

Characterizing Opportunistic Pathogens in Drinking Water Supplied by Private Wells

Kris M. Mapili

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Marc A. Edwards, Chair
Amy Pruden
Kelsey J. Pieper
Leigh-Anne Krometis

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ABSTRACT

Private wells are understudied potential sources of opportunistic pathogen (OP) infections. OPs, including *Legionella* and *Mycobacterium*, are of particular concern for immunocompromised individuals and are known to proliferate in drinking water systems. Much of our knowledge surrounding OP occurrence and growth in drinking water relates to municipal drinking water systems, which primarily use surface water sources and are always treated with primary and secondary disinfection in United States. However, OP occurrence and growth in private wells is not well understood and it is unclear how the knowledge developed in municipal systems will translate to private well systems with rare and infrequent exposure to chemical disinfectants. In addition, because private wells are more susceptible to microbial contamination than municipal systems, the impact of flooding on OP occurrence is of particular concern.

Two private well field surveys were conducted to document the incidence of OPs in private well systems. One survey conducted in North Carolina private wells with no history of recent flooding was focused on molecular and culture-based detection of *Legionella* spp. and *Legionella pneumophila*. The other survey was a broader molecular (i.e., DNA-based) characterization of the incidence of *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *Mycobacterium avium* (the most commonly nontuberculous mycobacteria associated with disease), and *Naegleria fowleri* in private wells with recent history of flooding (i.e., Hurricanes Harvey and Irma in 2017, or the Great Louisiana

Floods in 2016, extending to Texas, Florida, and Louisiana). All samples in both studies were analyzed for total bacterial 16S rRNA genes, indicator bacteria (e.g., total coliform and *Escherichia coli*) and inorganic constituents. Information about well system characteristics were obtained through questionnaires sent to participating residents.

Widespread detection of OP DNA markers were noted in the flooded well survey. *Legionella* spp. (detectable in 50-100% of well waters, depending on the flood event) and *Mycobacterium* spp. (detectable in 13.2-45.0% of well waters) were the most commonly detected among the OPs targets. At the genus level, *L. pneumophila* (7.9-65.5%) and *M. avium* (7.9-32.5%) were less commonly detected, but still highly variable. It is not possible to judge whether these OP levels were elevated as a result of the flooding because the sampling was carried out as an emergency response and background levels were not previously tested. Also of interest was whether well characteristics could predict OP levels, including well depth, well type, or treatment. However, none of these emerged as significant predictors of OP detection frequency or levels. Similarly, these OP DNA markers were not elevated in homes reporting submerged wellheads or system damage, suggesting that detection of these OPs is more dependent on the groundwater that supplies these private wells than influx of contaminated surface water. The incidence of DNA markers pertaining to *N. fowleri*, the “brain eating amoeba” that causes rare incidences of primary amebic meningoencephalitis (PAM), tended to be lower (5.0-12.7%) than that of other OPs targeted, but was more frequently detected in wells reporting submerged well heads, suggesting its occurrence was related to contamination from flood water.

A positive association between total bacteria and occurrence of both *Legionella* spp. and *Mycobacterium* spp., was observed in private wells of all surveyed areas, contrary to observations in municipal drinking water systems with secondary disinfectant residuals. On the other hand, *Legionella* reportedly has an optimal growth range of 20-42 °C in municipal systems and recent surveys of municipal systems reported a strong association between *Legionella* spp. and temperature that was not observed for private wells in this study. We speculate that the essentially “infinite” water age and lack of disinfectant for well water, may contribute to these differences relative to municipal water supplies. The results presented in this work are likely an overestimation of OPs numbers in private wells, as molecular detection of OPs does not distinguish between live and dead cells. In addition, sample sizes were limited by laboratory throughput and budget. Identifying key variables impacting the occurrence of OPs in private wells, given that our study shows that these pathogens are relatively common, might someday help limit the risk of infections.

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GENERAL AUDIENCE ABSTRACT

Non-fecal pathogens that are capable of growth in drinking water systems and causing illness primarily in individuals with compromised immune systems are referred to as opportunistic pathogens (OPs). OPs of concern in drinking water systems in the United States include members of the *Legionella* genus (i.e., *Legionella* spp.) and *Mycobacterium* genus (i.e., *Mycobacterium* spp.), as well as *Naegleria fowleri* (also known as “the brain-eating amoeba”). Much of our knowledge surrounding OP occurrence and growth in drinking water relates to municipal drinking water systems. Under the right conditions, OPs have the ability to grow in municipal drinking water systems and in building plumbing systems despite the use of disinfectants. However, OP occurrence and growth in private wells is not well understood and it is unclear how the knowledge developed in municipal systems translates to private well systems that rarely utilize chemical disinfectants. In addition, because private wells are more susceptible to microbial contamination than municipal systems, the impact of flooding on OP occurrence is of particular concern.

Two private well surveys were conducted to document the incidence of OP DNA markers and culturable OPs in private well systems. The first survey was conducted in North Carolina private wells with no history of recent flooding and focused on quantification of DNA markers for *Legionella* spp. and *L. pneumophila*, as well as culturable *L. pneumophila*. The second survey was conducted in flood-impacted private wells in

Texas, Florida, and Louisiana following Hurricanes Harvey and Irma in 2017 and the Louisiana Floods of 2016, quantifying five DNA markers for OPs (*Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, and *N. fowleri*). All water samples in both studies were also analyzed for total bacterial numbers (i.e., total number of copies of a gene present in all bacteria), certain bacteria that indicate environmental and fecal contamination (e.g. total coliform bacteria and *Escherichia coli*), and inorganics. Information about well system characteristics was obtained through questionnaires sent to participating residents.

While it was not possible to sample before the flooding events and determine the background detection rates, this survey found that detection of the target OPs in the flood-impacted wells was widespread, but highly variable. Both culture-based and DNA-based testing methods were used to verify the results because each approach has strengths and weaknesses. Detection of DNA markers indicates the genetic material of the organism being tested for is present and detects DNA of organisms that are both dead and alive. Thus, DNA detection may overestimate the presence live (and infectious) pathogens. Detection of culturable pathogens indicates the organism is alive, but only detects pathogens that readily grow on culture media. There may be other pathogens in the water that do not readily grow on the media. Thus culture may underestimate the occurrence of pathogens.

DNA markers for *Legionella* spp. (detectable in 50-100% of well waters) and *Mycobacterium* spp. (detectable in 13.2-45.0% of well waters) were the most commonly

detected among the targets in this study. The detection of DNA markers for *L. pneumophila* (7.9-65.5%) and *M. avium* (7.9-32.5%) was less common. There were no private well characteristics, such as well depth, well type, or treatment that emerged as significant predictors of these OP detection or levels. Similarly, these OPs were not elevated in recently flooded homes reporting submerged wellheads or system damage. Thus, detection of these OPs was found to be widespread and sporadic. Detection rates of *N. fowleri*, which causes rare incidences of primary amebic meningoencephalitis (PAM), DNA was lower (5.0-12.7%) than other OPs, and was also not related to private well characteristics. However, *N. fowleri* DNA was more frequently detected in wells with wellheads that were submerged due to flooding than in wells with unsubmerged wellheads, as were total coliform bacteria (an indicator of environmental contamination) and total bacterial numbers. This demonstrates concern that submergence of wellheads during flooding is a concern for the introduction of microbial contamination in private wells.

This work also explored two trends characteristic of municipal systems that were not observed in these surveys of private wells. First, positive associations between overall bacterial numbers and DNA markers for both *Legionella* spp. and *Mycobacterium* spp. were observed in private wells in all surveyed areas. This is contrary to what has been reported for overall bacterial numbers in municipal drinking water systems with a secondary disinfectant residual. Second, *Legionella* has been known to have an optimal growth range of 32 to 42 °C (90 to 108 °F) in municipal systems and recent surveys of municipal systems with both a free chlorine and chloramine secondary disinfectants

showed a strong association between *Legionella* spp. and temperature. These associations were not observed in private wells in this work. Continuous disinfection treatment in municipal drinking water systems may eliminate certain microbes, lowering overall levels of bacteria, while OPs may persist by resisting disinfection, resulting in no correlation between total bacteria and OPs. Private wells do not generally use continuous disinfection and represent low-nutrient environments where naturally occurring OPs can grow alongside other bacteria. The results of this study are likely an overestimation of OPs infection risk associated with private wells, as infection can only be caused by live OPs, which cannot be directly determined by measuring DNA markers. In addition, sample sizes were limited by laboratory throughput and budget. Identifying key variables that impact the occurrence of OPs in private wells is necessary to minimize the risk of associated infections linked to private wells. This work provides strong preliminary evidence that OP occurrence in private wells is relatively commonplace. Science-based options for at-risk (e.g., immunocompromised) individuals to decrease their exposure to OPs in private well water need to be developed.

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Chapter 1. Introduction

Groundwater as a Source of Drinking Water

Groundwater has been considered to be a more pristine source of water than surface water for human consumption due to the protective effects of overlying soil and subsoil layers, such as increased residence times as well as physical, chemical, and microbial attenuation [1]. In the United States, 90.5 million people use a community groundwater system and 48 million use a private well as their source of drinking water [2, 3]. While the United States Environmental Protection Agency (USEPA) requires monitoring and compliance of community groundwater systems to drinking water standards, private wells are not regulated by the USEPA. Thus, the responsibility falls on the homeowner to ensure that their well water is safe to drink [3]. Private well water quality is not commonly tested [4, 5] and private wells tend to be more susceptible to microbial contamination than municipal systems [6, 7] due to a combination of a lack of proper source maintenance, periodic testing, and water treatment by homeowners [8, 9]. Environmental factors such as floods, which can cause damage to well casing and plumbing and introduce microbial contaminants [10, 11], as well as geology, which can influence the probability of surface-groundwater interactions [12, 13], can make private wells more susceptible to contamination. Thus, groundwater may not always be entirely free of harmful chemical or microbial contaminants.

The presence of total coliform bacteria is monitored to indicate that the water source may contain environmental contamination and may be unsafe to drink. The presence of *Escherichia coli* indicates fecal contamination, rendering the water unsafe as certain strains of *E. coli* can cause gastrointestinal illness. Multiple studies have reported

varying amounts of total coliform and *E. coli* presence in groundwater [14, 15].

Detection of indicator bacteria tends to be higher in private wells compared to public water supplies, with 17.6-46% of samples from private wells positive for total coliform compared to 0.5-4.7% samples from public water supplies, and 8-14% of samples from private wells positive for *E. coli* compared to less than 0.07% samples positive from public water supplies [4, 12, 16-18].

Fecal pathogens as contaminants in groundwater and private wells

While the effective and consistent removal of fecal pathogens from water through the development of modern drinking water treatment is considered one of the greatest achievements of engineering in the last century [19], fecal contamination still remains a concern for residents reliant on private wells as their primary source of drinking water. Fecal pathogens are microorganisms that are derived from feces and are known to cause various types of gastrointestinal diseases if ingested. In England and Wales, rates of gastroenteritis (i.e., the stomach flu) associated with water supplies were 34.5 times higher than in private water supplies compared to public water supplies [20]. Fecal pathogens of key concern for private wells in the United States include *Salmonella*, *Shigella*, *E. coli* O157, *Giardia*, *Cryptosporidium*, and Hepatitis A [21]. While *E. coli* may be used to indicate fecal contamination in private wells, there may not always be a significant association between the detection in sampled wells containing fecal pathogens and sampled wells containing fecal indicators [22]. Flooding can increase transport of total coliform and *E. coli* from various sources, including a broken septic system and cattle manure [23, 24] and can carry them into private wells if wellheads are submerged.

Surface water flooding and groundwater contamination has also been linked to outbreaks caused by microorganisms of fecal origin [25].

E. coli O157, a subset of *E. coli* that produces shiga toxins (termed shiga toxin-producing *E. coli*) and can infect humans, is one of the most investigated fecal pathogens in private wells. *E. coli* O157 presence in wells has been predicted to be linked proximity to livestock, hydraulic connection to water main breaks, and septic tanks [26-28]. Shiga toxin-producing *E. coli* has been found in 9% of sampled wells in rural Austria [28] and in 4% of 180 sampled wells in the United States [29]. In the United States study, *E. coli* O157 was found to have no association with generic *E. coli* in the sampled private wells [29].

Opportunistic pathogens as a concern in municipal drinking water systems

Opportunistic pathogens (OPs), including *Legionella* spp., *Mycobacterium* spp., and *N. fowleri*, are an important source of waterborne disease in the United States and other developed countries and are of particular concern to immunocompromised individuals [30]. OPs are not derived from fecal matter, which municipal drinking water systems are generally designed to protect against, and instead are naturally found in various environments and are capable of survival and growth within the distribution system itself. Thus it is not surprising that fecal indicator bacteria in private wells do not always correlate with non-fecal pathogens [22], including opportunistic pathogens [31-33]. The exposure routes for OPs (e.g., inhalation of aerosols, aspiration of water into the lungs, eye contact, and skin contact) differ from those for fecal pathogens (e.g., ingestion), and disproportionately infect individuals with compromised immune systems.

Opportunistic pathogens can persist throughout drinking water treatment operations, in the distribution system, and throughout premise plumbing in low-nutrient conditions.

Legionella spp.

Species of *Legionella* are known to cause Legionnaires' Disease, a severe type of pneumonia, in individuals with compromised immune systems. *L. pneumophila* is the most commonly reported species associated with Legionnaires' Disease outbreaks [30]. Environmental sources of community-acquired Legionnaire's disease include cooling towers, hot springs, windshield wiper fluid, air conditioners, and humidifiers, among others [34]. *Legionella* has also been detected in large buildings and residential drinking water supplied by municipal water systems [35-38]. Individuals who acquire Legionnaires' Disease are exposed to *Legionella* through inhalation of aerosols that contain *Legionella* and through aspiration. *Legionella* is the most commonly reported pathogen in identified drinking water-associated outbreaks in the United States, with 57% of 42 drinking water outbreaks in years 2013 to 2014 known to be associated with *Legionella* [30]. While there were approximately 7,100 cases reported in 2017, the true incidence is estimated to be 8,000-18,000 cases annually [39, 40]. However, reported outbreaks of Legionnaire's Disease in the United States account for only about 4% of all cases that occur [34]. The other 96% of Legionnaires' Disease cases are sporadic, with a large proportion of these sporadic cases (~63%) having an unidentified source [41]. The detection of *Legionella* spp. and *L. pneumophila* in municipal systems and premise plumbing have been linked to water temperatures [42-45], water hardness [45], inorganic contaminants [42-44], heater type [35, 42], and presence of chlorine residuals [42, 44].

Mycobacterium spp.

