Published in final edited form as:
Environ Sci Pollut Res Int. 2017 January ; 24(3): 2326–2336. doi:10.1007/s11356-016-7921-5.

Annual variations and effects of temperature on *Legionella* spp. and other potential opportunistic pathogens in a bathroom

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Abstract

Opportunistic pathogens (OPs) in drinking water, like *Legionella* spp., mycobacteria, *Pseudomonas aeruginosa* and free-living amobae (FLA) are a risk to human health, due to their post-treatment growth in water systems. To assess and manage these risks, it is necessary to understand their variations and environmental conditions for the water routinely used. We sampled premise tap (N_{cold}=26, N_{hot}=26) and shower (N_{shower}=26) waters in a bathroom and compared water temperatures to levels of OPs via qPCR, and identified *Legionella* spp. by 16S rRNA gene sequencing. The overall occurrence and cell-equivalent quantities (CE L^{-1}) of *Mycobacterium* spp. were highest (100%, 1.4×10^5), followed by *Vermamoeba vermiformis* (91%, 493), *Legionella* spp. (59%, 146), *P. aeruginosa* (14%, 10), and *Acanthamoeba* spp. (5%, 6). There were significant variations of OP's occurrence and quantities, and water temperatures were associated with their variations, especially for *Mycobacterium* spp., *Legionella* spp. and *V. vermiformis*. The peaks observed for *Legionella*, mainly consisted of *L. pneumophila* sg1 or *L. anisa*, occurred in the temperature ranged from 19 to 49°C, while *Mycobacterium* spp. and *V. vermiformis* not only co-occurred with *Legionella* spp. but also trended to increase with increasing temperatures. There were higher densities of *Mycobacterium* in 1st than 2nd draw water samples, indicating their release from faucet/shower head biofilm. *Legionella* spp. were mostly at detectable levels and mainly consisted of *L. pneumophila, L. anisa, L. donaldsonii, L. tunisiensis* and an unknown drinking water isolate based on sequence analysis. Results from this study suggested potential health risks caused by opportunistic pathogens when exposed to warm shower water with low chlorine residue and the use of *Mycobacterium* spp. as an indicator of premise pipe biofilm and the control management of those potential pathogens.

Keywords

Legionella; opportunistic pathogen; tapwater; shower water; qPCR

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Conflict of interest
No conflict of interest exists.

Introduction

Exposure to opportunistic pathogens (OPs) within aerosols generated from use of taps and showers within healthcare facilities is an increasingly recognized drinking water risk (Beer et al. 2015). *Legionella pneumophila*, non-tuberculous mycobacteria (NTM), *Pseudomonas aeruginosa* and *Acanthamoeba* spp. may cause opportunistic infections, such as legionellosis by *L. pneumophila*, pulmonary infections by various *Mycobacterium* spp., skin infection from *Pseudomonas aeruginosa* and *Acanthamoeba* keratitis in susceptible humans (Ashbolt 2015). *L. pneumophila* is the leading water-associated pathogen causing severe pneumonia and death in immuno-compromised individuals (Neil &Berkelman 2008). Pulmonary disease and other health risks are associated with inhalation of both viable and non-viable bacteria or their components (Falkinham III 2003, Thorn 2001). There are increasing numbers of humans who are at risk from developing life-threatening opportunistic infections, such as women elders, AIDS patients, individuals undergoing therapy for solid organ transplantation, cancer chemotherapy etc. where immune-suppressants may be administered. Hence, to better protect such immune-compromised people there is a need to identify anthropogenic reservoirs of OPs (Exner et al. 2005, Falkinham III 2003). Considering OPs are often amoeba-resisting microorganisms (ARMs) and their growth in amoebae may be a critical feature of OP life cycles, the presence of opportunistic pathogenic free-living amoebae (FLA), and bacteria in building plumbing systems is an important but poorly studied public health issue. Particularly lacking are studies over periods of months to years. OPs have been detected in tap and shower water, including NTM, *L. pneumophila* and *P. aeruginosa* generated within shower heads and hot-water faucets (Aumeran et al. 2007, Falkinham III 2003, Falkinham et al. 2015, Rohr et al. 1998, Vianelli et al. 2006, Zacheus &Martikainen 1994, Zichichi et al. 2000). Some OP exposures were associated with waterborne infections (Bartley et al. 2015, Bédard et al. 2016, Demirjian et al. 2015, Exner et al. 2005), Legionnaires' disease (Alary &Joly 1991) and other pulmonary and wound infections (Falkinham III et al. 2008, Nishiuchi et al. 2007). Several studies have traced both *L. pneumophilia* and *M. avium*, leading water-associated NTM infections in hospitalized patients, to microbes in their home showers, implying home exposures were the source of their infections (Falkinham III et al. 2008, Nishiuchi et al. 2007, Pedro-Botet et al. 2002). Those findings indicated that tap and shower water (specifically, pipe biofilms) are potential sources of daily exposure to OPs.

However, year around sampling of OPs to ascertain trends or possible hot-spots of contamination within household plumbing are rarely reported (Chen et al. 2005, Cooper et al. 2008), yet more information is available from healthcare settings (Chen et al. 2005, Stout 2007). In the present study, we examined the annual occurrences and quantities of OPs with emphasis on *Legionella* spp. and analyzed the relationships between the OPs and some environmental parameters for tap water routinely used and within a recently built tap/shower unit to simulate a house bathroom, in which water temperature appears to play a major selective role.

Materials and methods

Sample collection and processing

Water samples were collected from an engineered bathroom of a research building in Cincinnati, OH. The bathroom was constructed with a cold and hot faucet/tap and a shower water mixer connected to a showerhead in January 2011. Both taps and shower were fed with the same building tap water. Total 13 sample dates across 2012 (Mar 28-Apr. 24 and Oct. 24 – Dec. 27) and 2013 (Jan. 30 – Apr. 23, Jun. 5 – Jul. 25 and Dec. 18) were used. For each sample date (usually on Wednesday and occasionally on Tuesday or Thursday), two 1-L tap and shower water samples were taken into sterile flasks at early morning (around 7:00). For taps, the first sample (1st draw) was taken immediately after turning tap on, while the second sample (2nd draw) was done after 3 min of flow. For shower water, which flows through showerhead, both samples were collected after 3 min of flow. Thus, the samples included 1st draw cold/hot, 2nd draw cold/hot tap and shower water. A number of non-bath cold/hot tap water samples, which were taken after 3 min of flow from taps of a laboratory in the same building and within the circulation system as the bath unit, were also included as reference for phylogenetic analysis. All samples were measured for total chlorine, free chlorine and temperature. Each water sample was filtered onto a sterile 0.4-μm pore-size polycarbonate membrane, then placed into a bead-beating tube with lysing matrix A (MP Biomedicals, Santa Ana, CA) and stored at −80°C, until use.

DNA extraction

DNA was extracted as described previously for filter-concentrated drinking water samples (Lu et al. 2016). Briefly, the 1.5 mL micro-tubes containing a filter and 300 μL 1 × T&C (cell and tissue) solution was disrupted and lysed using a Mini-Beadbeater-16 (BioSpec Products, Inc., Bartlesville, OK) for 2 × 30 s. The mixture was then centrifuged at 10,000 *g* for 8 min, supernatant transferred to a sterile tube and DNA extracted and purified using the MasterPure Complete DNA Purification Kit™ (Epicentre Biotechnologies, Madison, WI) per manufacture's instruction. DNA concentrations were estimated with a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware). DNA extracts were stored at −80°C until qPCR and PCR were performed in the same period with the same prepared reagents, standards, supplies and equipment.

