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Legionella DNA Markers in Tap Water Coincident with a Spike in Legionnaires' Disease in Flint, MI

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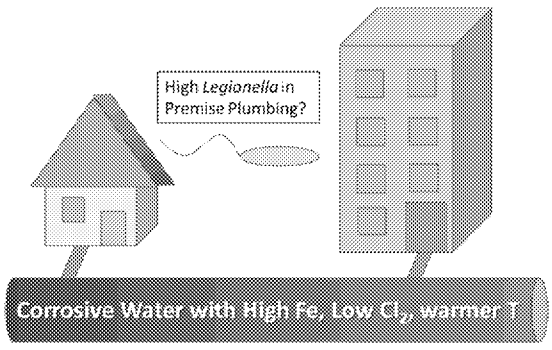
Supporting Information

ABSTRACT: Two clusters of Legionnaires' disease occurred in Flint, MI, subsequent to switching to a corrosive potable water source from April 2014 to October 2015. We hypothesized that the interrupted corrosion control and associated release of iron, nutrients, and depleted chlorine residual in the distribution system would lead to high levels of *Legionella*. A tap water survey conducted throughout Flint in August and October 2015 confirmed *Legionella pneumophila* in two hospitals (mean of 1890 ± 2220 gene copy numbers/mL, 48% positivity), but not small single-story buildings. The hospitals frequently had optimal *Legionella* growth temperatures and were located in high-water age zones of the distribution system (3 to >6 days). Relatively high concentrations of iron were present (mean of 51.0 ± 37.2 ppb), and Cl_2 residual was sporadic (mean of 0.700 ± 0.775 mg/L) throughout the Flint distribution system. This study addresses knowledge gaps linking legionellosis outbreaks to changes in municipal water quality and distribution system operation.



INTRODUCTION

In April 2014, the City of Flint, located in Genesee County, Michigan, began to use the local Flint River as their drinking water source (chlorinated, with no corrosion inhibitor), instead of the municipal water that they had purchased for decades from the City of Detroit (Lake Huron water, chlorinated and treated with orthophosphate for corrosion inhibition).[1] Failure to implement a federally mandated corrosion control program triggered an array of water quality issues, including rampant corrosion and lead contamination throughout the distribution system. Other consequences included increased frequency of main breaks,[2] rapid loss of disinfectant residual,[2] and high levels of total trihalomethanes.[3] Microbial aspects of water quality also deteriorated, with elevated fecal coliform bacteria and multiple boil water advisories.[2] After a public health emergency was declared, Flint returned to Detroit water in October 2015.

In January 2016, it was announced that cases of Legionnaires' disease, a deadly pneumonia caused by *Legionella* bacteria (usually *Legionella pneumophila*), had spiked in Genesee County during the period of Flint River usage.[4,5] Forty-five cases with five deaths occurred from June 2014 to March 2015[4] and 46 cases with seven deaths from May to October 2015.[5] A high percentage of cases occurred in people receiving Flint drinking water at home or that had visited a Flint hospital (65 of 91),[4,5] suggesting the outbreaks may have been tied to the municipal water system.

The water switch and subsequent legionellosis cases in Flint, MI, presented an opportunity to investigate linkages between municipal water quality and abundance of *Legionella* in premise plumbing. Here we report the findings of two field surveys of single-story homes and businesses and multistory hospitals in Flint, conducted during August and October 2015, while the drinking water was still sourced from the Flint River. Levels of DNA markers corresponding to *Legionella* spp. and *L. pneumophila* (23S rRNA and *mip* gene copies per milliliter, respectively) are compared to results of similar U.S. surveys performed in the absence of documented outbreaks[6,7] and considered alongside various physiochemical characteristics of the water to provide insight into contributing factors in the Flint outbreaks and inform strategies for minimizing the likelihood of waterborne disease.