Species of *Mycobacterium* are known to cause nontuberculous mycobacteria (NTM) infections, a severe lung disease, with *M. avium* complex (MAC) being the most common cause. *M. avium* complex are composed mainly of *M. avium* and *M. intracellulare*. *M. avium* is the most prevalent species of NTM that causes disease, especially in immunocompromised individuals [46] and is of increasing concern in drinking water [47]. Reported rates of NTM infection cases were 16 per 100,000 in the United States in 2014, nearly 10 times higher than for Legionnaire's Disease, and this has been increasing annually [47]. Drinking water is a potential route of exposure for NTM infections, as multiple studies have linked NTM infection to drinking water systems [48, 49]. *Mycobacterium* spp. are common inhabitants of drinking water systems and are known to survive and proliferate in biofilms [50, 51] and to be resistant to chlorine [52]. NTM abundance in distribution systems has been linked to disinfectant type and the presence of amoebae [53, 54].

Naegleria fowleri

Naegleria fowleri, also known as the "brain-eating amoeba", can cause primary amoebic meningoencephalitis (PAM), a rare but highly lethal (97%) brain disease [55-57]. The infection route of *N. fowleri* is through nasal passages into the brain, where it can destroy brain tissue [55, 56, 58]. Most cases of PAM worldwide have been contracted through contact with warm bodies of water including use of contaminated tap water in a neti pot, ponds, irrigation ditches, lakes, and hot springs [59-61]. *N. fowleri* DNA has been commonly detected in warm freshwater but has also been detected in treated drinking water distribution systems [60, 62]. Water must be forced up the nose,

allowing *N. fowleri* access to the brain via the optic nerve, for a PAM infection to occur. While there have been no known cases of PAM acquired from ingesting drinking water, PAM infections have been linked to getting water up the nose while taking a bath with water [63] and playing on a water lawn slide wetted with hose water [62] that contained the organism.

Opportunistic pathogens as contaminants in groundwater and private wells

While much of the current knowledge about opportunistic pathogens in drinking water pertains to their occurrence in municipal systems, there have been instances of disease cases linked to opportunistic pathogens in private wells. However, research surrounding opportunistic pathogen occurrence in private wells is lacking. The existing literature related to opportunistic pathogens in groundwater, the primary source of water for private wells, primarily focus on *Legionella* spp. and *N. fowleri*.

Legionella spp.

Private wells have been linked to Legionnaires' Disease in a few documented cases [36, 64]. This has been thought to be due to inadequate shock chlorination treatment (i.e. one-time chlorine disinfection), but could also be due the natural presence of *Legionella* in groundwater. *Legionella* spp. and *L. pneumophila* have been sporadically detected in groundwater over several years and their occurrence has differed between sampling locations that share the same aquifer, likely due to the generation of localized environments within the aquifer that may promote the development of *Legionella* [65]. Studies have reported varying rates of detection of *Legionella* in

untreated groundwater, which may not be indicative of their occurrence in modern private well systems with premise plumbing. For example, culturable *Legionella* was detected in 9.8% of private wells on an Indian Reservation, but only first-draw cold water samples were taken [66]. *Legionella* has been found in 29.1% of groundwater and 28.2% of biofilm samples from municipal well heads where there was believed to be no surface water interaction and there was no treatment [67]. Two studies in Poland found 20-30% of household wells were positive for *Legionella* spp. and *L. pneumophila*, even in cold well water samples, but these systems were shallow wells, open to the air without caps or pressurization [32, 68]. Higher rates of culturable *Legionella* positivity were found in another study, with 83% of water samples and 75% of biofilm samples from 12 private wells, but limited information was provided regarding the construction of wells and sample collection methodology [69]. Given the limitations of these studies, we do not know if factors that influence *Legionella* spp. occurrence in municipal systems also extend to their occurrence in private wells.

Mycobacterium spp.

The risk of NTM infections linked to private wells is unknown. To the knowledge of the author, only one study to date surveyed culturable *Mycobacterium* spp. in private wells, reporting 12 of 41 (29.3%) samples positive from homes supplied by untreated groundwater wells on an Indian Reservation in the United States [66].

Naegleria fowleri

While most PAM infections occur as a result of recreational exposure to *N. fowleri*, PAM cases have been linked to groundwater sources. One young female child died from PAM after being exposed to contaminated water from a borehole [70]. *N. fowleri* was found in 17 of 19 samples collected from sink traps in two homes supplied with groundwater where two separate cases of PAM occurred [71]. While most cases of PAM in the United States have occurred in the summer months, more recently PAM cases have been reported farther north than in previous years, likely due to increasing groundwater temperatures as a result of climate change [72]. Multiple studies have reported detection of *N. fowleri* in groundwater wells in Arizona, a state with a very warm climate. One study found 11 of 143 (7.7%) water samples collected at well heads were positive for *N. fowleri* DNA [59]. Another study found 12 of 113 wells (10.6%), which were constructed in compliance with well regulations of Arizona as of 2008, positive for *N. fowleri* DNA [31]. Six public water supply wells in the Phoenix metropolitan area were sampled in different seasons of the year, with 12 of 45 (26.7%) samples positive for *N. fowleri*, and all positive samples taken in late summer or early August [73]. However, while it is known that *N. fowleri* is a surface water organism, it is unclear in these studies whether or not surface water infiltrated groundwater and transported *N. fowleri* into the aquifers, or if *N. fowleri* naturally occurs in groundwater. A survey of homes supplied by private wells sampled in Louisiana following the Louisiana Floods in 2016 found 20% of homes positive for *N. fowleri* DNA, however baseline levels of *N. fowleri* prior to the storm were unknown [33]. It is possible that

surface water infiltration is a contamination route for *N. fowleri* in groundwater and private wells.

Thesis Overview

The goals of this research was to increase knowledge regarding the prevalence of opportunistic pathogens (OPs), specifically *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, and *N. fowleri*, in private wells and to identify factors that influence their prevalence. Chapter 2 is a survey of *Legionella* spp. and *L. pneumophila* in homes supplied by private wells with no recent history of flooding in Wake County, North Carolina utilizing a molecular method (i.e., real time quantitative polymerase chain reaction [qPCR]) and a relatively new culture method (i.e., IDEXX Legiolert) for the detection of *L. pneumophila*. Chapter 3 investigates the prevalence of OPs in private wells in areas that have been impacted by major flood events caused by three storms, the Louisiana Floods of 2016 and Hurricanes Harvey and Irma in 2017.

The specific objectives of the research summarized in this thesis are as follows:

- Quantify *Legionella* spp. and *L. pneumophila* in home plumbing networks supplied by private wells (Chapter 2)
- Compare molecular- and culture-based methods for detection and quantification of *Legionella* spp. and *L. pneumophila* in private wells (Chapter 2)
- Assess the prevalence of OPs in private wells (Chapter 2 and Chapter 3)

- Identify water quality and well system characteristics that impact the prevalence of OPs in private wells (Chapters 2 and 3)
- Investigate how floods impact the prevalence of OPs in private wells (Chapter 3)

The overall thesis expands knowledge of OPs associated with drinking water to include private wells and to improve our understanding of how factors that influence the presence of OPs in municipal systems may differ from factors that influence their presence in private wells.

Attributions

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- Kris Mapili - Participated in experimental design, conducted the majority of the lab analyses, conducted the majority of the data analysis, and wrote the thesis
- Marc Edwards – Primary thesis advisor. Assisted in experimental design, reviewed project progress and provided feedback, provided guidance in data

analysis, contributed to the writing of the manuscripts. PI on RAPID grants 1661496 and 1760296.

- William Rhoads - Participated in the experimental design, and oversaw the execution of the laboratory analyses, data analysis, and writing of the thesis. Co-PI on RAPID grant 1760296.
- Kelsey Pieper - Participated in the experimental design, and oversaw the execution of the laboratory analyses, data analysis, and writing of the thesis. Coordinated field sampling and other efforts between collaborators in Texas, Florida, and North Carolina. Co-PI on RAPID grants 1661496 and 1760296.
- Amy Pruden - Assisted in experimental design, reviewed project progress and provided feedback, provided guidance in data analysis, contributed to the writing of the manuscripts. Co-PI on RAPID grant 1760296.
- Adrienne Katner - Co-PI on RAPID grants 1661496 and 1760296. Coordinated field sampling in Louisiana.
- Dongjuan Dai – Led efforts in planning and executing the sampling campaign and subsequent processing for Chapter 2.
- Min Tang – Participated in experimental design and assisted efforts in planning and executing the sampling campaign for the manuscript described in Chapter 2.
- Mary Coughter – Participated in lab analysis for Chapter 3.
- Drew Gholson, Diane Boellstorff, & Andrea Albertin – led efforts in planning and executing the sampling campaigns in Texas and Florida for Chapter 3.

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Chapter 2. Survey of *Legionella pneumophila* in North Carolina private wells¹

Kris Mapili, Kelsey Pieper, Dongjuan Dai, Amy Pruden, Marc Edwards, Min Tang,

William Rhoads

Virginia Tech, Civil and Environmental Engineering, 418 Durham Hall, Blacksburg, VA

24061

Significance and Impact of the Study

The present knowledge regarding incidence of *Legionella*, the leading cause of waterborne disease in the United States, in drinking water and associated risk factors with plumbing infrastructure is primarily related to municipal systems. While *Legionella* has been found to occur naturally in groundwater, efforts to characterize its occurrence have been limited to sampling at the wellhead (i.e., not considering the household plumbing) and reported findings may not be representative of modern private wells. This research explores *Legionella* incidence in private wells and the risk factors associated with these unregulated plumbing systems.

Abstract

Private wells are potential understudied sources of community-acquired Legionnaires' disease, which is caused by species of *Legionella*, primarily *L. pneumophila*. To quantify *Legionella* and *L. pneumophila* in private wells and identify water quality and system characteristics that predict their presence, 100 drinking water samples were collected

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from 44 homes supplied by private wells in Wake County, North Carolina. Samples were analyzed using real-time quantitative polymerase chain reaction (qPCR) and IDEXX Legiolert to evaluate the incidence of *Legionella* spp. DNA, *L. pneumophila* DNA, and culturable *L. pneumophila*. Samples were also analyzed for total coliform, *E. coli*, and inorganics, and participants completed a household questionnaire. Total coliform and *E. coli* were detected in 20.4% and 0.0% of homes, respectively. *Legionella* spp. DNA, *L. pneumophila* DNA, and culturable *L. pneumophila* were detected in 100%, 65.5%, and 15.5% of homes. Within certain subgroups of samples, *Legionella* and *L. pneumophila* were linked to total bacteria, construction year, and softener use, but not with indicator bacteria, other water quality parameters, or system characteristics. These findings illustrate that *Legionella* and *L. pneumophila* incidence in private wells may be more sporadic than in municipal systems due to the heterogeneity of groundwater and household system characteristics.

Keywords

Legionella pneumophila, private wells, drinking water, groundwater, water quality, premise plumbing

Introduction

Private wells are understudied potential sources of community-acquired Legionnaires' disease, a severe pneumonia caused by *Legionella* spp. bacteria. Incidence of Legionnaires' disease is under-reported – while there were approximately 7,100 cases reported in 2017, incidence is estimated to be 8,000-18,000 cases annually [1, 2].

Sporadic cases (i.e., not being associated with an outbreak) comprise 96% of all cases [3], and the source of approximately 63% of cases is never determined [4]. Home plumbing is a potential source of Legionnaires' disease due the high potential for exposure to aerosols, inhalation of which is the primary exposure route for *Legionella*. Legionnaires' disease has been linked to private well water in the past [5], but because private wells are unregulated sources of drinking water, water quality in wells is not commonly tested [6, 7]. Microbial monitoring of private wells is typically limited to only total coliform and *E. coli* bacteria, which are used to indicate surface water and fecal contamination. It is well documented that indicator bacteria do not always correlate with non-fecal pathogens [8], including opportunistic pathogens [9-11]. Therefore, because private wells tend to be more susceptible to microbial contamination than municipal systems [6, 12], there is a need to characterize the incidence of *Legionella* species in private wells.

Legionella occurrence in large buildings and residential drinking water supplied by municipal water systems has been well documented [5, 13-15]. In these systems, detection of *Legionella* spp. and *L. pneumophila* have been linked to water temperatures [16-19], water hardness [19], inorganic contaminants [16-18], heater type [13, 16], and presence of chlorine residuals [16, 18]. The extent to which these risk factors extend to private wells is not known.

Although *Legionella* spp. have been documented in groundwater, these efforts are mainly limited to collecting samples at wellheads and, in many cases, are not representative of modern private well premise plumbing systems in the United States (US). For instance, culturable *Legionella* was detected in 9.8% of private wells on an Indian Reservation, but only first draw cold water samples were taken; no hot water

samples were taken and analyzed [20]. In Poland, 9-28% of household wells were positive for culturable *L. pneumophila*, but samples included shallow, open air wells without caps or pressurization [10, 21]. Higher rates of positivity have been identified in other US studies, with 28-29% of water and biofilm samples taken from municipal wellheads [22] and 83% of water samples and 75% of biofilm samples in another study with limited information reported about well construction and sample collection methodology [23]. Thus, reporting of *Legionella* incidence in private wells with modern plumbing is lacking.

The objectives of this study are to (i) quantify *Legionella* spp. and *L. pneumophila* in home plumbing networks supplied by private wells using molecular and culture methods, (ii) compare detection rates among the two methods; and (iii) identify water quality and system characteristics that are associated with the presence of *Legionella* spp. and *L. pneumophila* in private wells.

Results and Discussion

Two sampling events took place as part of a survey of *Legionella* and associated factors in private wells in Wake County, North Carolina – one on October 10, 2017 and the second on November 18, 2017. In both sampling events, two types of sampling kits – “basic” and “advanced” – were randomly distributed to residents (Appendix A, Table SI-1) along with sampling instructions. The basic kit included three cold water samples, including first draw sample for inorganic analysis, 5-minute flushed sample for inorganic analysis, and flushed sample for microbial culture analysis collected immediately following the second sample. The advanced kit included 5 samples, including a first draw

cold water sample for inorganic analysis, 5-minutes flushed cold water for inorganic analysis, flushed cold water sample for culture and molecular analysis collected immediately following the second sample, first draw hot water sample for culture and molecular analysis, and flushed-to-hot water sample for molecular analysis.

Survey of culturable *L. pneumophila* and molecular indicators of *Legionella* spp. and *L. pneumophila*

Culturable *L. pneumophila* was detected in 15.5% of houses (n=7 of 44) and 11.1% of all samples (n=8 of 72). Specifically, culturable *L. pneumophila* was detected in 9.1% (n=4 of 44) of flushed cold water and 14.3% (n=4 of 28) of first draw hot water samples (Table 1). Frequency of detection was not significantly different among the sample types (Test of Equal Proportions, p=0.76) (Figure 1A). The highest level was 4.74 MPN/mL, which is estimated to be equivalent to less than 3 CFU/mL [24]. The detection rate of culturable *L. pneumophila* observed in this study was similar to what has been reported in some municipal systems, but the quantifiable levels tended to be lower. Culturable *L. pneumophila* was detected in 74 of 491 (15.1%) of U.S. potable water samples collected from large buildings (e.g. offices, medical buildings, nursing homes, sports facilities, rehabilitation centers), using the same method, with a mean of 3.74 MPN/mL and a maximum of 146 MPN/mL [24]. In 290 positive samples collected from potable water systems in Germany, were positive with the mean and maximum reported were 1.3 MPN/mL and 22.7 MPN/mL, respectively [25].