Quantitative PCR (qPCR) for screening, quantification and PCR cloning

The PCR and qPCR assays used and reactions performed for detection of microbial pathogens are described in detail previously (Lu et al. 2016). In brief, the qPCR assay was performed with a 7900 HT Fast Real-Time Sequence Detector (Applied Biosystems) with reaction mixtures (20 μL) contained 10 μL 2× qPCR Master Mix (Applied Biosystems), 0.2 μM primers, 0.08 μM probe (final concentration) and 2 μL of template DNA. Initial DNA treatment consisted of 50°C for 2 min with UNG (Uracil-N-Glycosylase) to prevent carryover contamination, then 95°C for 10 min for DNA denaturing. The following quantification cycling protocol was used: 40 cycles at 95°C for 15 s and at 60°C or at the Tm (°C) specified by the assay developers referred by Lu et al. (2016) for 30 s with an extension at 72°C for 30 s and a final hold at 72°C for 5 min, with qPCR reactions for each DNA sample undertaken in duplicate. The standard curves of targets were constructed with the

surrogates; *Bacteroides thetaiotaomicron*, *Campylobacter jejuni, Legionella pneumophila, Salmonella enterica, Pseudomonas aeruginosa*, *Mycobacterium avium, Vermamoeba vermiformis*, *Acanthamoeba polyphaga* trophozoites, *Cryptosporidium parvum* oocysts and *Giardia duodenalis* cysts. Target cells in the extracts are reported as numbers of spiked cell or cyst equivalents (CE). Each DNA extract was assayed for potential qPCR inhibitors with 10-fold dilution vs. neat DNA and with the addition of the TaqMan Exogenous Internal Positive Control Reagents (a VIC-labeled probe) manufactured by ABI™. Furthermore, each qPCR run included a DNA standard curve in the first row of the 96-well plate, and a no-template control for each row of each 96-well plate assayed. For qPCR positive *Legionella* samples, PCR products from five reactions were pooled and cloned into pCR4.1 TOPO (Invitrogen). Individual clones (total 5 plates or 12 colonies per sample selected) were sequenced by using BigDye Terminator chemistry and an Applied Biosystems PRISM 3730XL as described by Lu et al.(Lu et al. 2008). Almost all of the clone sequences were proved true positive. Raw sequences were processed using Sequencher 4.9 software (Gene Codes, Ann Arbor, MI) for editing, comparing and alignment, using protocols, homology searches, chimera check and phylogenetic analysis as previously described (Lu et al. 2008). Representative *Legionella* 16S rRNA gene sequences from clone libraries were deposited in GenBank with accession numbers (KX238910-KX238948).

Data analysis

For data management and calculations, Microsoft Excel 2003 and SAS Systems version 9.2 (SAS, Cary, NC) were used. For each pathogen in each sampling category, the occurrence was expressed as frequency of detection (FOD) and calculated as the ratio of the number positive to the total sample number, while the mean was calculated as a geometric mean using log (base 10) transformed data from different sample dates for each site. Correlations used to determine the relationship among OPs and between the densities of OPs and other parameters (temperatures and chlorine) were analyzed using generalized linear models (GLM).

Results

The qPCR targets: *Salmonella, Campylobacter jejuni*, *Escherichia coli* O157, *Giardia duodenalis* and *Cryptosporidium* spp., and human faecal indicator *Bacteroides* were not detected, while potential opportunistic pathogens, such as *P. aeruginosa*, *L. pneumophila*, *Mycobacterium* spp., and possible host amoeba *Acanthamoeba* spp. and *V. vermiformis* were consistently detected (Table S1). The overall occurrence and cell-equivalent quantities (CE L^{-1}) of *Mycobacterium* spp. were highest (100%, 1.4×10^5), followed by *Vermamoeba vermiformis* (91%, 493), *Legionella* spp. (59%, 146), *P. aeruginosa* (14%, 10), and *Acanthamoeba* spp. (5%, 6).

OPs in the bath tap water

Fluctuations in OPs detections in the tap water were mainly evident with *Legionella*, *Mycobacterium* and *V. vermiformis*, and the densities of *Mycobacterium* spp. and *V. vermiformis* showed less variation annually compared to *Legionella* in the samples from both the tap and shower water (Fig. 1, 2 and 3). There appeared to be a consistent peak in

Legionella spp. in the cold tap (19–36°C), hot tap (21–49°C) and shower water (32–44°C) in December 27. Their densities showed significant differences between sample types (cold vs. hot and 1st draw vs. 2nd draw). Comparing the densities (geometric mean for each category) between the cold tap and hot tap waters, total *Legionella* were higher in the cold tap water (11 CE L^{-1}) than in the hot tap water (2 CE L^{-1}) ($P_{T\text{-}test}$=0.002), while *Mycobacterium* were higher in the hot (9.7×10^4 CE L^{-1}) than in the cold tapwater (3.8×10^4 CE L^{-1}) ($P_{T\text{-}test}$=0.011). Also, *Legionella* were significantly more numerous in the 2nd draw cold tap water (42 CE L^{-1}) than in the 1st draw cold tap water (3 CE L^{-1}) samples ($P_{T\text{-}test}$=0.004) (Table 1). Conversely, *Mycobacterium* were significantly higher in the 1st draw cold tap water (6.4×10^4 CE L^{-1}) than in the 2nd draw cold tap water (2.3×10^4 CE L^{-1}) samples ($P_{T\text{-}test}$ =0.032) (Table 1). For the hot water samples *Legionella* were at low levels (4~5 CE L^{-1}) close to the detection limit, while *Mycobacterium* and *V. vermiformis* were significantly higher in the 1st draw (2.1×10^6 and 1.6×10^3 CE L^{-1}, respectively) than in 2nd draw (4.6×10^5 and 4.1×10^2 CE L^{-1}, respectively) samples (both $P_{T\text{-}test}$ <0.001) (Table 1). The significant correlations of quantities between 1st and 2nd draw samples (R^2 = 0.87 and 0.98, respectively) indicated that levels of both *Mycobacterium* and *V. vermiformis* were source-related. The highest frequency of detection (FOD) and mean maximum densities of *Legionella* occurred in the 2nd draw cold tap water, while those of *Mycobacterium* presented in the 1st draw hot tap water,

OPs in the bath shower water

The mean densities of *Legionella* in the shower water (204 CE L^{-1}) were higher than those in tap water, but those of *Mycobacterium* (8.6×10^4 CE L^{-1}) and *V. vermiformis* (242 CE L^{-1}) in the shower water were lower than those in the hot tap water samples (Table 1), indicating *Legionella* increased in the shower water and *Mycobacterium* and *V. vermiformis* were probably released/sourced to a greater extent from hot tap water than the shower water. Similarly to the 2nd draw cold tap water samples, the highest density of *Legionella* spp. (1.5×10^3 CE L^{-1}) (Fig. 3), which mainly consisted of *L. pneumophila* sg1 (Fig. 4), also occurred in Dec. 27 (31°C), indicating high *Legionella* densities were attributed to the mixing of the cold tap water with the hot tap water, with the former likely being the main source of these legionellae. Interestingly, variations in *Legionella* were highly correlated with *V. vermiformis* (R^2=0.79) and *Mycobacterium* (R^2=0.57). Specific qPCR for *L. pneumophila* (Lp) and serum group 1 (sg1) showed that mean density of Lp or sg1 was 95±17 CE L^{-1}. There were significant close correlation between the densities of *Legionella* and Lp or sg1 ($R^2_{\text{(Leg-Lp or sg1)}}$ =0.93~0.97). Sequence data showed that the presence of Lp or sg1 corresponded to the dominant qPCR signals, especially during the peak *Legionella* period (Fig. 4).