MATERIALS AND METHODS

Study Site and Water Sampling. Two surveys of tap water in Flint, MI, and the vicinity were conducted in 2015. The first, occurring on August 18 and 19, focused on small buildings: 16 single-story homes and businesses within Flint and four businesses within nearby Flint Township (which was maintained on Detroit water). The second, occurring on October 15 and 16, focused on two health care centers in Flint (hospitals 1 and 2), immediately prior to the city switching back to Detroit water. These hospitals were selected because of their location in high-water age zones in the distribution system and because large institutional buildings are known to be particularly susceptible to *Legionella* colonization.[8]

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Cold water samples were collected from all buildings in the first survey, with hot water additionally sampled in three Flint homes. Hot and cold samples were collected in the second survey, from public restroom hand wash sinks, representing a cross section of various buildings and floors from both hospitals. A total of 60 samples were collected from 30 outlets in three buildings in hospital 1, while 40 samples were collected from 20 outlets in hospital 2.

First flush tap samples of approximately 1000 mL were collected for microbiological analysis in sterile polypropylene bottles pretreated with 24 mg of sodium thiosulfate and 292 mg of ethylenediaminetetraacetic acid (in solution, adjusted to pH 8.5) and kept on ice until processing. Subsequently, two 10 mL cold water samples were collected in polyethylene tubes for measurements of chlorine and analyzed immediately, and metals were analyzed later following storage at room temperature. All microbiological samples were either shipped overnight or transported by ground the same day to Virginia Tech. These procedures were modified slightly for hospital sampling to include additional sample collection. Two samples were collected at each tap, starting with a first flush cold water sample. After this, the hot water lines were flushed for 30 s, followed by collection of hot water.

Water Quality Analysis. Outlet water temperature and flow rate were measured at the time of sample collection. Total chlorine (detection limit of 0.1 mg/L) was measured in field using a DR2700 portable spectrophotometer (Hach, Loveland, CO). Metals, including iron (10 ppb detection limit), were measured by inductively coupled plasma mass spectrometry according to Standard Method 3125B.[9] Samples were not filtered prior to analysis, yielding a measurement of total metals (dissolved + particulate).

Sample Processing and Quantitative Polymerase Chain Reaction (q-PCR). Within ~24 h of sample collection, 1000 mL water samples were concentrated onto sterile 0.22 μm pore size mixed cellulose ester membranes (Millipore, Billerica, MA). Sample bottles were weighed before and after filtration to determine the volume filtered. Filters were torn to pieces, transferred to DNA extraction tubes, and stored at −20 °C until extraction using FastDNA SPIN Kits (MP Biomedicals, Solon, OH). q-PCR was used to quantify *Legionella* spp. 23S rRNA and *L. pneumophila mip* genes following previously established methods[10,11] on a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Further details of q-PCR methods are available in the Supporting Information.

Data Analysis. Two-tailed Mann−Whitney tests were used to test for significant differences in data distributions ($p < 0.05$). Spearman's rank correlation coefficients were calculated to examine correlations between *Legionella* spp./*L. pneumophila* and physiochemical water parameters.

RESULTS AND DISCUSSION

High Levels of *Legionella* and *L. pneumophila* in Flint, MI, Tap Water. *Legionella* spp. and *L. pneumophila* gene copies were detected in tap water from buildings at various locations throughout the Flint drinking water distribution system during the period of corrosive water usage (Table 1). No *Legionella* were detected in the control group samples of small Flint Township buildings ($n = 4$), which maintained usage of Detroit water. Gene copies of *Legionella* spp. and *L. pneumophila* in the Flint hospitals (Table 1) were high relative to levels previously reported in U.S. tap water.[6,7] *Legionella* ($p = 2.37 \times 10^{-9}$) and *L. pneumophila* ($p = 7.85 \times 10^{-10}$) levels were significantly

Table 1. Occurrence of *Legionella* spp. and *L. pneumophila* Gene Copies in Various Flint, MI, Locations[a] and Comparison to Published Surveys of Tap Water