A subset of residents (n=29) collected additional samples for molecular testing. *Legionella* spp. DNA was detected in 100% of homes (n=29) and 82.4% of all samples

(n=70 of 85). Specifically, DNA was detected in 75.9% of flushed cold water samples, 82.1% of first draw hot water samples, and 89.3% of flushed to hot water samples (Table 1). The incidence of *Legionella* spp. DNA was not significantly different among sample types (Test of Proportions, p=0.41; Figure 1C). *L. pneumophila* DNA was detected in 65.5% of homes (n=19 of 29) and 31.8% of all samples (n=27 of 85; Table 1; Figure 1D). Detection rate of *L. pneumophila* gene copies in first draw hot water samples (50.0%) were significantly higher than in flushed cold

Table 2. Summary of detection and quantification of targets of interest.

	<i>Legionella</i> spp.		<i>L. pneumophila</i>		Culturable <i>L. pneumophila</i>	
	n	%	n	%	n	%
All Samples	n=85	-	n=85	-	n=72	-
Detected	70	82.4	27	31.8	8	11.1
>QL [†]	41	48.2	7	8.2	8	11.1
<QL	29	34.1	20	23.5	0	0
Not detected	15	17.6	58	68.2	64	88.9
Flushed cold	n=29	-	n=29	-	n=44	-
Detected	22	75.9	4	13.8	4	9.1
>QL	14	48.3	3	10.3	4	9.1
<QL	8	27.6	1	3.4	0	0
Not detected	7	24.1	25	86.2	40	90.9
First draw hot	n=28	-	n=28	-	n=28	-
Detected	23	82.1	14	50	4	14.3
>QL	8	28.6	2	7.1	4	14.3
<QL	15	53.6	12	42.9	0	0
Not detected	5	17.9	14	50	24	85.7
Flushed to hot	n=28	-	n=28	-	Not Tested	
Detected	25	89.3	9	32.1		
>QL	19	67.9	2	7.1		
<QL	6	21.4	7	25		
Not detected	3	10.7	19	67.9		

[†]The quantification limit (QL) is the same as the detection limit (DL) for the IDEXX Legiolert; QL: 5 or 10 gc/mL for *Legionella* spp. and *L. pneumophila* and 0.1 MPN/mL for culturable *L. pneumophila*

water samples (13.8%; p=0.04; Figure 1D), but the detection rate in flushed hot water samples (32.1%) were not different from first draw hot water (p=0.28) or flushed cold water samples (p=0.18). Observing higher detection in first draw hot water samples

compared to flushed to hot water and cold water is consistent with the observations related to *Legionella* growth in large building systems, as stagnant hot water plumbing is more suitable for *L. pneumophila* growth than the hot water tank or cold plumbing [26, 27].

The reported incidence of *Legionella* spp. in private wells in this study was higher than observed in untreated groundwater from municipal wells (28% of 114 samples) [22] and in stagnant cold samples taken from homes supplied by private wells (9.8% of 41

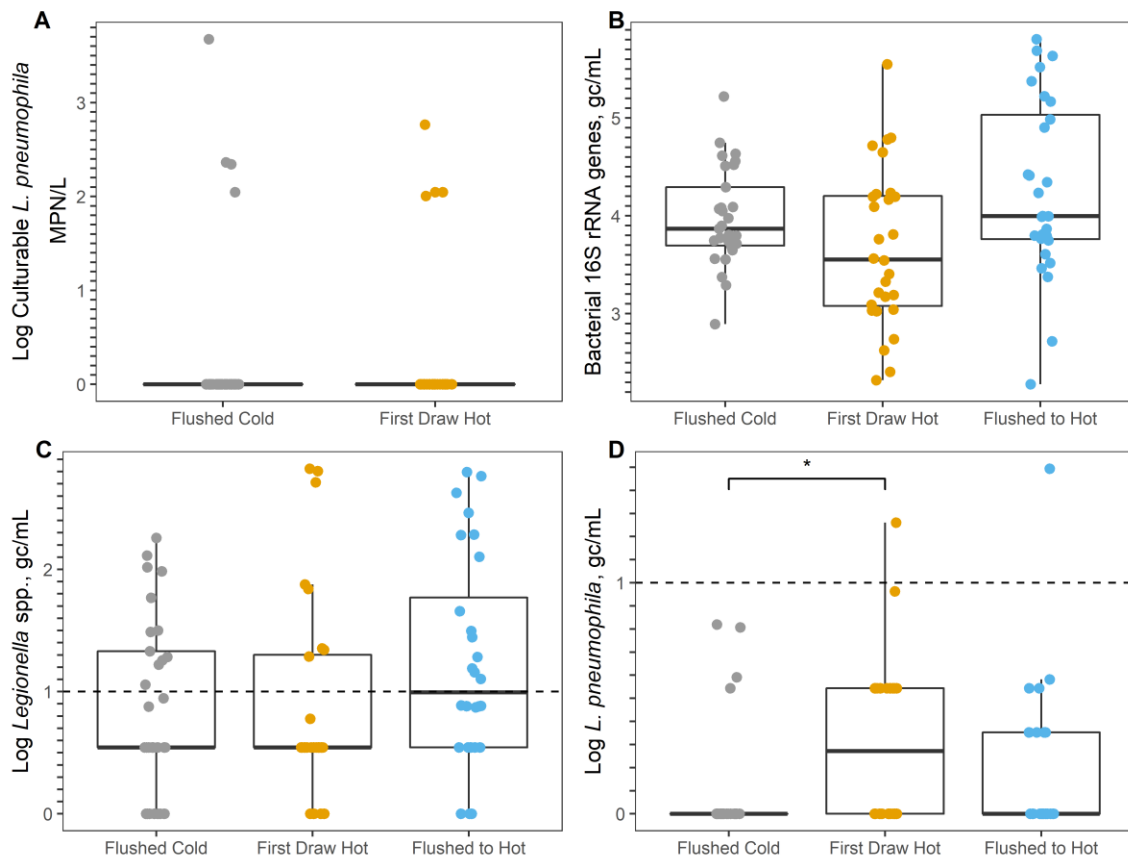


Figure 1. Enumeration of target microbes in water samples. Numbers of: A) culturable *L. pneumophila*, B) total bacterial 16S rRNA gene copies, C) *Legionella* spp. gene copies, and D) *L. pneumophila* gene copies in flushed cold, first draw hot, and flushed to hot water samples. Data points reflect all measurements made, while boxplot limits reflect data with the median \pm 1.5 times the inter-quartile range. Samples with gene copies below the quantification limit (BQL) are reported as half of the corresponding limit of quantification (LOQ). Dashed line in C) and D) represent the LOQ (10 gc/mL). Samples with no detection are reported as 0. * denotes significant difference (Test of Proportions, $p < 0.05$) in detection between sample types.

samples) [20]. In keeping with *Legionella* spp., the incidence of *L. pneumophila* in this study was lower than observed in hot water samples from municipal systems distributing chlorinated groundwater (48.9% of 45 samples) [28], lower than observed in open air wells (22.2% of 45 samples), and comparable to untreated and unheated groundwater private supply systems (9.1% of 109 samples) [10]. Although these studies have reported detectable levels of culturable *L. pneumophila* in groundwater, there is no reported baseline levels of *L. pneumophila* DNA in groundwater or private wells. There is a need to improve monitoring of *Legionella* and *L. pneumophila* incidence and levels in private wells with modern plumbing systems.

Relationship of *Legionella* spp. and *L. pneumophila* to total bacteria, total coliform, and *E. coli*

Total coliform bacteria were detected in 20.4% of flushed cold water samples (n=9 of 44) and *E. coli* was not detected. Total coliform in positive samples ranged from 0.01 to 24.2 MPN/mL, with a median of 0.932 MPN/mL. Total coliform MPNs were not correlated with culturable *L. pneumophila* MPNs (Spearman's ρ , $p=0.66$; $n=44$), *Legionella* spp. DNA ($p=0.08$; $n=29$), or *L. pneumophila* DNA gene copies ($p=0.25$; $n=29$). The lack of correlation suggests that the source of *Legionella* in private wells was not linked to the cause of total coliform bacteria, which is consistent with previous reports [10].

Total bacteria (16S rRNA gene) were detected in all samples and gene copies ranged from 1.89×10^2 to 6.39×10^5 gc/mL (Figure 1B). The mean 16S rRNA gene copies were not different based on sample types (Kruskal-Wallis, $p=0.12$), which was consistent with *Legionella* spp. observations. For samples with detectable *Legionella* spp. (n=70 of 85),

Legionella spp. was correlated with 16S rRNA ($\rho=0.69$) (Figure 2). This association was observed within all three sample types ($\rho=0.49$ to 0.69) (Appendix A, Figure SI-1). Such correlation has been previously observed in private wells that were impacted by severe flooding in the aftermath of the Louisiana Floods in 2016 [11]. Studies in municipal systems have reported a relationship between total bacteria, specifically heterotrophic aerobic bacteria, and culturable *Legionella* spp. was weak or non-existent when a disinfectant residual was present [29], but strong when a disinfectant residual was absent [30]. The significant relationship observed in private wells, with a recent history of flooding [11] and herein with no flooding, suggests that areas in private wells with no disinfectant that support higher total bacteria levels could also support higher *Legionella*

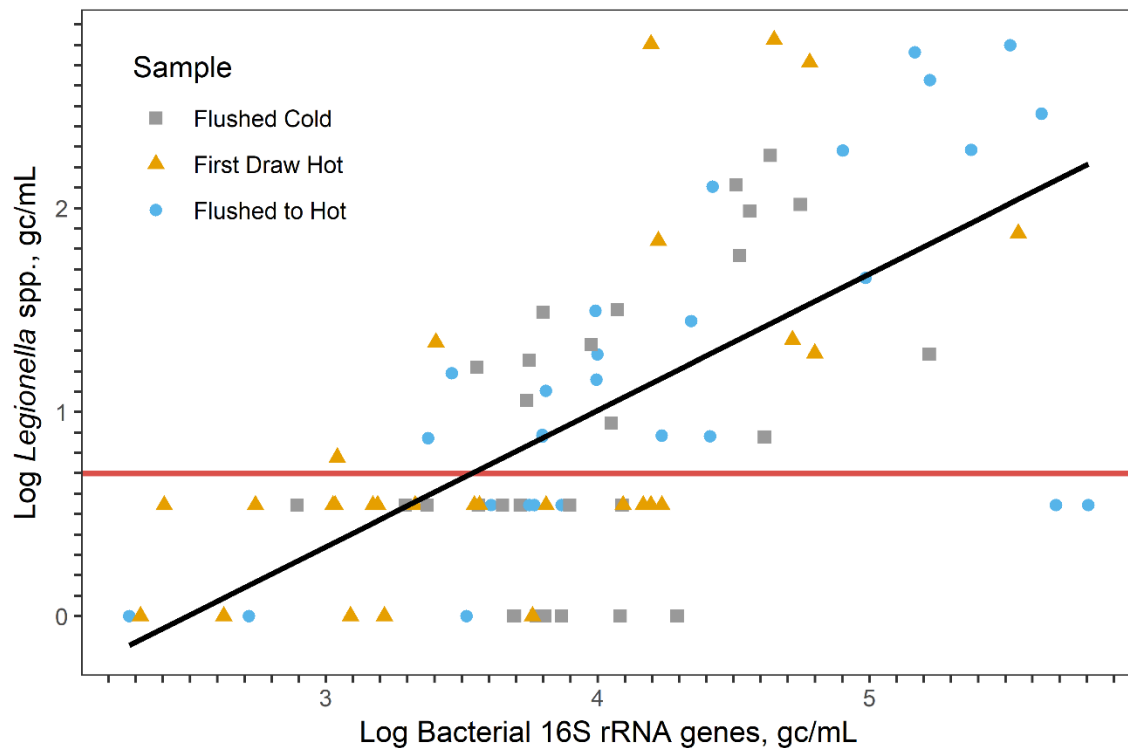


Figure 2. Positive correlations between *Legionella* spp. and total bacterial 16S rRNA gene copy numbers across all samples. Red line represents limit of quantification (LOQ=5 gc/mL). Samples that were above detection, but below LOQ are plotted as half the LOQ (2.5 gc/mL). Samples below detection are plotted as zero.

spp. levels when *Legionella* spp. are present.

Impact of water temperature and water heater type

Water temperature was not related to the occurrence of total bacteria or *Legionella*. For total bacteria (16S rRNA) and *Legionella* spp. qPCR, where there were enough quantifiable samples to make determinations, there was no correlation with temperature ($p=0.88$ and 0.41 ; Appendix A Figure SI-2). In addition, water temperature distributions were not different in samples that were positive compared to negative by *Legionella* culture, *Legionella* spp., or *L. pneumophila* gene markers across all sample types (Wilcoxon Test, $p=0.12-1$; Figure 3). Further, detection rates of culturable *L. pneumophila*, *Legionella* spp. DNA, or *L. pneumophila* DNA were not more likely to occur within the permissive growth ranges for *Legionella* in cold (>20 °C; Figure 3; Test of Equal Proportions, $p=0.36$ to 0.91) or hot (<50 °C; $p=0.47$ to 1.0) water samples. This is counter-intuitive to conventional wisdom where warmer cold water temperatures (>20 °C) and cooler hot water temperatures (<50 °C) would be more likely to be colonized that has recently been observed in two household studies with a secondary disinfectant residuals [31, 32]. In these studies, quantification of *Legionella* spp. generally increased from approximately 20 °C to 49 °C and then begins to decrease with increasing hot water temperatures, falling off more rapidly at temperatures >52 °C. The lack of association between temperature and *Legionella* spp. in this study could have been influenced by the limited sample size and sample collection by untrained citizen-scientists. In addition, there were only 2 samples (5.0%) with reported temperatures above 55 °C, the minimum strongly inhibitory temperature recommended for *Legionella* growth [33].