Phylogenetic tree of identified *Legionella* spp.

Two distinct phylogenetic clades of *Legionella* OTU were categorized from 16S rRNA gene sequences (Fig. 5). The first clade on the top of the tree was 99% identical to clinically-relevant strains *L. anisa*, *L. pneumophila*, *L. geestiana*, *L. massiliansis, L. drozanskii, L. tunisiensis or L. donaldsonii*. The sequences mainly derived from the samples of shower, 2nd draw cold tap and some non-bath hot/cold tap water. The other clade, being mostly from non-bath cold (major) and hot (a few) tap water, was 99% identical to those previously

described from drinking water distribution systems (Lu et al. 2016) or drinking water biofilms (Keinänen-Toivola et al. 2006) and low temperature drinking water (Wullings &van der Kooij 2006), respectively.

Impact of water temperature on OP presence

Overall, water temperatures were positively associated with the densities of *Legionella* (R^2=0.33, P<0.001), *V. vermiformis* (R^2=0.31, P<0.001) and *Mycobacterium* (R^2=0.38, P<0.001). The linear regression (including the samples of non-bath water) of *Legionella* and temperatures showed a very low slope and three points, which occurred in the same period, beyond upper 95% confident interval (corresponding to the peaks in the 2nd draw cold tap and shower water, respectively) (Fig. 6). Most of the *Legionella* positive samples (71%) were detected between 25–40°C (Fig. 6). However, there were different temperature trends in the density distributions of the three OP targets in 1st draw versus 2nd draw bath tap water (Fig. 7). Generally, both *Mycobacterium* and *V. vermiformis* densities increased with temperature from 19 to 50°C, but the former had a higher correlation than the latter (R^2=0.42 and 0.17 in the 1st draw tap water and 0.52 and 0.19 in the 2nd draw tap water, Fig. 7). However, in the 1st (21–47°C) and 2nd draw hot tap water (45–49°C) samples, the densities of *Legionella* were at low levels (<34 CE L^{-1}), while *V. vermiformis* and *Mycobacterium* maintained much higher densities than *Legionella*. *Mycobacterium* spp. were higher in the 1st than 2nd draw tap water and in the hot than cold tap water, respectively. Only in the 2nd draw cold tap (20–36°C) and shower water (32–42°C) were there high densities of *Legionella* (Table 1).

Discussion

Variations, factors and potential risk

Legionella spp. appear ubiquitous in drinking water and building plumbing systems, albeit at generally very low concentrations, where they survive and some strains at least replicate inside several species of amoeba that colonize biofilms (Brooks et al. 2004, Dupuy et al. 2016, Fields et al. 2002). However, unlike *Legionella* in cooling tower biofilms, where *Legionella* densities reached up to 10^5 cell L^{-1} at near optimal temperatures of ~35°C (Ikedo &Yabuuchi 1986, Yamamoto et al. 1992), they are normally in low densities to non-detectable in drinking water (< 10^3 CFU or cell L^{-1}). For example, the mean *Legionella* spp. were 187±458 in two chlorinated drinking water systems in southwest Virginia (Wang et al. 2012) and 85 ±154 CE/L in a recently investigated chlorinated metropolitan distribution water system (Lu et al. 2016), and 290±190 /L in a unchlorinated drinking water supply in the Netherlands (Wullings et al. 2011). According to previous documents, the normal quantity of *L. pneumophila* in water samples should not exceed 10^3 CFU L^{-1} (Guillemet et al. 2010, WHO 2007). Hence, our results with qPCR values < 10^3 CE L^{-1} for *Legionella* spp. seem within normal levels for sampled drinking water. A recent report for hospital tap systems, where legionnaires' disease outbreaks occurred and *L. pneumophila* was detected > 10^3 CE L^{-1} (Bédard et al. 2016), implicated that high densities of *L. pneumophila* (> 10^3 CE L^{-1}) could be associated with health risk. Furthermore, our building legionellae clustered within two phylogenetic groups previously reported in drinking water distribution systems/

chlorinated biofilm or an engineering biofilm (Keinänen-Toivola et al. 2006, Lu et al. 2016, Wullings &van der Kooij 2006).

What is of potential concern, however, was the peak period (e.g. December 27) when higher concentrations of *L. pneumophila* sg1 were detected. The peak of OPs might have caused by stagnant pipe water, because of less water use during Christmas holiday season, and appropriate water temperatures. *L. pneumophila* sg1 along with *Mycobacterium* and *V. vermiformis* in the cold water and shower mixer of the unit (2nd draw cold tap water and shower water) reached peak, which potentially increased risk to water users, especially during morning showering events. This *Legionella* risk appeared to be reduced, if water temperatures are low (<20°C) (Ashbolt 2015, Buse et al. 2012), and high enough (>42°C) (Ohno et al. 2003), although they would still survive under high temperatures up to 70°C (Allegra et al. 2008, Bédard, 2016 #135, Allegra, 2011 #138). The significant low densities of *Legionella* (4 CE L^{-1}) detected in the hot tap (46–49°C) in this study indicated *Legionella* spp. were suppressed. However, *Mycobacterium* and *V. vermiformis* demonstrated to be resistant to high temperature in this study (46–49°C) and previous studies (Schulze-Röbbecke &Buchholtz 1992). According to Ohno et al.(2003), *L. pneumophila* becomes non-cultivable at 42°C along with significantly reduced metabolic activity at >45°C, while for *Mycobacterium* spp., 10 isolates from water supply systems examined by Schulze-Röbbecke and Buchholtz (1992) showed cultivable at high temperatures ranged from 50–70°C. It has been hypothesized that the rise in pulmonary infections by NTM over recent decades is linked to increased use of showers (O'Brien et al. 2000), possibly because various *Mycobacterium* spp. can cause pulmonary disease both in healthy people and those predisposed to pulmonary infection, and they are highly resistant to various treatments. In many hospitals, *Mycobacterium* (mostly NTM) infections now outnumber *M. tuberculosis* detections reported by clinical laboratories (Heifets 2004).