	hospital 1 hot water ($n = 29$)	hospital 1 cold water ($n = 30$)	hospital 2 hot water ($n = 20$)	hospital 2 cold water ($n = 19$)	Flint small buildings ($n = 19$)	Flint Township small buildings ($n = 4$)	U.S. cold water survey[b] ($n = 269$)	Virginia[c] ($n = 90$)	Florida[c] ($n = 54$)
Legionella concentration range (GC/mL)	42.8−66000	32.2−119000	119−5170	28.5−61300	11.9−2460	ND	NR	10.4−2300	<5−760 ± 286
Legionella mean concentration (gene copies/mL ± SD)	15500 ± 16900	17600 ± 27000	775 ± 1560	6590 ± 16700	699 ± 1020	ND	NR	187 ± 458	101 ± 184
L. pneumophila concentration range (gene copies/mL)[d]	195−5410	201−13200	103−131	56.3−3300	ND	ND	0.04−365[e]	<5−13.7 (max)	20.3−219 (max)
L. pneumophila mean concentration (gene copies/mL ± SD)[d]	1790 ± 1300	2480 ± 3180	112 ± 13.0	1170 ± 1500	ND	ND	2 ± NR	9.80 ± 4.40	90.4 ± 111.9
Legionella positivity (%)	100	90	45	63	53	0	NR	30	68
L. pneumophila positivity (%)	93	60	15	16	0	0	28.6	4.4	5.0
mean chlorine concentration (mg/L ± SD)	NR	0.474 ± 0.613	NR	0.889 ± 0.999	0.931 ± 0.585	0.263 ± 0.046	NR	1.99 ± 0.64	1.81 ± 1.12
mean temperature (°C ± SD)	35.0 ± 6.43	23.8 ± 4.62	41.2 ± 4.41	24.0 ± 6.49	23.7 ± 2.24	38.9 ± 6.90	NR	26.0 ± 9.9	33.2 ± 9.0
municipal water age range (h)	>144	>144	73−144	73−144	<24 to 144	NR	NR	72−408	72−408
mean iron concentration (ppb)	NR	37.6 ± 32.3	NR	52.9 ± 25.3	72.3 ± 46.4	0 ± 0	NR	NR	NR

[a]Mean *Legionella* and *L. pneumophila* values for Flint hospital and small building samples represent only quantifiable data. NR, not reported, no data available, or not assayed. ND, assayed, but not detected. [b]From ref 7. [c]From ref 6, which used q-PCR methodology in the same laboratory identical to that used in the study presented here. [d]*L. pneumophila* carries one copy of the *mip* gene;[32] thus, gene copy numbers and GU are interchangeable. [e]Values represent data for *L. pneumophila* serogroup 1 in GU/mL.

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higher at hospital 1, where all hot water outlets sampled were positive for *Legionella* spp. and >10^4 gene copies/mL of *L. pneumophila* were observed at some sites. The lower detection rates and concentrations observed at hospital 2 might be attributed to *Legionella* remediation efforts implemented several months prior to the field survey, including enhanced monitoring, temperature management, and installation of a hot water monochloramine treatment system.[12] Hospital 1 reported no history of on-site treatment until 2016.

Trends in *Legionella* Occurrence by Buildings. Building type was of interest, as larger and older buildings, such as the two hospitals surveyed, tend to be susceptible to colonization by *Legionella* and other opportunistic pathogens[13] because of factors such as infrequent flow, longer residence time, higher temperatures, difficulty maintaining sufficiently high temperature/disinfectant to kill pathogens, and increased surface area to bulk water ratio for biofilm growth.[14] *Legionella* spp. were detected in all building types, with rates of detection higher in hospitals, while *L. pneumophila* were observed only in hospitals (Table 1). Concentrations of *Legionella* spp. were higher in hot and cold water from hospitals than from Flint small buildings, significantly so for hospital 1 cold water ($p = 2.68 \times 10^{-5}$) and hot water ($p = 3.33 \times 10^{-8}$), but not for hospital 2 (where remediation was implemented) in either cold water ($p = 0.080$) or hot water ($p = 0.833$). The two hospitals were located in high-water age zones (Table 1), indicating longer periods of time before treated water reached these buildings. When compounded by the greater water residence time within hospital plumbing, relative to the time in smaller buildings, the resultant net elevated water age could have contributed to the higher levels of *Legionella*.

Examining trends for the two hospitals on an outlet-by-outlet basis (Figure 1) provided insight into patterns of occurrence.