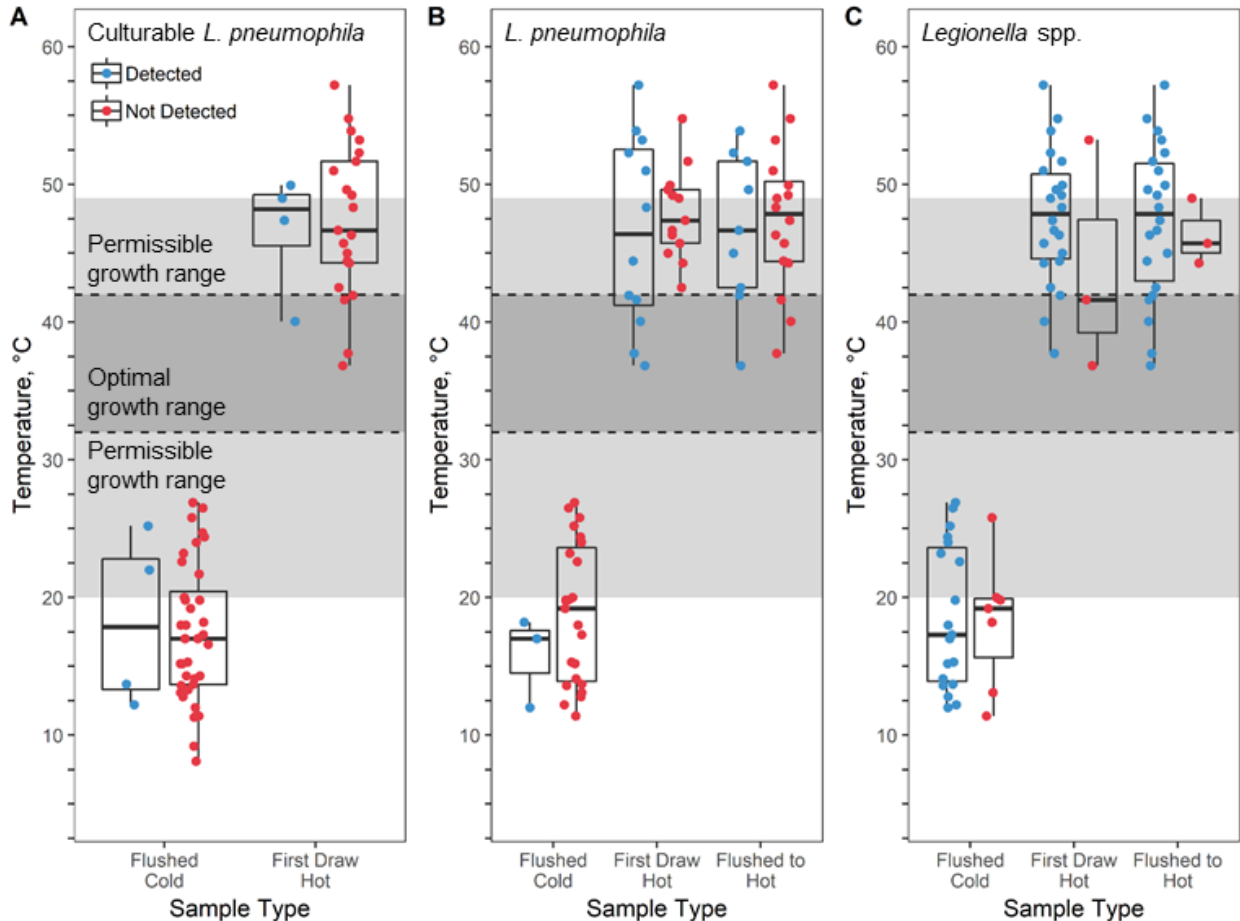


Figure 3. Temperatures reported by residents in samples types by detection of A) culturable *L. pneumophila*, B) *L. pneumophila*, and C) *Legionella* spp. Light gray shaded area: permissive growth range for *Legionella* spp. (20 to 49 °C). Dark gray shaded area: optimal growth range for *Legionella* spp. (32 to 42 °C). White area: outside optimal and permissive growth range. Blue data: organism detected. Red data: organism not detected. Three samples were removed due to the temperature recorded being greater than the maximum reportable temperature of the thermometer deployed in the first sampling campaign (49 °C).

Of 44 households that reported their water heater type, 12 (27.3%) reported using electric heaters, 6 (13.6%) reported using gas heaters, and 26 (59.0%) were unsure of their type. There was no difference in the detection of culturable *L. pneumophila*, *Legionella* spp. DNA, or *L. pneumophila* DNA based on heater type (Test of Equal Proportions, $p=0.59-1.0$). Previous studies have reported that *L. pneumophila* was more frequently detected in electric heaters than gas or oil heaters [13, 16] due to internal thermal stratification within the tank [34]. Though this conclusion may be influenced by

the small sample size, electric heaters are a possible risk factor for contracting Legionnaires' disease [35] and for household colonization of *Legionella* that has been observed in municipal systems [13]. However, this could not be confirmed in this study.

Associations with water quality characteristics

Of the 44 participating houses, 36 had soft water (hardness of less than 60 mg/L as CaCO₃), 6 had moderately hard water (60 – 120 mg/L as CaCO₃), 1 had hard water (120 – 180 mg/L as CaCO₃), and 1 had very hard water (more than 180 mg/L as CaCO₃). Hardness was not correlated with culturable *L. pneumophila*, *Legionella* spp. DNA, or *L. pneumophila* DNA gene copies (p=0.31 to 0.99, n=29 or 44), suggesting that hardness did not impact the presence of *Legionella* spp. and *L. pneumophila* in plumbing systems in homes supplied by private wells in this study. One study across 146 homes supplied with water from municipal systems with both groundwater and mixture water (e.g. combined surface water and groundwater) as sources in Italy found a negative association between hardness and *Legionella* in hot water samples; however, this study did not report the association in samples from only groundwater-sourced homes [16].

Copper, zinc, and iron levels in flushed cold water samples ranged from <1 to 473.1 µg/L, <5 to 678.8 µg/L, and <10 to 24.9 mg/L, respectively. Culturable *L. pneumophila*, *Legionella* spp., and *L. pneumophila* were not correlated to any of the inorganics measured (p=0.61 to 0.96, n= 29 or 43).

Associations with well characteristics

Of 39 households that reported well type, 34 (87.2%) reported having a drilled well and 5 (12.8%) reported using a dug or bored well. Reported well depths (n=30; 76.9%) ranged from 40 to 565 feet, with a median of 245 feet. Reported years of construction (n=38) ranged from 1975 to 2015, with a median of 1999.

In 1986, well construction regulations, which specified requirements for casing construction to prevent rock fragments, sand, and gravel from falling into the well shaft, as well as grouting to prevent the leakage of contaminants into the aquifer, were implemented in Wake County to help protect well water and groundwater quality [36]. Twelve wells (31.6%) were constructed before the 1986 and 26 wells (68.4%) were constructed after 1986. There was no significant difference in the detection of culturable *L. pneumophila* and *L. pneumophila* DNA before and after 1986 (Test of Proportions, $p=0.070-1$). Levels of *Legionella* spp. were significantly higher in samples from wells constructed before 1986 (96% positive of 27 samples) than those constructed after 2008 (75% positive of 48 samples) ($p=0.022$), suggesting that improper construction could be a contamination route for *Legionella* in wells. However, these differences did not hold when considering individual sample types ($p = 0.10-1$).

Reported forms of treatment included 5 homes (12.8%) using iron removal, 2 (5.13%) using and activated carbon pitcher filter, 3 (7.69%) using an acid neutralizer, 12 (30.8%) using a water softener, 5 (12.8%) using a granulated activated carbon filter, 19 (48.7%) using a sediment filter, and 3 (7.69%) using a reverse osmosis (RO) unit. No households reported using UV treatment or a chlorinator, and 11 homes (28.2%) reported not using any type of treatment. *Legionella* spp. DNA was significantly lower in cold

water samples from homes that used a water softener than from homes that did not use a water softener overall (Wilcoxon Test, $p=0.02$, $n=27$) but no significant difference was seen in first draw hot water samples ($p=0.63$, $n=26$) or flushed hot water samples ($p=0.91$, $n=26$) (Figure 4). While one study found that extremely high hardness (> 250 mg/L CaCO_3) in municipal systems using groundwater or mixed (surface and groundwater) was protective against *Legionella* spp. colonization [16], the impact of the presence of a softener was not assessed. Given that most of the sampled homes in this study had water hardness below 180 mg/L CaCO_3 , the meaningfulness of this result is unclear. No significant differences were seen for *L. pneumophila* DNA or culturable *L. pneumophila* in samples from homes that used a softener than from homes that did not use a softener in all sample types (Test of Equal Proportions, $p=0.41$ to 1, $n=79$).

Culture and molecular methods comparison

A total of 57 samples (29 flushed cold and 28 first draw hot water) were tested for *L. pneumophila* using both IDEXX Legiolert and qPCR. Of these, 18 samples (31.6%; 4 flushed cold and 14 first draw hot) tested positive for *L. pneumophila* DNA and 6 samples (10.5%; 2 flushed cold and 4 flushed hot) tested positive for culturable *L. pneumophila*. Only one sample tested positive for *L. pneumophila* via both methods (Appendix A, Table SI-2). The 5 samples that were positive for *L. pneumophila* via IDEXX Legiolert but negative via qPCR ranged from 0.1 to 4.74 MPN/mL, with a median of 0.11 MPN/mL. The 17 samples that were positive via qPCR but

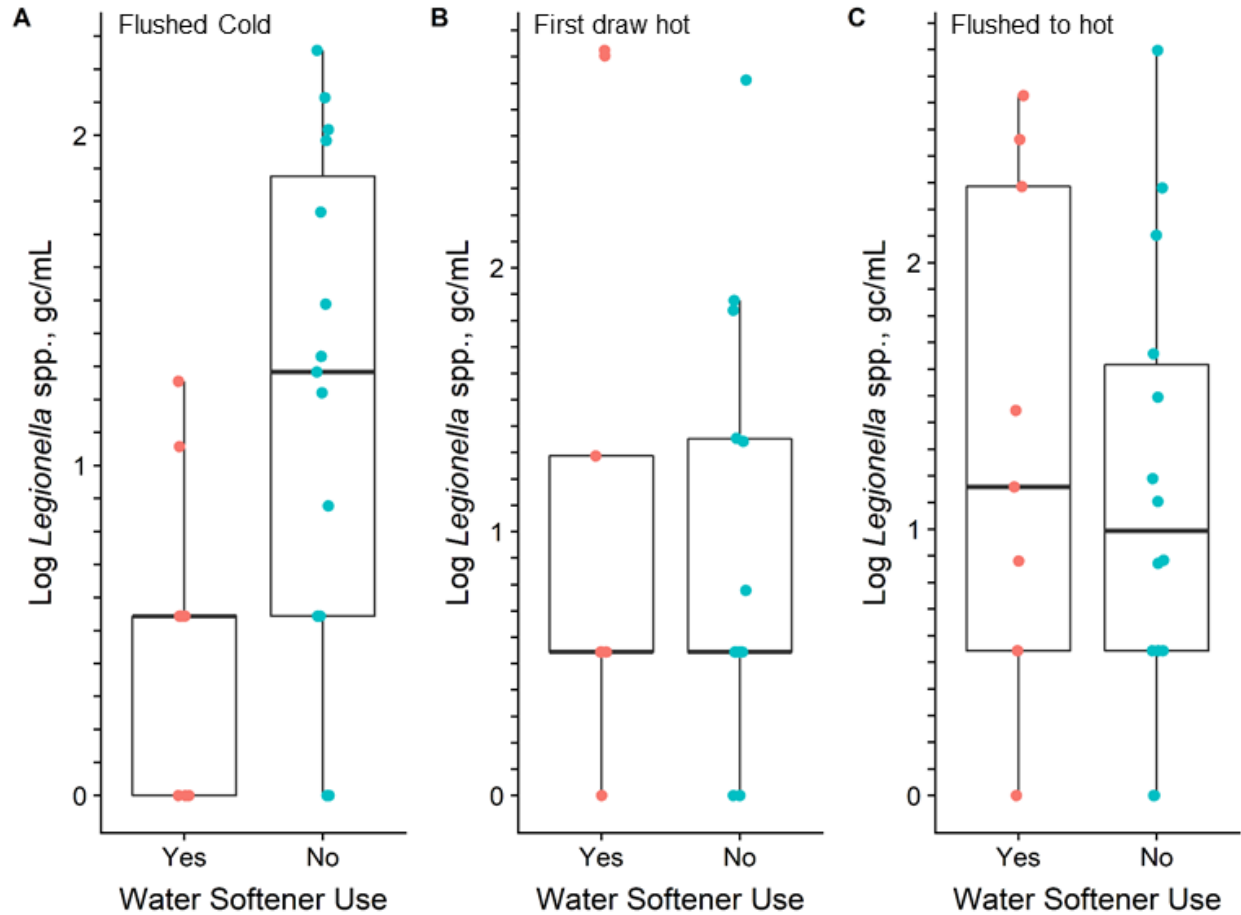


Figure 4. Enumeration of *Legionella* spp. among water softener treatment use in A) flushed cold, B) first draw hot, and C) flushed hot water samples. *Legionella* was significantly lower in cold water samples from homes with water softener than from homes without water softener. Samples with gene copies BQL were shown as half of the corresponding LOQ values.

negative for IDEXX Legiolert ranged from BQL to 17.2 gc/mL, with the median BQL.

The one sample that was positive in both methods was 0.11 MPN/mL via IDEXX

Legiolert and BQL via qPCR.

There are several hypotheses that could explain the discordance in the 5 samples that were positive according to IDEXX Legiolert but were negative according to qPCR.

While IDEXX Legiolert measures viable *L. pneumophila* that grow in the broth media, qPCR does not distinguish between live and dead cells [37]. These methodological differences resulted in a higher proportion of positive detection by qPCR overall, as

expected. A literature review of 28 articles found that of 3,967 samples analyzed concurrently by qPCR and traditional culture methods, 50% more likely to be positive by qPCR than culture [37]. Attempts were made to obtain isolates from the 5 samples with culturable *L. pneumophila* but no detectable *L. pneumophila* DNA, but isolates were not able to be recovered (Appendix A, Section SI-1). qPCR was also repeated at higher dilutions (up to 1:100) for DNA extracts to reduce inhibition but the target gene was still below detection in all five samples. Differences in culture and qPCR positivity could be due to inefficiencies of recovering *L. pneumophila* DNA through the filter concentration and DNA extraction processes, as well as the relatively high detection limit of the qPCR assay (theoretically approximately 1 gc/ mL) relative to the IDEXX Legiolert method one order of magnitude lower (0.1 MPN/mL). In addition, there have been reports of false positivity with Legiolert ranging from 0 to 3.3% [24, 25, 38, 39]. The organisms that may cause false positivity have not been specified, and therefore could not be determined here. IDEXX Legiolert is a relatively newly developed culture method used for the detection of *L. pneumophila*. When compared to conventional culture methods using agar, Legiolert yielded higher counts (0 – 0.668 MPN/mL) than a membrane filter-concentrated culture method (0 – 0.01 CFU/mL) in potable water samples from municipal systems [25]. Legiolert exhibited higher sensitivity in potable water samples [24] and equivalent sensitivity in non-potable water samples compared to plate culture methods [24, 38].

Limitations

The overall lack of correlation between parameters associated with increased incidence of *Legionella* detection in large building or residential systems supplied by municipal utilities, indicates that the sample size of this study may have been too small to confirm significant trends or that *Legionella* positivity in private wells is more strongly influenced by different factors than in municipal water. Previous studies investigating *Legionella* occurrence in groundwater supplies reports that *Legionella* is widely detected, but the incidence of *L. pneumophila* – the most commonly reported pathogenic species of *Legionella* – is less common, similar to what we report herein. In addition, the heterogeneity of groundwater supplies between geographically similar private wells [40] also may indicate there is more randomized positivity in groundwater than in municipal systems with distinct characteristics that increase risk of *Legionella* positivity, such as having an electric relative to gas water heater [13, 35], having low disinfectant levels, or other uncontrolled system disruptions (e.g., water main break) [41].