As showed in the results, the generally detrimental impact of higher water temperatures in giving higher densities of major OPs were significant, but the effect seemed to differ by potential OPs, the sample type and the range of temperatures. *Mycobacterium* (presumably including various NTM) increased with temperatures in a linear trend (R^2: 0.41~0.52, $P<0.0001$) within the range (7–49°C) surveyed for bath and non-bath water. There might have been higher mycobacteria densities in locally sloughed biofilms (more from 1st draw tap water than from 2nd draw tap water) and these same trends might have been across different water sample types (non-bath cold/hot, and bath 1st and 2nd draw tap water). *Legionella* acted differently with more variations than other OPs. It seemed that most sampled water (non-bath cold: 7–28°C, 1st draw and 2nd draw hot tap water: 46–49°C) were not favorable to *Legionella* development, but the water temperatures (37–39°C) possibly favored both *Legionella* and *Mycobacterium* compared to the water temperatures (7–29°C). Our findings are in agreement with previous studies, for example, *Legionella* is able to survive or/and grow at temperature ranging from 20 to 50°C in distribution systems (Stout et al. 1985, Wadowsky et al. 1985). Wadowsky et al. (1985) also reported that naturally occurring *L. pneumophila* multiplied at a temperature between 25 and 37°C (Wadowsky et al. 1985). Rodgers (1994) noted that *L. pneumophila* accounted for a low proportion of biofilm microbiota at 20°C, while it was most abundant at 40°C in biofilms, where it accounted for up to 50% of the total bacterial biomass (Rogers et al. 1994). As for the major

source of actual OP colonization, *Legionella* and *Mycobacterium* could be different. Considering the higher densities of *Mycobacterium* in 1st than 2nd draw samples, they were probably from the biofilm at the tap faucet or shower head and hot water released more organisms as their quantities were 1st > 2nd draw tap and shower. These findings were in agreement with biofilms from various faucets and shower heads, which showed *Mycobacterium* spp. to be the major members (Feazel et al. 2009). By contrast, the major factor contributing to *Legionella* contamination could be from cold water or pipes and increased due to elevated temperatures in the re-circulating hot tap water and the warm basement (2nd draw cold tap water). Therefore, higher concentrations in the 2nd draw cold vs. hot tap water samples were possibly due to appropriate temperatures (19–36°C) for *Legionella* replication. These findings were different from those reported from hospital building tap water, where *Legionella* in tap and shower water generally were thought to derive from hot water or sediment of a hot water tank (Allegra et al. 2011, Bartley et al. 2015, Demirjian et al. 2015, Stout et al. 1985, Wadowsky et al. 1982).

Chlorine disinfectant was used in the drinking water distributed to the building and kept at the level regulated by US EPA (~ 1 mg/L, Table S2) at the non-bath and bathroom outlets in our study. However, all *Legionella* detections occurred at the free chlorine concentrations < 0.4 mg L^{-1}, mostly at <0.1 mg L^{-1} (Fig. S1). The peak of *Legionella* co-occurred with no detectable free/total chlorine, and there was a negative correlation between *Legionella* densities and free chlorine after excluding the peak values, indicating that maintaining a chlorine residual should also be a factor controlling *Legionella*. Considering chlorine concentrations measured were lower in 1st than 2nd draw water sampling sites (Table S2), chlorine residual alone could not be the causal factor in the *Legionella* densities being less in 1st than 2nd draw water. Regarding the potential effects of amoeba hosts on *Legionella* and *Mycobacterium*, it was frequently observed that *V. vermiformis* correlated with *Legionella* and *Mycobacterium* spp. presence, especially with the latter. Previously it has been demonstrated that the growth of pathogenic *Legionella* is positively supported by free-living amoebae, biofilm and algae (Greub &Raoult 2004, Tison et al. 1980, Wadowsky et al. 1988). In a distribution system, positive correlations between *V. vermiformis* and total bacteria, mycobacteria and *V. vermiformis*, and *Legionella* and *V. vermiformis* have been reported (Delafont et al. 2014, Wang et al. 2012). As of *P. aeruginosa*, low level of occurrence and densities compared to the major three OP groups were also observed previously in distribution system (Lu et al. 2016) and storage tank sediments (Lu et al. 2015). It was reported that *P. aeruginosa* were more prevalent in drains (51%) than manual faucet water (14%), in the 1st draw than the 2nd draw (Charron 2014), and in outlet point than plumbing system (Christina 2015).

Indicators of problematic biofilm growth: *Mycobacterium* spp.

A previous study on drinking water distribution system showed that total *Legionella* could be used to indicate saprozoic (environmental) opportunistic bacterial growth for piped microbial water quality (Lu et al. 2016). In the current study, mycobacteria occurred more frequently and at higher density in the 1st than in the 2nd draw samples, possibly because they were released from biofilms within faucets or shower heads. Mycobacteria, including NTM, readily form persistent biofilms due to their generally waxy cell walls and slow

growth rates, favorable attributes to resist shear forces generated during shower operation (Wadowsky et al. 1988, Wadowsky et al. 1982) and as evident in numerous tap and shower water and biofilm samples (Covert et al. 1999, du Moulin et al. 1988, Kusnetsov et al. 2003, O'Brien et al. 2000, Rusin et al. 1997, van der Wielen &van der Kooij 2013, Wang et al. 2012) using culture-based methods. For example, the relative abundance of mycobacteria accounted for 38%, 32% and 12% of total bacterial community in drinking water tubing biofilm, shower head biofilm and tap water, respectively (Feazel et al. 2009, Lu et al. 2014). However, sampling of biofilm in distribution systems or on premise is challenging in most cases. Yet, so-called first flush water samples (1st draw samples in this study) yield one estimate for biofilm-derived mycobacteria, which may provide an indication of biofilms of potential concern in premises. In addition to the common presence of various mycobacteria in drinking water biofilms, biofilms are subject to detachment due to abrasion effects from sediments (Brading et al. 2003), and the rate of erosion and sloughing of biofilm increases with increased biofilm thickness and fluid shear at the biofilm-bulk liquid interface (Characklis &Marshall 1990). Furthermore, *Mycobacterium* spp. presence also correlates with *V. vermiformis* (Liu et al. 2012), presumably present with general increases in bacterial biomass in biofilms. We also report that increased mycobacteria correlated with *Legionella* and *V. vermiformis*. These characteristics might make total *Mycobacterium* and *Legionella* ssp. suitable indicators for problematic biofilm and water quality, respectively, noting that both genera include non-pathogenic and environmental members.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements & Disclaimer