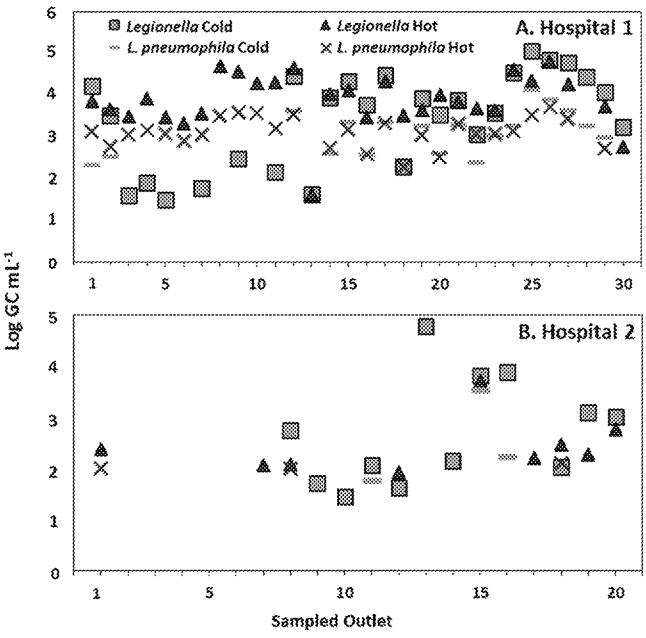

Figure 1. Concentrations of *Legionella* spp. and *L. pneumophila* gene copies as determined by q-PCR in water samples collected from public tap water outlets in two Flint, MI, hospitals (A, hospital 1; B, hospital 2). One hot and one cold water sample were collected from each outlet. Outlets with no data points correspond to samples with concentrations below the limit of quantification.

When quantifiable, *L. pneumophila* consistently accounted for a similar percentage of the total *Legionella* genus in both cold and hot water at hospital 1 (cold water, 12.2 ± 6.7% SD; hot water, 13.6 ± 7.9% SD). The cases in which *L. pneumophila* were not detected tended to have low *Legionella* spp. levels; all nine samples with ≤312 gene copies/mL had no measurable *L. pneumophila*. *L. pneumophila*:*Legionella* spp. ratios were more variable at hospital 2, likely because of the effects of on-site remediation and lower levels of *Legionella* spp., which could have obscured trends.

Role of Physiochemical Water Quality Characteristics. We hypothesized that rampant corrosion in the Flint water distribution system would stimulate an increased abundance of *Legionella* by three general mechanisms: (1) loss of disinfectant residual due the high chlorine demand of iron corrosion, (2) elevated levels of iron and other microbial nutrients, and (3) increased temperature due to the warmer Flint River source water.[15] Additionally, we predicted that conditions in large buildings would be conducive to *Legionella* growth and survival, as is known to be problematic in hot water lines,[16,17] especially when outlet line temperatures are <60 °C.[18,19]

Chlorine dosing by the municipality was increased after the switch to Flint River water (~2−2.5 mg/L exiting the treatment plant vs ~0.9 mg/L prior to April 2014)[15] in an attempt to manage water quality problems but was still frequently absent (>50% of samples were below detection) or present at levels much lower at the tap than those reported in prior U.S. surveys (Table 1). Low levels of chlorine are consistent with expectations of corrosion-induced decay. No negative associations between *Legionella* spp./*L. pneumophila* and chlorine were observed (Figure S1), but this analysis was constrained by high rates of nondetection and low levels of chlorine. Positive correlation was noted between *Legionella* spp. and chlorine in hospital 1 hot water ($r_s = 0.518$). Inconsistent or low chlorine levels may be beneficial to *Legionella*, potentially reducing the level of competition with microorganisms that are less tolerant of chlorine.[17,20] Nonetheless, it is well-known that disinfectant residual maintenance is critical for reducing risk of Legionnaires' disease.[21] Hospital 2, which had implemented on-site monochloramination, had detection rates and levels of both *Legionella* spp. and *L. pneumophila* lower than those of hospital 1.

In addition to more consistent disinfection, outlet temperatures appropriate for *Legionella* control were more common in hospital 2 and likely acted to reduce occurrence. Several sites at hospital 2 were recorded near the upper limit for *Legionella* growth of 48 °C,[22] with a maximum of 51 °C. Here, where temperatures reached a growth-inhibitory range, negative correlation was observed between temperature and *Legionella* spp. in hot water samples ($r_s = -0.725$). At hospital 1, a significant positive relationship was observed between water temperature and *Legionella* spp. for both hot and cold water ($r_s = 0.596$). This was not surprising, given that the majority (all but two) of the samples ranged from 20 to 48 °C, which is in a range permissive of *Legionella* growth.[22] It is noteworthy that distribution system water temperature increased during Flint River water use compared to prior experience with Detroit water (treatment plant effluent mean of 20.9 °C in August 2013 vs 23.3−24.0 °C the two following years),[23−25] which could have pushed temperatures toward the optimal range for *Legionella* growth while accelerating chlorine decay.