Materials and Methods

Site location and sampling procedures

Two citizen-science sampling campaigns occurred on October 10, 2017 and November 18, 2017 in Wake County, North Carolina. In both sampling events, two types of sampling kits, a “basic” or “advanced” kit, were randomly distributed to residents (Appendix A, Table SI-1). All sampling kits included sampling instructions, a thermometer, sampling bottles, and a questionnaire. For the basic kit, residents collected three water samples: (1) a 250 mL first draw cold water sample for inorganic analysis, (2)

a 250 mL flushed cold water (after a 5-minute flush) for inorganic analysis and temperature measurement, and (3) a 120 mL flushed cold water sample for microbial culture analysis immediately following collection of the second sample. For the advanced kit, residents collected five samples: (1) a 250 mL first draw cold water sample for inorganic analysis, (2) a 250 mL flushed cold water (after a 5-minute flush) for inorganic analysis, (3) a 1 L flushed cold water sample for culture and molecular analysis immediately following collection of the second sample, (4) a 1 L first draw hot water sample for culture and molecular analysis, and (5) a 1 L flushed-to-hot water sample for molecular analysis and temperature measurement. It was not feasible to process all samples for molecular analysis, thus only a subset of homes (29 of 44) received the advanced kit. Residents were asked to complete a questionnaire about the characteristics and maintenance history of their private wells (Appendix A, Section SI-2). Participation in this campaign was voluntary and all procedures were approved by Virginia Tech Institute Review Board (#17-805).

Water quality analysis

Inorganics were analyzed at Virginia Tech using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) per methods 3030 D and 3125 B [42]. Blanks and/or spikes of known concentrations were processed every 10 samples for QA/QC. Detection limits were 1 µg/L for copper, 5 µg/L for zinc, and 10 µg/L for iron. Inorganic parameters below minimum reporting level were treated as half of the limit for non-parametric statistical analysis. Total coliform and *E. coli* were quantified using the IDEXX Colilert 2000 method (Westbrook, MN), with a detection limit of 1.00 MPN/100 mL. Trip and

field blanks and laboratory controls were included in both sampling campaigns.

Culturable *L. pneumophila* was quantified using the IDEXX Legiolert method (Westbrook, MN). Testing was performed per the manufacturer's instructions for US potable water samples (IDEXX Laboratories, Inc., Westbrook, U.S.).

Molecular analysis for advanced water sampling kits

All 1 L water samples were filtered through mixed-cellulose ester membranes (0.22 μm , Millipore, Billerica MA), with DNA extracted directly from filters using a FastDNA SPIN kit (MP Biomedicals, Solon OH). DNA extractions were diluted 1:5 or 1:10 with nuclease-free water for quantitative polymerase chain reaction (qPCR) to minimize potential PCR inhibition, as appropriate. Filters, DNA extracts, and diluted samples were stored at -20 °C until processed or analyzed. Gene copies of total bacteria (16S rRNA gene), *Legionella* spp. (23S rRNA gene), and *L. pneumophila* (*mip* gene) were determined by qPCR on a CFX96 Realtime System (Bio-Rad, Hercules CA). Primers, reagents, and qPCR protocols are described in detail elsewhere (Appendix A, Table SI-3). Serially-diluted standards (from 10^8 to 10^2 gene copies (gc) per reaction for 16S rRNA and from 10^6 to 5 gc per reaction for *mip* and 23S rRNA) were included in each qPCR run. The limit of quantification (LOQ) was 100 gc/reaction for total bacteria, and 5 or 10 gc/reaction for *Legionella* spp. and *L. pneumophila*. qPCR reactions for each sample, standards, and a non-template control were run in triplicate on each qPCR plate. Samples with positive amplifications in at least two of the three replicate reactions and with gene copy values above LOQ were considered quantifiable. Samples with positive amplification, but not meeting the above quantifiable criteria, were considered detectable

but below quantification limit (BQL), which is <5 or <10 gene copies/reaction. These samples were treated as half of LOQ in non-parametric analyses, while samples with no positive amplification were considered as non-detectable (ND) and treated as zero.

Statistical Analysis

Data analysis was performed in RStudio using R (version 3.4.3). Wilcoxon test was used to compare median gene copies among sample types, use of treatment and water temperature within *Legionella* optimal growth range (20 °C to 42 °C) and between detection of *Legionella* spp. or *L. pneumophila* and water quality. Spearman's rho (ρ) was used to evaluate the association between *Legionella* spp. and *L. pneumophila* quantities and water quality and system characteristics. For data sets with a high (>50%) proportion of non-detects, the Test of Equal Proportions was used.

Acknowledgements

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Conflict of Interest

No conflict of interest declared.

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Chapter 3: Occurrence of opportunistic pathogens in private wells: a three state molecular survey

Kris Mapili, Kelsey Pieper, Amy Pruden, Marc Edwards, Mary Coughter, William Rhoads

Virginia Tech, Civil and Environmental Engineering, 418 Durham Hall, Blacksburg, VA 24061

Abstract

Private wells serve as a drinking water source for 13 million households in the United States and are susceptible to microbial contamination following floods. Opportunistic pathogens (OPs) pose public health concerns in municipal drinking water systems, but their occurrence in private wells are understudied. Here, a molecular survey of OPs (*Legionella*, *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *Naegleria fowleri*, and shiga toxin-producing *Escherichia coli* DNA markers) in homes supplied by private wells in flood-impacted areas of Texas, Florida, and Louisiana following Hurricane Harvey (2017), Hurricane Irma (2017), and the Louisiana Floods (2016), was conducted. Samples were also analyzed for total coliforms, *E. coli*, and inorganic constituents, and well system characteristics were collected via a questionnaire sent to participating residents. Of 139 drinking water samples, 36.7% and 7.9% of private wells were positive for total coliform and *E. coli*, respectively, and DNA markers for genera *Legionella* and *Mycobacterium* were detected in 54.7% and 30.9% of well systems. DNA markers for *L. pneumophila* and *M. avium* were detected in 12.9% and 19.4% of all samples. *N. fowleri* DNA was detected in 8.6% of samples and no samples had detected gene markers for shiga toxin-producing *E. coli*. Total bacteria was positively associated with *Legionella* and *Mycobacterium* in all sampled wells, and total coliform was associated with *N. fowleri* in submerged wells. *Legionella* was also inversely correlated with well age in

unsubmerged wells. This work provides strong preliminary evidence that OP occurrence in private wells is relatively commonplace. Science-based options for at-risk individuals (i.e., immunocompromised) to decrease their exposure to OPs in private well water need to be developed.

Introduction

The extent to which opportunistic pathogens (OPs) are found in drinking water supplied by private wells is largely unknown. OPs are the leading cause of waterborne disease in the United States – *Legionella* (specifically *L. pneumophila*) and nontuberculous mycobacteria (specifically the nontuberculous *Mycobacterium avium* complex, with *M. avium* being the most common source of disease) can cause severe pneumonia primarily in immunocompromised individuals via inhalation or aspiration of aerosol entrained bacteria [1, 2] while *N. fowleri* can cause primary amoebic meningoencephalitis (PAM), a rare but highly lethal brain disease, via nasal aspiration [3-5]. *Legionella*, *Mycobacterium*, and *N. fowleri* have been observed to occur naturally in groundwater, as studies report that 7.7-83% samples detected at least one of these OPs [6-11]. While Legionnaires' disease (LD) has been linked to private well systems in the past, the etiology of the 64% of LD is undetermined [12] and we lack baseline incidence of *Legionella* in home plumbing systems, particularly in private well systems, to determine potential disease burden. Only one study has documented mycobacteria occurrence in private wells to our knowledge and reported 29% of samples positive from 57 homes by culture [8]. Despite this occurrence, this still has been limited direct monitoring of OPs in

private wells used for consumption, which is likely because drinking water quality in private wells is not regulated [13].

After flooding events, drinking water supplied by private wells can become contaminated with surface water contaminants [14-17]. Contamination is typically assessed only by the presence of coliform bacteria (i.e., total coliform and *E. coli*), which are indicators for surface water and fecal contamination. These bacteria do not always adequately predict other sources of microbial risks, as they have been found to sometimes be associated with OP occurrence [16] and other times not [18]. OPs are common inhabitants of drinking water systems, as *Legionella* spp. and *Mycobacterium* spp. are oligotrophic bacteria known to survive and proliferate in biofilms associated with drinking water systems [19-22]. *N. fowleri* is commonly detected in warm freshwater, and has been detected in surface water sourced drinking water distribution systems with low chlorine residuals [23]. After the Louisiana Floods of 2016, our molecular survey of drinking water from private wells reported 77.5% of homes were positive for *Legionella* spp., 15% for *L. pneumophila*, and 20% for *N. fowleri* DNA markers [16]. Without baseline OPs monitoring data in Louisiana, the source of these OPs, whether naturally occurring or introduced during flooding, was unknown.

Determining the source of OPs contamination in well water is a high priority due to the health implications associated with potential exposure and infection. While there is substantial knowledge about OPs in municipal water systems, well water studies have highlighted contradicting findings, suggesting that knowledge cannot always be extrapolated. For example, in municipal systems, it is widely recognized there is no relationship between the occurrence of *Legionella* and heterotrophic aerobic bacteria, an

indicator for total bacterial growth [24], yet a correlation between *Legionella* spp. and the total bacterial 16s rRNA gene markers was documented in private wells and associated home plumbing, suggesting that conditions that favor general bacteria are capable of supporting some OPs as well [16]. We speculate that this difference might be due a typical “water age” of years for ground water, versus hours, days or weeks for treated and disinfected municipal water, and could be limited to OPs with ecological advances for surviving in oligotrophic environments, such as the host-pathogen relationship *Legionella* has with amoeba [25, 26]. Such a relationship was not observed for *N. fowleri* [4]. In addition, water temperature is documented to strongly correlate with the incidence and levels of *Legionella* spp. and *L. pneumophila* gene markers in home plumbing served by municipals systems [27, 28]. However, temperature in drinking water from private wells was not correlated with *Legionella* [29]. Clearly, private wells may have large difference in nutrient loading, water age, and lack of disinfection residuals, which may impact the survival and growth of OPs.

As background knowledge of OP incidence in private wells is limited and studies suggest that flooding event may increasing OP loading, we conducted a multi-state well water assessment of *Legionella*, *L. pneumophila*, *Mycobacterium*, *M. avium*, and *N. fowleri*. Specifically, the objectives of this study were to (i) assess the prevalence of OPs in private wells following three severe flooding events and (ii) identify post-flood damage, water quality, and well system characteristics that increase the likelihood of detecting OPs in private wells.

Methods and Materials

Study area description

Post-flooding water quality was evaluated after three different natural disasters: (1) Great Louisiana Flood of 2016 (August 2016); (2) Hurricane Harvey (August 2017); and (3) Hurricane Irma (September 2017) (Table 1). All storms caused widespread flooding and structural damage throughout impacted counties, but there substantially higher rainfall in Texas. While percent of private wells in flood-impacted counties was relatively consistent among the states (12-16% of population), the number of well users varied considerably. Private wells sampled during our campaigns in Texas and Louisiana were likely drawing groundwater from the Coastal Lowlands aquifer system, which generally contains layers of clay, silt, sand, and gravel, but varies locally [30-32]. Private wells sampled during our campaign in Florida were either drawing from the Surficial aquifer system which is unconsolidated sand aquifer or Floridan aquifer system which is a carbonate bedrock aquifer [30].

Citizen science water sampling

Three citizen science well water testing campaigns were conducted in the aftermath of a severe rainfall event and two hurricanes. Advertisement for participation in the sampling campaigns was conducted via radio, newspaper, and local word-of-mouth through extension agencies and/or community partners. Participants picked up sampling kits provided by our research team at specified pick-up locations. Each kit included: sampling instructions (Appendix B, Section SI-1, SI-2, and SI-3), sampling bottles, and a questionnaire about the characteristics, maintenance history, and flood-induced damages of their private wells. Participants returned the sampling kits on predetermined mornings

to specified drop-off locations. Samples were collected by our research team or extension agents, packaged on ice in secondary containers, and delivered the next day to Virginia Tech for processing. Participants received water quality results via email and USPS mail, which included the detection and concentration of total coliform and *E. coli* bacteria, inorganic concentrations (e.g., lead, copper, iron), and anion concentrations (e.g., nitrates).

Table 1: Natural disaster characteristics, flood and damage characteristics, for each state.

Storm characteristics	Florida	Louisiana	Texas
Name of natural disaster	Hurricane Irma ²	Louisiana Floods	Hurricane Harvey ¹
Date of natural disaster	September 10-13, 2017	August 9–14, 2016	August 26-30, 2017
Cost of damage	\$50 billion (2017 USD) ²	\$10.7 billion (2016 USD) ⁴	\$125 billion (2017 USD) ¹
Number of counties that declared a state of emergency	67 ¹⁰	22 ⁹	60 ¹¹
Primary causes of damage	Wind, flooding, storm surge	Flooding	Flooding, storm surge
Rainfall	10-15 inches ¹	26 inches ³	60 inches ¹
Highest inundation levels	6 to 10 feet above ground level ²	6 feet above ground level	6 to 10 feet above ground level ¹
Number of private wells potentially impacted	2,460,295 private wells, 12% of impacted population ⁵	314,870 private wells, 16.1% of impacted population ⁵	870,903 residents, 12% of impacted populations ⁵
Aquifer systems	<u>Surficial aquifer system</u> ⁶ Unconsolidated; sand <u>Floridan aquifer system</u> ⁶ Bedrock with solution channels; carbonate rocks	<u>Coastal Lowlands aquifer system</u> ^{7,8} Poorly consolidated to unconsolidated; layers of clay, silt, sand, and gravel	

References: ¹[33] ²[34] ³[35] ⁴[36] ⁵[37] ⁶[30] ⁷[31] ⁸[32] ⁹[38] ¹⁰[39] ¹¹[40]

The primary focus of each sampling campaign was to determine the rates of microbial contamination in the aftermath of major flooding events. In each campaign, there were two types of sample kits provided: (1) “basic” kit assessed coliform bacteria and inorganic and anion concentrations and (2) “advanced” kits assessed coliform bacteria and inorganic and anion concentrations as well as analyzed for waterborne pathogens DNA. This study focuses exclusively on analysis from the advanced kits. More details about the results from entire sampling campaign can be found elsewhere [41]. Cold water samples were collected after 5+ minutes of flushing to represent water beyond

the home plumbing (i.e., in pressure tanks, pipes from the well the home, or in well casings depending on system size which was not documented).

In Louisiana, residents in Ascension and Livingston Parishes were recruited to participate in October 27-30, 2016 [16]. A total of 100 basic and 50 advanced kits were randomly distributed to participating residents. In the advanced kit, sequential 250 mL and 1 L samples were collected after 5 minutes of flushing. The 250 mL samples were used to measure inorganic concentrations. The 1 L sample was split upon arrival at the lab, and 100 mL was used to perform total coliform and *E. coli* culturing while the remainder was filter-concentrated for molecular detection of DNA targets.