This research was supported by U-SSW2.2B for pathogen detection and Research Effort of SSWR 6.01C for Distribution System Indicators and Plumbing Water. The views expressed in this article are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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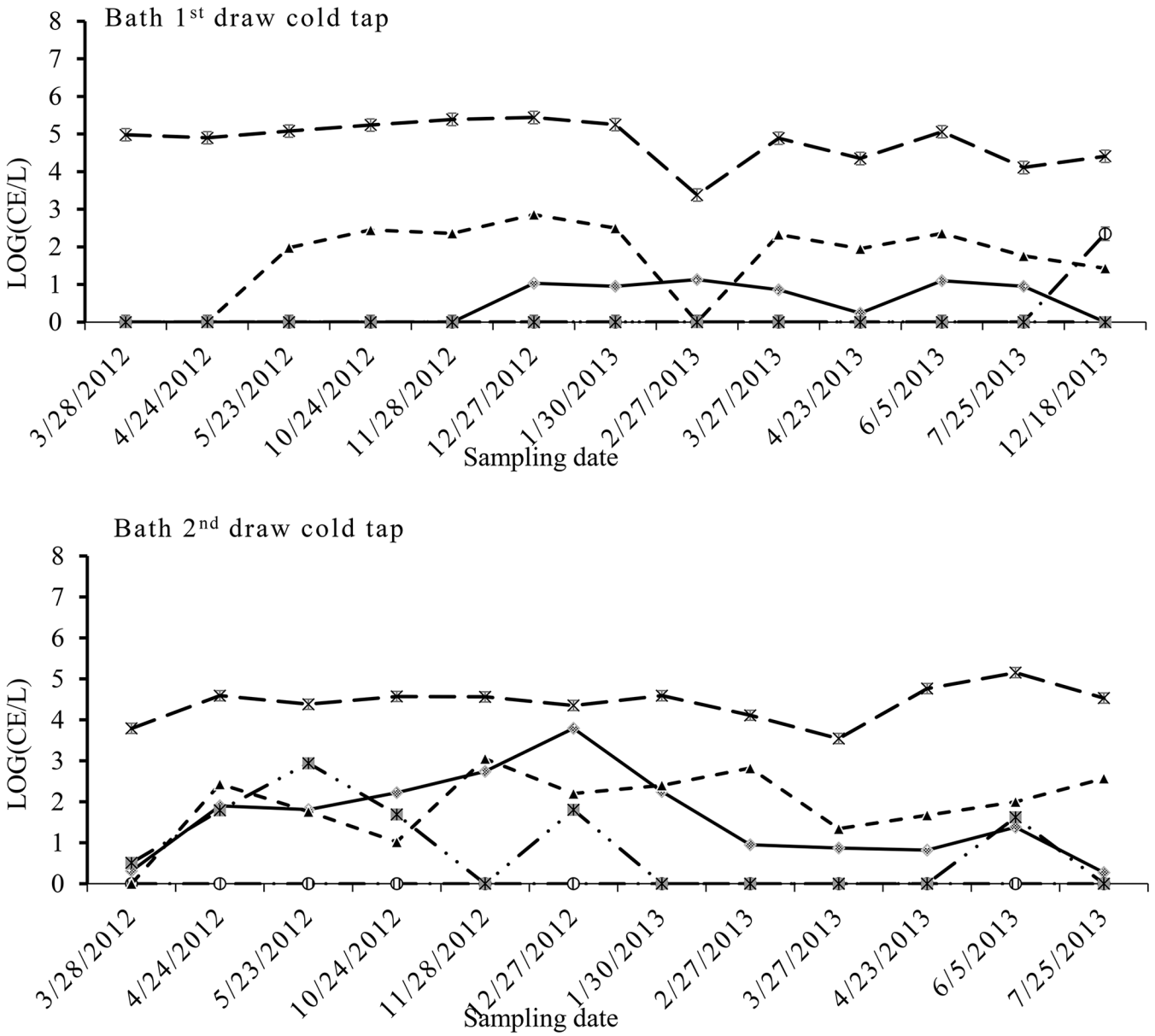


Fig. 1.

Quantity (CE L^{-1}) variations of *Legionella* spp. (—◆—), *Mycobacterium* spp. (—×—), *Acanthamoeba* (—○- -), *V. vermiformis* (- ▲ -) and *P. aeruginosa* (—✱ ·) along sampling dates in the bath 1st draw cold tap water and bath 2nd draw cold tap water

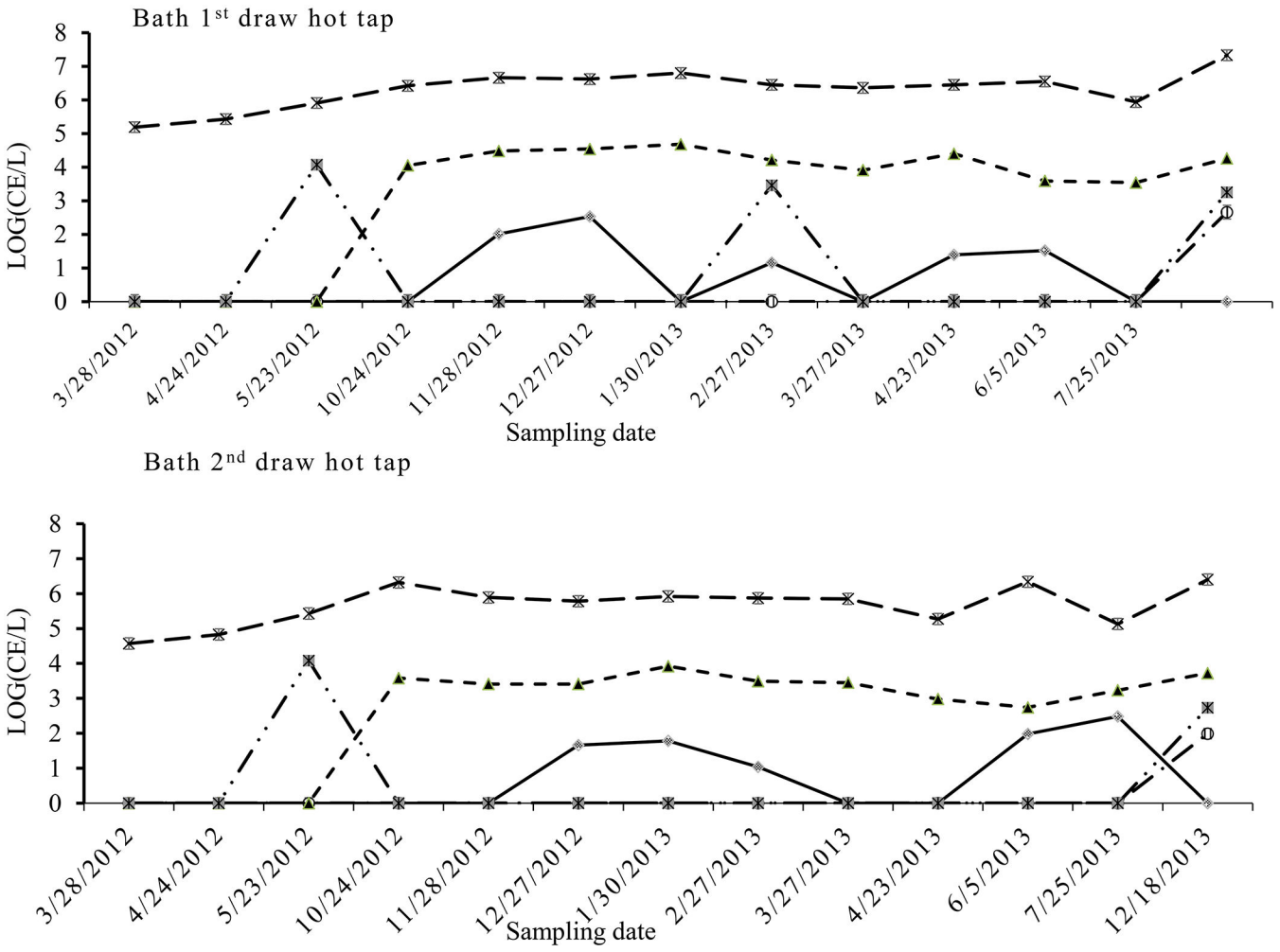


Fig. 2.