Iron, released because of corrosion, was consistently detected in all Flint buildings but was always below detection in Flint

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Township where *Legionella* were never detected (Table 1). Given the high temperatures and low chlorine levels in Flint Township samples, iron originating from corroding pipe may have been a key factor in facilitating high *Legionella* levels in Flint. Iron has been found to be a key nutrient for *Legionella*[26] and to be positively associated with their presence and concentrations in hot water systems.[27] However, we were not able to identify significant positive correlation between *Legionella* and iron concentration, possibly because of complex speciation of iron and poor bioavailability.[28]

Implications. Legionellosis accounts for more drinking water-related outbreaks in the United States than all other contaminants combined,[29] has a case-fatality rate of >9%,[30] and leads to annual health care-associated costs greater than $430 million USD.[31] This study fills an important knowledge gap linking the switch to Flint River water to the 2014 and 2015 clusters of Legionnaires' disease in Genesee County (the first reported major outbreaks of legionellosis associated with distribution of corrosive municipal water) and demonstrates the important role of water utilities in controlling proliferation of pathogens in premise plumbing. Our field results are consistent with a recent lab-scale study indicating synergistic effects of distribution system water chemistry and premise plumbing conditions in stimulating *Legionella* amplification.[32] Together, these results demonstrate a need for a multi-stakeholder and multibarrier model for managing *Legionella* risk that includes water utilities and other entities involved in the design, regulation, and management of premise plumbing systems[33] to avoid future outbreaks.[34] Examples include water utility guidelines for minimizing risk of community level outbreaks, formal communication channels to building operators regarding changes in water quality, and coordination of *Legionella* monitoring.

A major knowledge gap exists concerning the relationship between *Legionella* occurrence and risk of outbreak, especially when molecular methods are applied.[35] Quantitative microbial risk assessment can estimate infectious dose and safe levels in tap water,[36] with field studies such as this helping to validate models. On the basis of reported correlations between molecular and culture-based measurements from European monitoring guidelines, a recent study suggested a remediation action level of 4 genome units/mL for *L. pneumophila* in hot and cold water systems.[37] We detected mean concentrations 500 times greater than this in hospital 1.[38] While gene copy numbers are not a direct measurement of viable organisms, monitoring of molecular targets provides precise, quantitative information over several orders of magnitude about organisms viable at some point upstream in the distribution system, while also capturing viable but nonculturable organisms. This study helps address this critical knowledge gap by providing information about both *Legionella* and *L. pneumophila* gene copy numbers associated with an outbreak event.

Our field results support the overarching hypothesis that interrupted distribution system corrosion control can lead to high *Legionella* numbers in premise plumbing, though further research is necessary to confirm the specific mechanisms involved. Comparison of DNA sequences of clinical and water-derived *Legionella* isolates would be necessary to definitively link the outbreak to the water source. Collection of environmental isolates and other *Legionella* monitoring by public health agencies was extremely limited during the time of Flint River usage, emphasizing the value of the molecular field survey presented here. The very high levels of *Legionella* DNA markers relative to analogous environmental studies serve to highlight the value of routine monitoring for these pathogens in high-risk environments, particularly during events with potentially harmful or unknown impacts on the microbial quality of drinking water.[34] Unfortunately, federal guidelines contain no specific monitoring requirements for *Legionella* to be performed by water utilities,[39] providing little incentive for data collection, and failing to provide guidance to cities in crisis, such as Flint, wishing to proactively prevent outbreaks. Such monitoring might be conducted routinely by utilities in large publicly owned buildings representing a high risk for *Legionella* colonization. Although Flint is an extreme case, establishing a relationship between corrosive water and *Legionella* is highly relevant to other water distribution systems. Aging drinking water infrastructure is a serious problem across the United States; lessons learned from Flint can help other cities better prepare to avert public health crises in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.6b00192.

A description of q-PCR validation methods, Figure S1, and a discussion of relationships between *Legionella* and metals other than iron (PDF)

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Notes

The authors declare no competing financial interest.

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