Following Hurricanes Harvey and Irma in 2017, coolers containing sampling kits were shipped to extension offices in 10 counties in Texas and 6 in Florida. Sample collection in Texas occurred on 7 different dates between September 18 and October 11, 2017 [41]. Sample collection in Florida occurred on 6 different dates between October 9 and October 24, 2017 [42]. Each testing campaign included a mixture of basic and advanced sampling kits, which were randomly distributed to residents. In the advanced kits, a 1 L sample was collected after 5 minutes of flushing. The 1 L sample was split upon arrival at our lab – 10 mL was used to quantify inorganic concentrations, 100 mL was used to perform total coliform and *E. coli* culturing, and the remainder of the sample was filter-concentrated for molecular analysis as before. Participation in all campaigns was voluntary and all procedures were approved by Virginia Tech Institutional Review Board (#16-918).

Water quality analysis

Aliquots and the 250 mL samples were acidified with 2% nitric acid and digested for a minimum of 16 hours prior to analysis using inductively coupled plasma-mass spectrometry (ICP-MS) per methods 3030D and 3125 B. Blanks and/or spikes of known concentrations were processed every 10 samples for QA/QC purposes. The minimum reporting levels were 0.5 µg/L for arsenic; 1.0 µg/L for cadmium, chromium, lead, silver, copper, and manganese; 5 µg/L for zinc; 10 µg/L for iron, chloride, sulfate, and nitrate; and 50 µg/L for sodium, . Total coliform and *E. coli* were quantified using the IDEXX Colilert 2000 method (Westbrook, MN), with a detection limit of 1.01 MPN/100 mL.

Molecular analysis

All 1 L water samples were filtered through mixed-cellulose ester membranes (0.22µm, Millipore, Billerica MA), with DNA extracted directly from filters using a FastDNA SPIN kit (MP Biomedicals, Solon OH). DNA extractions were diluted 1:5 or 1:10 with nuclease-free water for quantitative polymerase chain reaction (qPCR) to minimize PCR inhibition, as appropriate. Filters, DNA extracts, and diluted samples were stored at -20 °C until processed or analyzed. Gene copy numbers of total bacteria (16S rRNA gene), *Legionella* spp. (23S rRNA gene), *L. pneumophila* (*mip* gene), *Mycobacterium* spp. (16S rRNA gene), *M. avium* (16S rRNA gene), and *N. fowleri* (ITS) were determined by qPCR. Detection of shiga toxin-producing *E. coli* (*stx1* and *stx2* genes) were determined using PCR. Primers, reagents, qPCR and PCR protocols are described in detail elsewhere (Appendix B, Table SI-1) [43-48]. Diluted standards (from

10⁸ to 10² gene copies (gc) per reaction for 16S rRNA and from 10⁶ to 5 gc per reaction for OPs were included in each qPCR run. The limit of quantification (LOQ) was 100 gc/reaction for total bacteria, 10 gc/mL for *Legionella* spp. and *L. pneumophila*, and 10 or 50 gc/mL for *Mycobacterium* spp., *M. avium*, and *N. fowleri*. The LOQ was applied on each qPCR run, as appropriate. qPCR reactions for each sample, standards, and a non-template control were run in triplicate on each qPCR plate. Samples with positive amplifications in at least two of the three replicate reactions and with gene copy values above LOQ were considered quantifiable. Samples with positive amplification, but not meeting the above quantifiable criteria, were considered detectable, but below quantification limit (BQL). These samples were treated as half of LOQ in non-parametric analyses, while samples with no positive amplification were considered as non-detectable (ND) and treated as zero.

Data analysis

Data analysis was performed in RStudio using R (version 3.4.3). Inorganic concentrations below the MRL were treated as half of the MRL and gene copy numbers were log₁₀-transformed for non-parametric analyses. The Wilcoxon and Kruskal-Wallis Tests were used to determine differences water quality between two or more groups. Where Kruskal-Wallis indicated a difference, the Dunn's test with Bonferroni correction was used. Spearman and Kendall correlations were used to determine relationships among water quality parameters. For water quality parameters with a high (>50%) proportion of non-detects, the Test of Equal Proportions was used.

Results and Discussion

Characteristics of private wells surveyed

The questionnaire was used to document private well design, maintenance, and operation characteristics that may have impacted well water quality after the flooding events (Table 2). Of the private wells sampled, 65.8-80.3% of reported wells were drilled, median well depth was 147.5-400 feet, and median year of construction was 1995-2002. However, wellhead submersion, a potentially primary route for well water contamination, was elevated in Texas (41.0%) compared to Florida (22.5%) and Louisiana (7.9%). Higher rates of wellhead submersion were not associated with more system damage. For instance, 31.6% residents in Louisiana reported a system damage compared to 26.2% in Texas. However, this finding may be attributed to the types of flooding events examined in this study. The most common type of system damage was electrical damage (n=15 of 127, 11.8%) or damage to pump (n=11 of 127, 8.7%), suggesting a primary barrier to well water recovery was re-instating the ability to supply groundwater to the home plumbing system. Shock chlorination (i.e., dosing and recirculating high concentrations of chlorine in private wells to achieve specified log-reduction of waterborne pathogens) is a primary remediation recommended after flooding. More than a third of residents in Texas (36.1%) shocked chlorinated their system after the storm compared to 7.9% in Louisiana and 10% in Florida.

Occurrence of total bacterial genes, indicator bacteria, and OP genes

A total of 139 samples were collected from private wells in Texas (n=61), Florida (n=40), and Louisiana (n=38) following flooding caused by Hurricane Harvey in 2017, Hurricane Irma in 2017, and the Great Louisiana Flood in 2016. To quantify the

Table 2. Summary of sampled well system characteristics in each state.

	Florida	Texas	Louisiana
Number of days after storm samples were collected	32-38	24-34	73-76
Number of samples analyzed for this study	40	61	38
Well type , n, % of total samples			
Drilled	32 (80%)	49 (80.3%)	25 (65.8%)
Dug or bored	1 (2.5%)	0 (0%)	2 (5.3%)
Don't know or not reported	7 (17.5%)	12 (19.7%)	11 (28.9%)
Well depth , feet			
n reported, % of total samples	18 (45%)	43 (70.5%)	21 (55.3%)
Median	147.5	200	400
Range	35-300	30-650	25-2300
Year constructed			
n reported, % of total samples	25 (62.5%)	41 (67.2%)	21 (55.2%)
Median	2002	2000	1995
Range	1955-2015	1965-2017	1951-2015
Submerged , n, % of total samples			
Yes	9 (22.5%) ^a	25 (41.0%) ^a	3 (7.9%) ^b
No	24 (60%)	26 (42.6%)	12 (31.6%)
Don't know or not reported	7 (17.5%)	10 (16.4%)	23 (60.5%)
Damaged , n, % of total samples			
Yes	5 (12.5%)	16 (26.2%)	12 (31.6%)
Electrical damage	5 (12.5%)	8 (13.1%)	2 (5.3%)
Pump damage	2 (5.0%)	4 (6.6%)	5 (13.2%)
Pipe damage	2 (5.0%)	3 (4.9%)	1 (2.6%)
Casing damage	0 (0%)	2 (3.3%)	NA
Cover damage	0 (0%)	1 (1.6%)	NA
No	30 (75%)	42 (68.8%)	21 (55.3%)
Don't know or not reported	5 (12.5%)	3 (4.9%)	5 (13.2%)
Shock chlorinated , n, % of total samples			
Yes	4 (10%)	22 (36.1%)	3 (7.9%)
No	28 (70%)	33 (54.1%)	35 (92.1%)
Don't know or not reported	8 (20%)	6 (9.8%)	0 (0%)

^aCheckbox survey question (yes, no, or don't know)

^bFree text survey question

NA: Not Applicable. This question was not included in the Louisiana questionnaire

microbial water quality of private well systems, total coliform and *E. coli* were measured using IDEXX Colilert with most probable number (MPN) quantification estimated.

Samples were also analyzed using qPCR to quantify gene targets associated with total bacteria (universal 16S rRNA gene marker), *Legionella* spp. (23S rRNA gene), *L. pneumophila* (*mip* gene), *Mycobacterium* spp. (16S rRNA gene), *M. avium* (16S rRNA gene), and *N. fowleri* internal transcribed spacer region (ITS).

Total coliform and *E. coli* monitoring have historically been used as an indicator for well water contamination [15, 49]. More than a third of wells tested (36.7%; 51 of 139 samples) were positive for total coliform, with quantifiable samples ranging from 1.00 to 1.01×10^3 MPN/100 mL. *E. coli* was detected in 11 of the 51 samples (21.6%) that were positive for total coliform and 7.9% of all samples, with quantifiable samples ranging from 1.00 MPN/100 mL to 7.76×10^1 MPN/100 mL. There were no differences in total coliform (Kruskal $p=0.49$) or *E. coli* (Kruskal $p=0.11$) incidence by state, potentially due to limited number of positive samples in Florida and Louisiana. Prior work has shown that total coliform and *E. coli* positivity rates can vary widely, even in the absence of a flooding event [17, 49-51]. State-level results in this study were not elevated in comparison to contamination rates reported in other states (total coliform: 14.6-46% positive; *E. coli*: 1.5-14%).

Total bacteria loading varied among the three states (Figure 1; Kruskal-Wallis, $p=0.00014$), ranging from 2.1×10^2 to 2.47×10^7 gc/mL (Table 3, Appendix B Figure SI-1). Florida had the highest level of total bacterial gene numbers with a median level of 5.99×10^5 gc/mL, which was approximately one order of magnitude higher than Texas or Louisiana. Measured total bacteria in the sampled areas in this study was up to approximately two orders of magnitude higher than total bacteria in flushed cold water samples collected from 29 private wells in Wake County, North Carolina in a non-flood scenario (median = 7.38×10^3 gc/mL) [29]. The differences in measured total bacteria may be due to environmental differences between each geographic location.

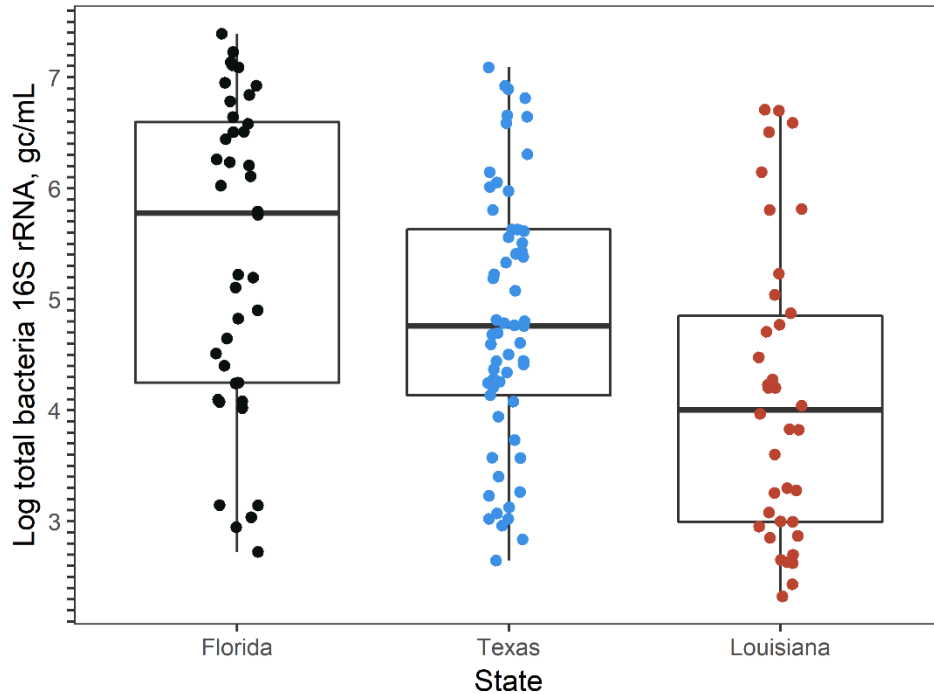


Figure 1: Boxplots of total bacteria (16S rRNA) for all samples in Florida (n=40), Texas (n=61), and Louisiana (n=38). Boxplots represent the interquartile range (IQR), whiskers extend to median $\pm 1.5 \times \text{IQR}$. 16S rRNA genes in private wells were significantly different among the three sampled locations (Kruskal-Wallis, $p=0.00014$).

Legionella spp. were the most commonly detected OP genus. Overall, 76 of 139 samples (54.7%) were positive for *Legionella* spp., with quantifiable samples ranging from 8.17 to 1.62×10^4 gene copies/mL. *L. pneumophila*, the most commonly reported pathogenic species of the genus *Legionella*, was detected in 18 of the 76 samples that were positive for *Legionella* spp. (23.7%) and 12.9% of all samples. Samples with quantifiable *L. pneumophila* ranged from 6.19 to 1.08×10^2 gene copies/mL. There were no differences in *Legionella* spp. (Kruskal Wallis, $p=0.24$) or *L. pneumophila* (Test of proportions, $p=0.48$) by state. The detection of *Legionella* spp. was similar to positivity rates found in other studies. For example, a survey of two chloraminated drinking water systems observed that 30-82% of samples were positive for *Legionella* spp. In this study, *L. pneumophila* was not the dominant species of *Legionella* detected, as *L. pneumophila*

represented less than 3% of the *Legionella* spp. detected in 90% of all samples collected. This is similar to studies conducted in municipal systems, where *L. pneumophila* accounted for 0.1-1.0% of the total *Legionella* spp. detected [52, 53], though there are also examples where *L. pneumophila* was the dominant species [54-57]. In groundwater supplies, the fraction of *L. pneumophila* may vary geographically, as *L. pneumophila* was reported the dominant *Legionella* spp. in one location but was not detected in another location, though both locations were sampled from the same geology [7]. Although there has been extensive focus on *L. pneumophila*, other species of *Legionella*, such as *L. longbeachae*, *L. micdadei*, *L. bozemanii*, and *L. dumooffii* are documented human pathogens [58]. Assessing which species of *Legionella* are most prevalent in groundwater supplies may need to be assessed on local basis.

Mycobacterium spp. was the next most frequently detected, as it was detected in 43 of 139 samples (30.9%). Samples with quantifiable *Mycobacterium* spp. ranged from 13.3 to 3.03×10^3 gene copies/mL. *M. avium*, the most common cause of MAC infections in immunocompromised individuals [1], was detected in 27 of the 43 samples (62.8%) positive for *Mycobacterium* spp. and 19.4% of all samples. However, all *M. avium* samples were all BQL. There were no differences in the levels of *Mycobacterium* spp. among states (Kruskal Wallis, $p=0.031$; Dunn Test with Bonferroni correction, $p=0.056$ -1). The levels of *Mycobacterium* spp. detected in this study were similar to reported levels in flushed samples collected in chloraminated municipal drinking water systems (e.g., $15\text{-}3 \times 10^3$ gene copies/mL; Wang et al., 2012), but incidence was lower than in homes supplied by untreated groundwater wells on an Indian Reservation in the United States (e.g., 29.3% of 41 samples by culture) [8]. *M. avium* dominated the

Mycobacterium genus in approximately 20% of samples collected in this study. Although *M. avium* is documented to be the most common species associated with MAC infections in immunocompromised individuals, there are other pathogenic nontuberculous mycobacteria, such as *M. intracellulare*, *M. kansasii*, *M. abscessus*, and *M. chelonae* [19, 59-62]. Again, assessing which are most prevalent species in groundwater supplies may need to be assessed on local basis.