Quantity (CE L^{-1}) variations of *Legionella* spp. (—◈—), *Mycobacterium* spp. (—×—), *Acanthamoeba* (—◇- -), *V. vermiformis* (- ▲ -) and *P. aeruginosa* (—✱ ·) along sampling dates in the bath 1st draw hot tap water and bath 2nd draw hot tap water

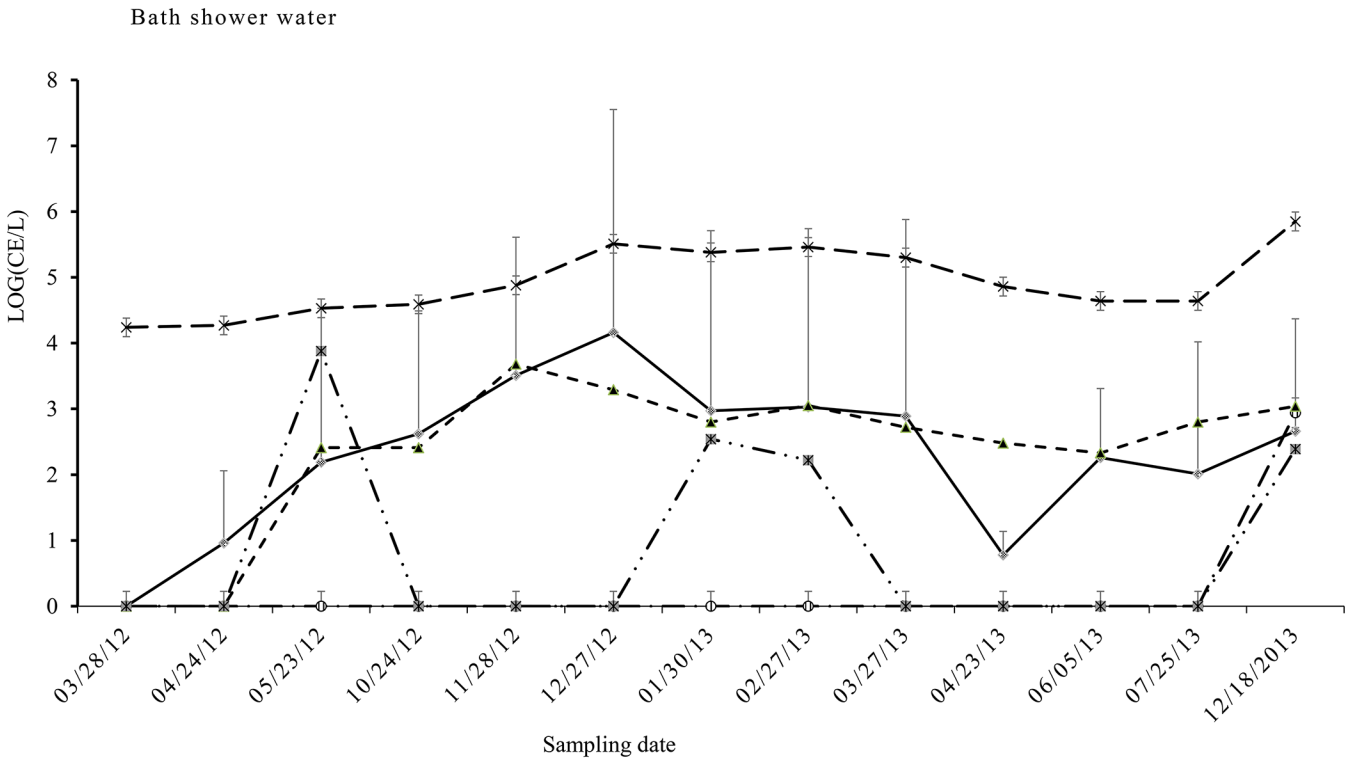


Fig. 3.

Quantity (CE L^{-1}) variations of *Legionella* spp. (—◆—), *Mycobacterium* spp. (—×—), *Acanthamoeba* (—○- -), *V. vermiformis* (- ▲ -) and *P. aeruginosa* (—✱ ·) along sampling dates in the bath shower water

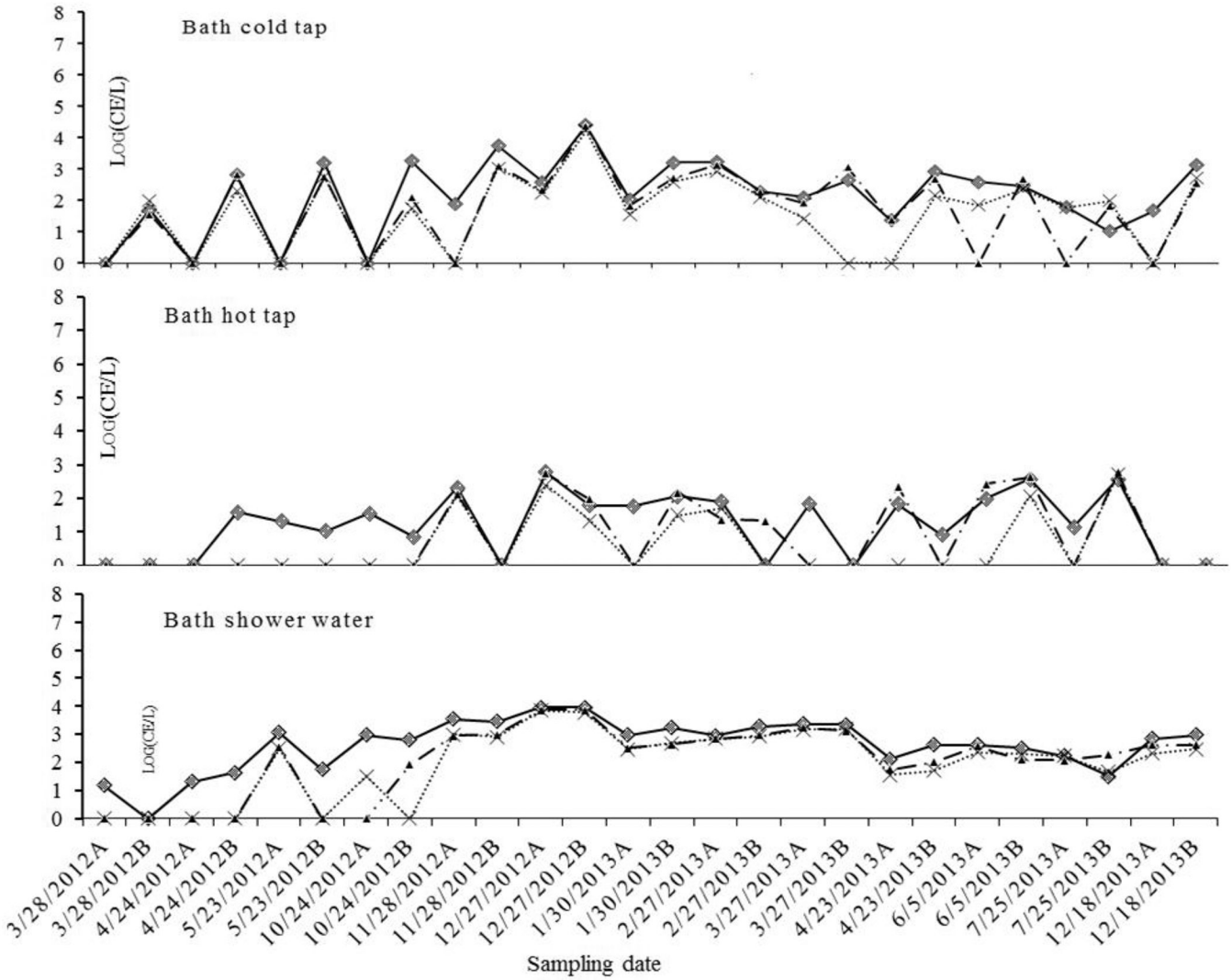


Fig. 4.

