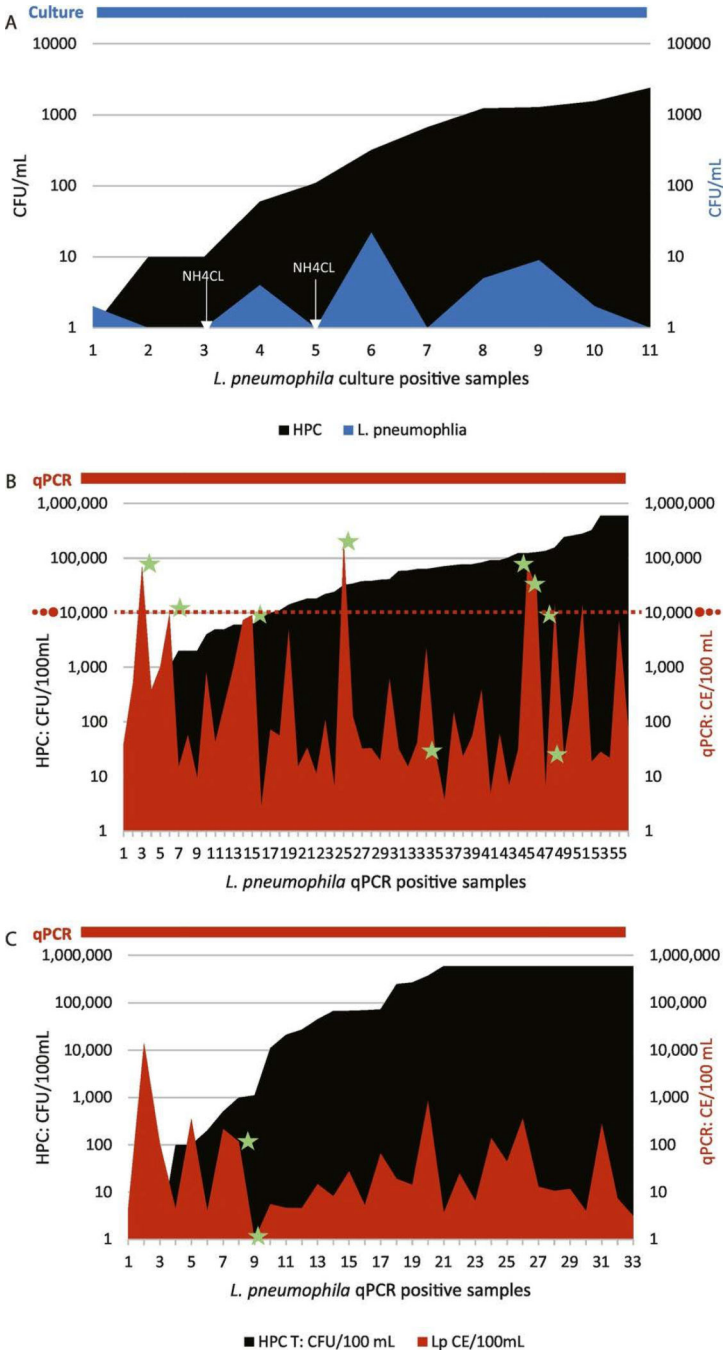


Fig. 5.

Legionella pneumophila positive samples: comparison of *L. pneumophila* culture and qPCR concentration to heterotrophic plate count (CFU/100 mL) values for each of the qPCR positive findings. A. *L. pneumophila* culture positive samples in either chlorine or chloramine treated water (as indicated). B. *L. pneumophila* qPCR positive samples in chlorine treated water C. *L. pneumophila* qPCR positive samples in chloramine treated

water. The green star represents samples that were also culture positive. The dotted line (Panel B) represents the threshold of higher culture likelihood.