The incidence of *N. fowleri* was low, with detection in only 12 of 139 samples (8.6%). Two samples had quantifiable levels of *N. fowleri* at 20.1 and 2.51×10^2 gc/mL. There was no statistically significant difference in the detection of these pathogens by state (Test of proportions, $p=0.25$). *N. fowleri* has been linked to several public water utility supplies, including fatal cases of PAM [63, 64], and has been detected wells used as a public drinking water supply [9, 18, 65]. However, very little is known about its occurrence in private wells. Our comprehensive survey of post-flood samples in Louisiana that included additional samples taken from within the home plumbing systems indicated that *N. fowleri* DNA is detected in 20% of homes [16]. Given that 12.7% of flushed cold water samples were positive in Louisiana (Table 3, Appendix B Figure SI-1), it is likely the positivity reported in Texas and Florida was underestimated.

Table 3: Detection and quantification rates of total bacteria, *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, and *N. fowleri* genes in all samples.

	Florida	Texas	Louisiana
Total bacteria (16S rRNA)	n=40	n=61	n=38
Detectable	40 (100%)	61 (100%)	38 (100%)
BQL	0 (0.0%)	0 (0.0%)	0 (0.0%)
Quantifiable	40 (100%)	61 (100%)	38 (100%)
Below Detection	0 (0.0%)	0 (0.0%)	0 (0.0%)
Range	$5.30 \times 10^2 - 2.47 \times 10^7$ gc/mL	$4.42 \times 10^2 - 1.23 \times 10^7$ gc/mL	$2.1 \times 10^2 - 3.90 \times 10^6$ gc/mL
Median	5.99×10^5 gc/mL	5.77×10^4 gc/mL	3.86×10^4 gc/mL
<i>Legionella</i> spp. (23s RNA)	n=40	n=61	n=38
Detectable	25 (62.5%)	32 (52.4%)	19 (50.0%)
BQL	7 (17.5%)	7 (11.5%)	8 (21.0%)
Quantifiable	18 (45.0%)	25 (41.0%)	11 (28.9%)
Below Detection	15 (37.5%)	29 (47.5%)	19 (50.0%)
Range	ND – 1.28×10^4 gc/mL	ND – 1.62×10^4 gc/mL	ND - 9.10×10^3 gc/mL
Median	BQL	BQL	BQL
<i>L. pneumophila</i> (mip)	n=40	n=61	n=38
Detectable	7 (17.5%)	8 (13.1%)	3 (7.9%)
BQL	6 (15.0%)	3 (4.9%)	3 (7.9%)
Quantifiable	1 (2.5%)	5 (8.2%)	0 (0.0%)
Below Detection	33 (82.5%)	53 (86.9%)	35 (92.1%)
Range	ND – 50.8 gc/mL	ND – 1.08×10^2 gc/mL	ND - BQL gc/mL
Median	ND	ND	ND
<i>Mycobacterium</i> spp. (16S rRNA)	n=40	n=61	n=38
Detectable	18 (45.0%)	20 (31.7%)	5 (13.2%)
BQL	14 (35.0%)	5 (8.2%)	1 (2.6%)

Quantifiable	4 (10.0%)	15 (24.6%)	4 (10.5%)
Below Detection	22 (55.0%)	41 (67.2%)	33 (86.8%)
Range	ND – 1.32×10^2 gc/mL	ND – 3.03×10^3 gc/mL	ND - 5.62×10^2 gc/mL
Median	ND	ND	ND
<i>M. avium</i> (16S rRNA)	n=40	n=61	n=38
Detectable	13 (32.5%)	11 (18.0%)	3 (7.9%)
BQL	13 (32.5%)	11 (18.0%)	3 (7.9%)
Quantifiable	0 (0.0%)	0 (0.0%)	0 (0.0%)
Below Detection	27 (67.5%)	50 (82.0%)	35 (92.1%)
Range	ND – BQL gc/mL	ND – BQL gc/mL	ND - BQL gc/mL
Median	ND	ND	ND
<i>N. fowleri</i> (ITS)	n=40	n=61	n=38
Detectable	2 (5.0%)	8 (12.7%)	2 (5.2%)
BQL	1 (2.5%)	8 (12.7%)	1 (2.6%)
Quantifiable	1 (2.5%)	0 (0.0%)	1 (2.6%)
Below Detection	38 (95%)	53 (86.9%)	36 (94.7%)
Range	ND – BQL gc/mL	ND – BQL gc/mL	ND – 2.51×10^2 gc/mL
Median	ND	ND	ND

ND=not detected

BQL=detected, but below quantification limit

Association between total bacterial genes and OP genes

Our previous comprehensive study in Louisiana documented a correlation between total bacteria and *Legionella* spp. gene numbers across all sample types, including stagnant and flushed samples collected from within home plumbing. We confirmed this trend in Texas and Florida flushed cold water. Total bacteria were positively correlated with *Legionella* spp., across all states (Spearman, $p < 2.2 \times 10^{-16}$, $\rho = 0.72$) and within each state (Texas: $\rho = 0.67$; Florida: $\rho = 0.77$; Louisiana: $\rho = 0.74$; Figure 2a). Similarly, total bacteria were correlated with *Mycobacterium* spp. across all states ($\rho = 0.41$) and within each state (Texas: $\rho = 0.48$; Florida: $\rho = 0.32$; Louisiana: $\rho = 0.56$; Figure 2b). Lower detection rates of *L. pneumophila* and *M. avium* prevented similar statistical comparisons. However, total bacteria gene numbers were elevated in both *L. pneumophila* positive (Wilcoxon, $p = 0.0018$; Figure 2c) and *M. avium* positive samples ($p = 6.52 \times 10^{-8}$; Figure 2d). No correlation or trend was observed between total bacteria and *N. fowleri* ($p = 0.33$; data not shown).

This observed relationship is contrary to observations from field work in municipal systems with a secondary disinfectant residual, wherein *Legionella* occurs independently of total bacterial numbers or heterotrophic plate counts [24]. It is well-documented that *Legionella* can resist chemical disinfectants [66, 67], while the majority of microbial members of total bacteria may be more susceptible to residual disinfection. In private wells, continuous disinfectant practices are rare [51, 68]. Therefore, conditions that supports the growth of total bacteria may also support the growth of OPs, particularly in systems where OPs are integral members of the background microbial ecology in groundwater supplies. In a simulated drinking water system with no secondary

disinfectant residual, rapid development of biomass was found to result elevated concentrations of *Legionella* [69]. Furthermore, a strong association between HPC bacteria and *Legionella* was observed in a municipal system that did not have a disinfectant residual [54]. These findings are in support of our comprehensive study in Louisiana, which suggested that areas in private wells without continuous disinfection that may support the growth of total bacteria may also support the growth of *Legionella* in private well water [16], and extends this finding to the presence of *Mycobacterium* spp.

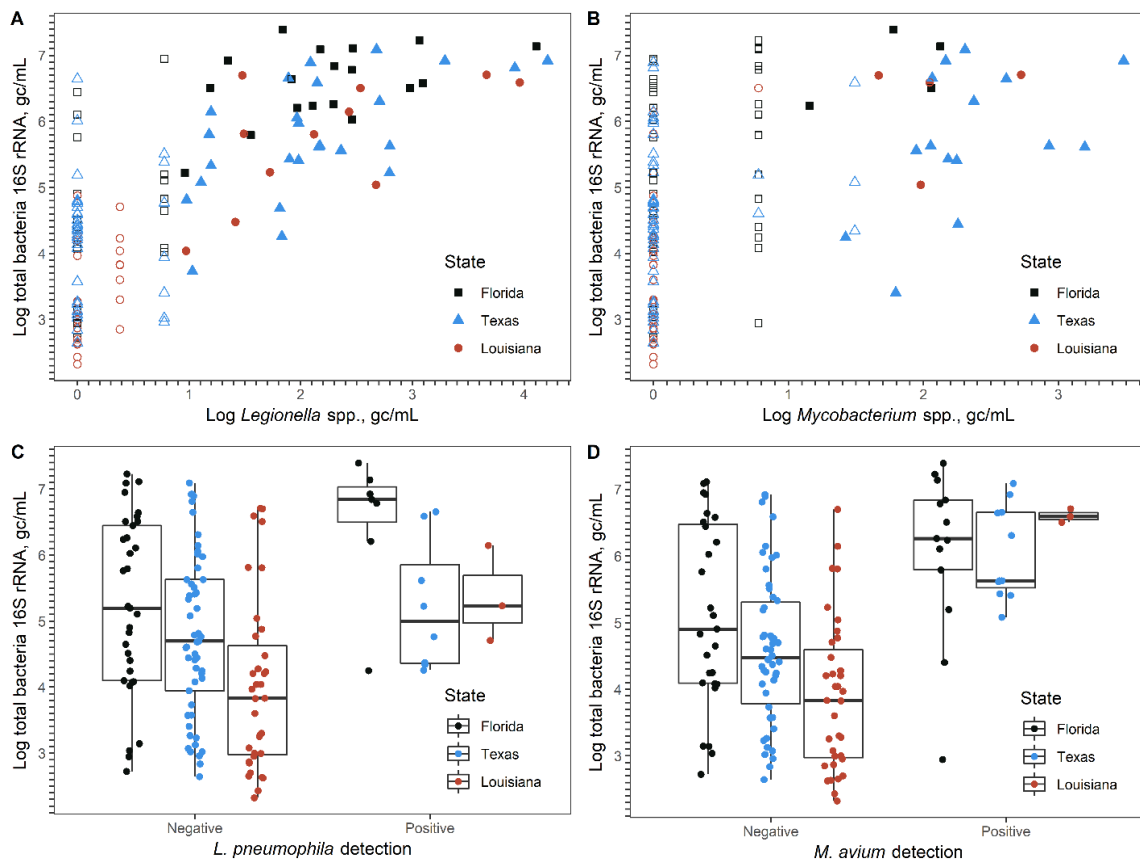


Figure 2. Positive correlations between total bacteria and A) *Legionella* spp. and B) *Mycobacterium* spp., and boxplot of total bacteria within samples that were positive and negative for C) *L. pneumophila* and D) *M. avium*. Quantifiable data represented with a filled-in point in scatter plots while BQL reported as half the LOQ and ND reported as 0 gc/mL in boxplots.

Impacts of wellhead submersion and well system damage

Private wells that reported having submerged wellheads during the flooding event tended to have an increased detection of surface water-associated contamination. Higher levels of total coliform rates (Test of Proportions, $p=0.02$), and *N. fowleri* detection rates ($p=0.01$) were detected in submerged compared to unsubmerged wells (Table 4). This trend appeared to be driven by the incidence of wellhead submersion in Texas, where the highest proportion of wells sampled were flooded (Table 1). Total bacteria gene numbers and detection of total coliform and *N. fowleri* were higher in submerged compared to unsubmerged in Texas ($p_{\text{total bacteria}}=0.01$; $p_{\text{total coliform}}=0.01$; $p_{N.fowleri}=0.046$), but not in Florida or Louisiana ($p=0.60-1.0$). This may be due to the lower rates of wellhead submersion in Florida and Louisiana (Table 2) as well as the longer lag time between flooding and sampling in Louisiana (Table 1).

While there is an increased rate of microbial contamination in flooded private wells, studies have traditionally focused primarily on indicator bacteria [14, 15, 70]. Given that *N. fowleri* is a thermophilic amoeba that is historically a public health concern in recreational surface waters [71], it is logical that submerged wells during flooding events would be at higher risk of being contaminated, similar to established knowledge of increases in total coliform and *E. coli* contamination discussed above [15, 17]. This is the first result to our knowledge that indicates that submersion of private wellheads may be associated with contamination of *N. fowleri* and deserves further scrutiny.

Reported damage to the well system was not significantly associated with higher total bacteria gene numbers or detection rates of total coliforms or *N. fowleri* ($p=0.12-1.0$). The two highest reported types of damage to well systems were electrical and pump-

related. This suggests that a primary barrier to well water recovery was the functionality of the system to deliver groundwater to the home. This type of damage would not be expected to be a source of surface water contamination and explains why reported well damage was not be associated with surface water related contamination. Thus, submersion of wellheads was a better indicator of surface water contamination than damage to the well system.

There was no relationship between submerged wellheads and the levels or detection of non-surface water associated OPs. The detection of *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., and *M. avium* were not different between submerged and unsubmerged wells overall (Table 4; Kruskal Wallis, $p=0.29-0.66$) or within each state ($p=0.48-1.0$). In keeping, there were no differences in the detection of *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., or *M. avium*, between damaged and undamaged well systems overall ($p=0.44-1.0$) or within each state ($p=0.24-1.0$). Both *Legionella* and *Mycobacterium* are commonly found in all aquatic environments [55, 72-74], including groundwater [6, 8, 75-77]. Therefore, detection of these genes may represent background detection rates. However, as OPs monitoring in private well is limited, there is no baseline data to confirm this. As such, there was also no significant difference in the detection frequency of *Legionella* or *Mycobacterium* in samples that were total coliform positive compared to total coliform negative ($p=0.57-1.0$).

Well system characteristics relationship with OP genes

Various characteristics of the well design and construction practices did not appear to have influenced the likelihood of detecting waterborne pathogens. Well depths

were similar among the three states and were not linked to the incidence of OPs in private wells (Table 2). *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium* and *N. fowleri* were not correlated with well depth overall (Spearman's, $p=0.51-0.98$) or in each individual state ($p=0.15-0.91$). Overall, *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *N. fowleri*, and total bacteria were not correlated to well construction year overall ($p=0.10-0.65$; *Legionella* spp. reported in Figure 3a) or in submerged wells ($p=0.32-0.73$). However, in unsubmerged wells, *Legionella* spp. was correlated with well construction year across all three states ($p=0.0030$, $\rho=0.46$, $n=40$; Figure 3b), as well as within each state with enough samples to evaluate trends in Texas ($n=17$, $p=0.014$, $\rho=0.58$; Appendix B Figure SI-2) and Florida ($n=15$, $p=0.025$, $\rho=0.45$; Appendix B Figure SI-2). This means that newer wells tended to have higher levels of *Legionella* spp. This trend conflicts with the traditional wisdom in municipal systems with secondary disinfection, wherein older buildings and homes are more frequently associated with the presence of *Legionella* [78]. Thus, results from this limited sampling highlight that further research into *Legionella* spp. in private wells is warranted to understand populations potentially at risk.