Quantity (CE L^{-1}) variations of *Legionella* spp. (—◆—), *rtxA* for *L. pneumophila* (– ▲ –) and P65/66 (—×—) for *L. pneumophila* sg1 along sampling dates in the bath cold tap water, hot tap water and shower water

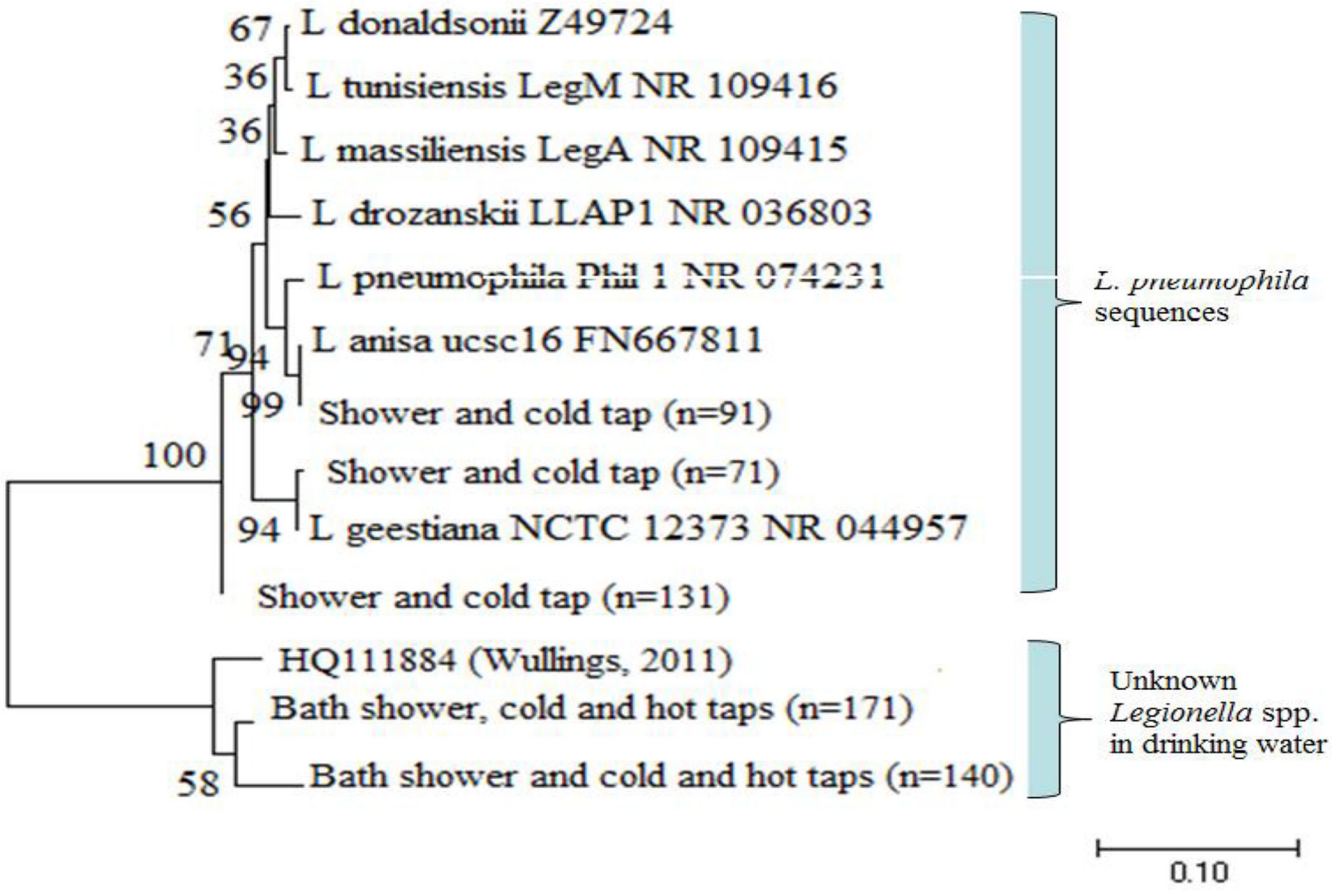
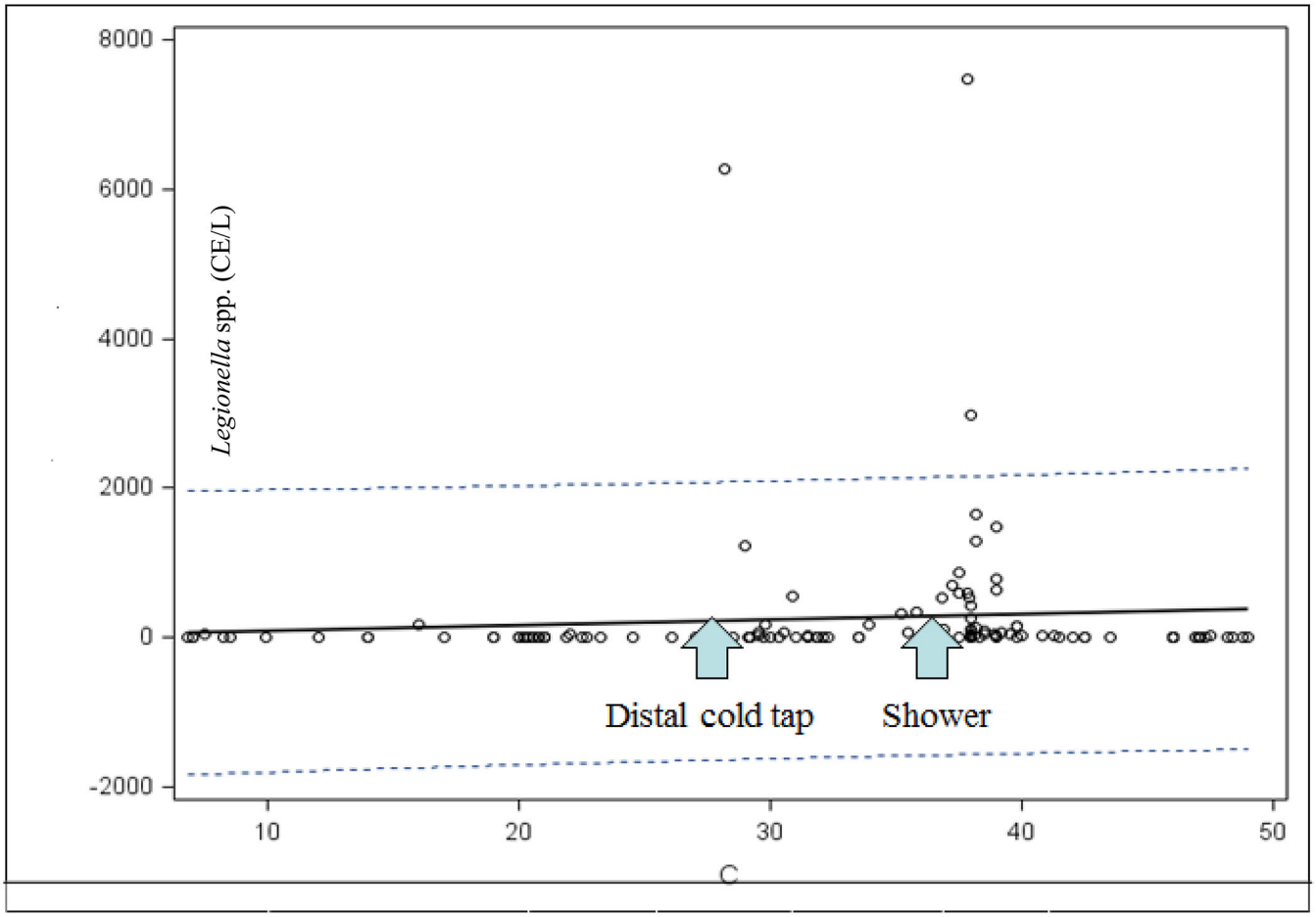


Fig. 5.

Unrooted neighbor-joining tree of 16S rRNA gene amplified for *Legionella* spp. sequences obtained from clone libraries of the building taps and bath water. Sequences were aligned, and a bootstrap consensus tree was created with MEGA6 (1% divergence). Bootstrap values greater than 50 (1000 replicate) are shown at the nodes



Temperature (° C)	< 20	20-24.9	25-29.9	30-34.9	35-39.9	≥ 40
Legionella (%)	3.7	14.5	16.4	14.5	40	9.1

Fig. 6.
Correlation of *Legionella* spp. with temperatures

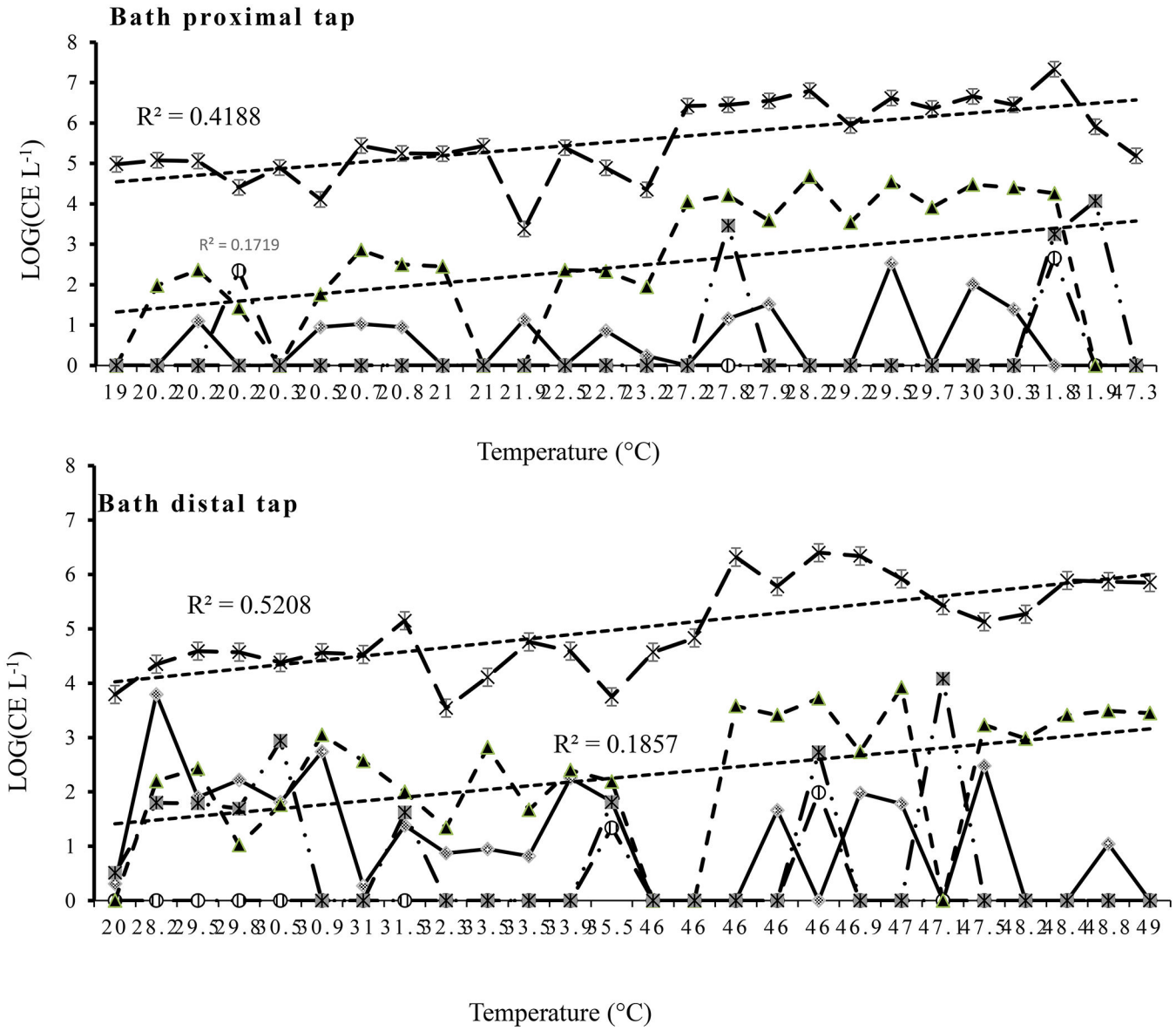


Fig. 7.

Correlations between temperatures and the quantity (CE L^{-1}) of *Legionella* spp. (—◆—), *Mycobacterium* spp. (—✕—), *Acanthamoeba* (—○—), *V. vermiformis* (—▲—) and *P. aeruginosa* (—✱—) in the bath water

Table 1.

Opportunistic microbial contamination based on frequency of detection (FOD: %), geometric mean concentration (MeC: cell equivalent or CE L^{-1}) and maximum concentration (MaC: CE L^{-1}) analyzed using qPCR from bathroom water (n=13) from 2012 (Mar 28-Apr. 24 and Oct. 24 – Dec. 27) to 2013 (Jan. 30 – Apr. 23, Jun. 5 – Jul. 25 and Dec. 18)

Site	1st draw cold tap	2nd draw cold tap	1st draw hot tap	2nddraw hot tap	shower
Legionella FOD (%)	37	95	46	38	77
Legionella MeC (StDev) (CE/L)	3(3)	42(10)	4(8)	4(9)	204 (14)
Legionella MaC (CE/L)	13	6262	34	30	1642
Acanthamoeba FOD (%)	4	4	4	4	8
Acanthamoeba MeC (StDev) (CE/L)	1(4)	1(2)	1(5)	1(3)	1(6)
Acanthamoeba MaC (CE/L)	225	21	46	10	150
Vermamoeba vermiformis FOD (%)	87	95	87	87	100
Vermamoeba vermiformis MeC (StDev) (CE/L)	49(10)	90(6)	1601 (72)	407 (32)	242 (13)
Vermamoeba vermiformis MaC (CE/L)	732	1135	4872	836	648
Mycobacterium FOD (%)	100	100	100	100	100
Mycobacterium MeC (StDev) (CE/L)	6.4×10^4(3)	2.3×10^4 (2)	2.1×10^6(3)	4.6×10^5 (3)	$8.6\times$lO^4 (3)
Mycobacterium MaC (CE/L)	2.8×10^5	1.4×10^5	2.2×10^6	2.6×10^5	7.2×10^4
Pseudomonas aeruginosa FOD (%)	0	37	12	8	12
Pseudomonas aeruginosa MeC (StDev) (CE/L)	0	8(10)	6(38)	3(20)	7(23)
Pseudomonas aeruginosa MaC (CE/L)	0	878	1176	878	1021
# of instances Rank 1 FOD		1			2
# of instances Rank 1 MeC	1	1			2
# of instances Rank 1 MaC	1	1	2	1	