Inorganics relationship with OP genes

There were no convincing associations between inorganics and OPs in this study. The concentration of some inorganics (i.e., arsenic, iron, manganese, sodium, chloride) varied among the three states (Kruskal-Wallis, $p=3.02\times 10^{-11}$ - 0.023; Table 5), and some inorganics varied between the Texas and Louisiana sampling locations (i.e., hardness, manganese) likely due to local variation the Coastal Lowlands aquifer system (Kruskal Wallis, $p=1.34\times 10^{-6}$ and 4.72×10^{-3}) [30-32]. Moreover, within the state datasets,

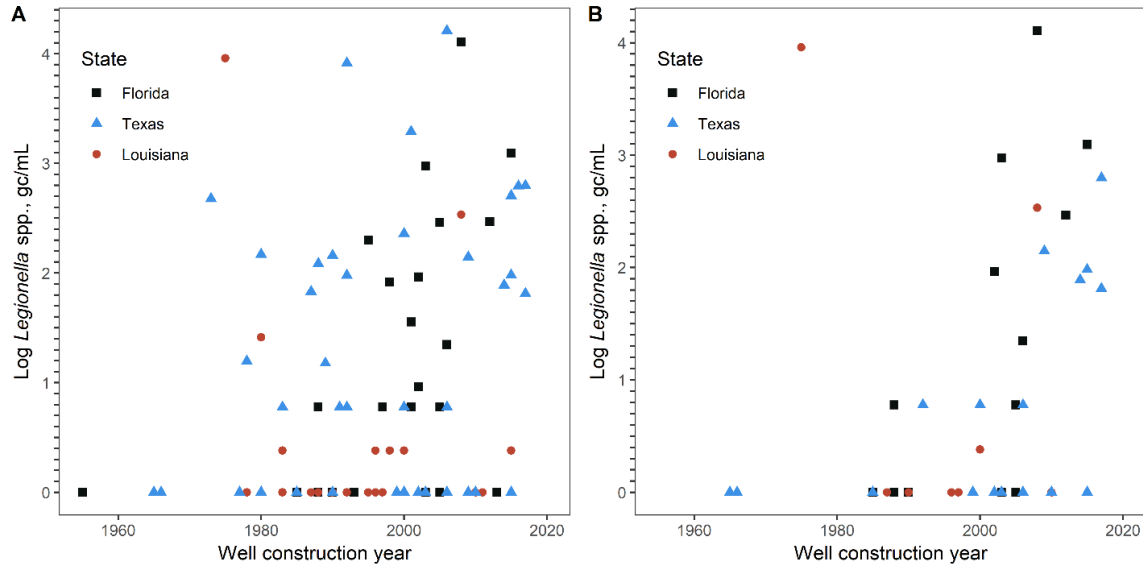


Figure 3. Relationship between *Legionella* spp. and well construction year in A) all well samples and B) in only unsubmerged wells. There is no significant correlation between *Legionella* spp. and well construction year when all samples are accounted for, but there is a significant positive correlation between *Legionella* spp. and well construction year in all unsubmerged wells as well as in unsubmerged wells Texas and Florida.

associations were found between some OPs and inorganics. For example, in Florida samples, sulfate, copper, and nitrate were significantly higher in samples with detected *Legionella* spp. than in samples without detected *Legionella* spp. (Wilcoxon, $p=0.027-4.76 \times 10^{-5}$), and iron was significantly lower in samples with detected *Legionella* spp. than in samples without detected *Legionella* spp. ($p=0.029$). However, the detection of OPs overall were sporadic and there were no overarching patterns or associations between inorganics and OPs.

Impact of shock chlorination

Shock chlorination did not appear to impact the occurrence of OPs and total bacteria in this study. Accounting for all samples, 29 well users reported shock chlorinating their well and 98 reported not shock chlorinating their well after the flooding

event. Total bacteria numbers were not significantly different between wells that reported shock chlorinating and those that did not (Wilcoxon, $p=0.17$). The proportions of detection for all investigated OPs were not significantly different between shock chlorinated wells and non-shock chlorinated wells (Test of proportions, $p=0.22-1.0$). There are numerous concerns associated with shock chlorination practices by well users and research is needed to explore the impact of shock chlorination on the presence of OPs in private wells [79]. Prior work in large buildings systems that shock chlorinate their plumbing systems after incidence of Legionnaires' disease associated with their plumbing system has been demonstrated to be ineffective [80]. Often, the same *Legionella* strain that was present before the shock chlorination reemerges several weeks afterwards [80]. Therefore, it would not be expected that shock chlorination is an effective remediation strategy unless it can be confirmed *Legionella* or *Mycobacterium* do not naturally occur in the groundwater.

Implications for private well stewardship practices

Overall, the risk for infections caused by OPs in private wells is not clear. While submersion of private wellheads may be associated with contamination of *N. fowleri*, the risk of infection following storms may still be low because contamination only leads to exposure if the residents are using the water, and because the exposure pathway requires the organism to forcefully enter the nasal passages (e.g., getting water up the nose in recreational waters or improper use of a neti pot). The risk of PAM could be elevated during summer months or in geographic areas with warmer climates as *N. fowleri* is a warm freshwater pathogen [64], or through bathing or swimming in pools that use

contaminated groundwater [18]. About 96% of Legionnaires' Disease cases are sporadic (i.e. non-outbreak related) [81] and there are limited data on *Legionella* in private wells to assess private wells as a source for Legionnaires' Disease. The risk for *Legionella* in homes supplied by private wells may be lower than the risk associated with larger buildings due to the relatively simpler building structure, but impact of the lack of exposure to chlorine may be a major contributing factor that offsets the potential benefits of smaller, simpler plumbing systems. *L. pneumophila* was detected in 12.9% of homes in this study, less than what was reported in a nationwide survey of taps in which most of the sampling sites were larger buildings (47% of 68 sites) [82]. The previous study in Louisiana found that positive detection and higher levels of *Legionella* spp. and total bacteria in well columns were more likely to yield detectable and higher levels of *Legionella* spp. and total bacteria at taps [16], and the results of this study suggest that wellhead submersion may not be a route of contamination for *Legionella* spp. in private wells. Thus, it is likely that the risk for *Legionella* in private wells is based on more background occurrence in groundwater than flood impacts. The risk for infections caused by *M. avium* complex and other species of *Mycobacterium* associated with private wells following floods are unknown. Only one study to our knowledge has surveyed *Mycobacterium* spp. background levels in groundwater wells, reporting 12 of 41 (29.3%) samples from homes supplied by untreated groundwater wells positive for culturable *Mycobacterium* spp. [8]. *M. avium* numbers were found to be correlated with turbidity in raw source waters for drinking water distribution systems, with one raw water sample having increased turbidity due to heavy rains and flooding yielding *M. avium* [19], so it is possible that mycobacteria incidence increases in systems with submerged wellheads.

Mycobacterium spp. are also known to be resistant to chlorine [83]. Therefore, shock chlorination may not be effective in treating private well water against opportunistic pathogens, especially following floods. More research is necessary to determine effective and continuous treatment options for well users to treat against opportunistic pathogens in private wells.

Limitations

The timing of the sampling campaign may have influenced the prevalence of OPs detected in private wells, and thus the results presented in this study may be an underestimation of exposure risk immediately following the storm. Sampling occurred 24-34 days after Hurricane Harvey in Texas and 32-38 days after Hurricane Irma in Florida, which were near the time when residents were able to return home. Sampling in Louisiana occurred 73-76 days after the storm, which was 9-11 weeks since residents may have returned home. While inactivation rate models have not yet been developed for the investigated OPs in private wells, one model that predicts the inactivation rate of *E. coli* in well water [84] suggests that the highest level of *E. coli* in the present study (776 MPN/L) measured 34 days following the flood could have been up to 152,000 MPN/L one day following the storm if water was completely stagnant before measurement, accounting for natural attenuation with time. The immediate risks of exposure to OPs in flood-impacted private wells are not well characterized, as baseline data regarding the prevalence of OPs in well water was not available for before the storm, rendering it impossible to differentiate the impact of the storm compared to normal conditions.

This survey relied heavily on molecular detection of OPs. Molecular detection of any target microorganism includes detection of live and dead cells. Culture methods of

the investigated pathogens were not undertaken in this study and thus it is not possible to assess the risk of infection caused by live pathogens to private well users following storm events. Thus, the overall detection rate of DNA markers for pathogens in this study is likely an overestimation of viable and infectious pathogens.

The limited sample size in this study prevented the development of conclusions regarding some risk factors of OPs in private wells following flooding events. The proportion of submerged wells reported was much higher in Texas than in Florida and in Louisiana. While the suggestion of wellhead submersion potentially introducing contaminants into wells, this finding could not be extended to wells in Florida and in Louisiana. Known wellhead submersion may also have been underreported in Louisiana due delay in sampling and to the self-reporting bias – the questionnaire sent to participating residents in Texas and Florida explicitly asked residents if their wells were submerged whereas the questionnaire sent to residents in Louisiana did not. The known specific types of damage to well systems were also limited in sample size, limiting conclusions on how specific types of damage to well systems could impact the presence of OPs in private wells following floods.

Table 4: Summary of total bacteria, OPs, and indicator bacteria in submerged and unsubmerged wells in each state.

	Florida (n=33)		Texas (n=51)		Louisiana (n=21)	
	Submerged (n=9) n (% of 9 samples)	Unsubmerged (n=24) n (% of 24 samples)	Submerged (n=25) n (% of 25 samples)	Unsubmerged (n=26) n (% of 26 samples)	Submerged (n=3) n (% of 3 samples)	Unsubmerged (n=12) n (% of 12 samples)
Total bacteria (16s rRNA)						
Detectable	9 (100%)	24 (100%)	25 (100%)	26 (100%)	3 (100%)	12 (100%)
BQL	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Quantifiable	9 (100%)	24 (100%)	25 (100%)	26 (100%)	3 (100%)	12 (100%)
Below Detection	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Range	884 - 1.23×10 ⁷ gc/mL	530 - 2.47×10 ⁷ gc/mL	442 - 8.35×10 ⁶ gc/mL	1.05×10 ³ - 4.53×10 ⁶ gc/mL	740 - 5.00×10 ⁶ gc/mL	210 - 3.90×10 ⁶ gc/mL
Median	6.19×10 ⁵ gc/mL	1.09×10 ⁶ gc/mL	3.64×10 ⁵ gc/mL	3.41×10 ⁴ gc/mL	1.20×10 ³ gc/mL	3.50×10 ⁴ gc/mL
Legionella spp. (23S rRNA)						
Detectable	6 (66.7%)	14 (58.3%)	15 (60%)	10 (38.5%)	1 (33.3%)	7 (58.3%)
BQL	3 (33.3%)	3 (12.5%)	1 (4.0%)	5 (19.2%)	0 (0%)	2 (16.7%)
Quantifiable	3 (33.3%)	11 (45.8%)	14 (56%)	5 (19.2%)	1 (33.3%)	5 (41.7%)
Below Detection	3 (33.3%)	10 (41.7%)	10 (40%)	16 (61.5%)	2 (66.7%)	5 (41.7%)
Range	ND - 2.88×10 ² gc/mL	ND - 1.28×10 ⁴ gc/mL	ND - 1.62×10 ⁴ gc/mL	ND - 6.26×10 ² gc/mL	ND - 29.1 gc/mL	BQL - 9.10×10 ³ gc/mL
Median	BQL	BQL	1.41×10 ¹ gc/mL	ND	ND	BQL
L. pneumophila (mip)						
Detectable	0 (0%)	4 (16.7%)	2 (8.0%)	4 (15.4%)	0 (0%)	1 (8.3%)
BQL	0 (0%)	3 (12.5%)	2 (8.0%)	1 (3.8%)	0 (0%)	1 (8.3%)
Quantifiable	0 (0%)	1 (4.2%)	0 (0%)	3 (11.5%)	0 (0%)	0 (0%)
Below Detection	9 (100%)	20 (83.3%)	23 (92%)	22 (84.6%)	3 (100%)	11 (91.7%)
Range	all ND	ND - 5.08×10 ¹ gc/mL	ND - BQL	ND - 1.08×10 ² gc/mL	all ND	ND - BQL
Median	ND	ND	ND	ND	ND	ND
Mycobacterium spp. (16S rRNA)						
Detectable	6 (66.7%)	10 (41.7%)	9 (36%)	10 (38.5%)	1 (33.3%)	2 (16.7%)
BQL	6 (66.7%)	6 (25%)	14 (56%)	4 (15.4%)	0 (0%)	1 (8.3%)
Quantifiable	0 (0%)	4 (16.7%)	8 (32%)	6 (23.1%)	1 (33.3%)	1 (8.3%)
Below Detection	3 (33.3%)	14 (58.3%)	16 (64%)	16 (61.5%)	2	10 (83.3%)
Range	ND - BQL	ND - 1.32×10 ² gc/mL	ND - 3.03×10 ³ gc/mL	ND - 8.49×10 ² gc/mL	ND - 4.55×10 ¹ gc/mL	ND - 110 gc/mL
Median	BQL	ND	ND	ND	ND	ND
M. avium (16S rRNA)						
Detectable	3 (33.3%)	8 (33.3%)	7 (28%)	3 (11.5%)	0 (0%)	2 (16.7%)

BQL	3 (33.3%)	8 (33.3%)	7 (28%)	3 (11.5%)	0 (0%)	2 (16.7%)
Quantifiable	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Below Detection	6 (66.7%)	16 (66.7%)	18 (72%)	23 (88.5%)	3 (100%)	10 (83.3%)
Range	ND - BQL	ND - BQL	ND - BQL	ND - BQL	all ND	ND - BQL
Median	ND	ND	ND	ND	ND	ND
<i>N. fowleri</i> (ITS)						
Detectable	1 (11.1%)	0 (0%)	6 (24%)	1 (3.8%)	0 (0%)	1 (8.3%)
BQL	1 (11.1%)	0 (0%)	6 (24%)	1 (3.8%)	0 (0%)	0 (0%)
Quantifiable	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)
Below Detection	8 (88.9%)	24 (100%)	19 (76%)	25 (96.2%)	3 (100%)	11 (91.7%)
Range	ND - BQL	all ND	ND - BQL	ND - BQL	all ND	ND - 251 gc/mL
Median	ND	ND	ND	ND	ND	ND
Total coliform						
Detectable	4 (44.4%)	9 (37.5%)	15 (60.0%)	6 (23.1%)	0 (0%)	3 (25.0%)
Below Detection	5 (55.6%)	15 (62.5%)	10 (40.0%)	20 (76.9%)	3 (100%)	9 (75.0%)
Range	ND - 4.11×10 ² MPN/100 mL	ND - 283 MPN/100 mL	ND - 1000 MPN/100 mL	ND - 416 MPN/100 mL	all ND	ND - 14.7 MPN/100 mL
Median	ND	ND	1.00 MPN/100 mL	ND	ND	ND
<i>E. coli</i>						
Detectable	1 (11.1%)	0 (0%)	6 (24.0%)	1 (3.8%)	0 (0%)	0 (0%)
Below Detection	8 (88.9%)	24 (100%)	19 (76.0%)	25 (96.2%)	3 (100%)	12 (100%)
Range	ND - 1.00 MPN/100 mL	all ND	ND - 77 MPN/100 mL	ND - 2.00 MPN/100 mL	all ND	all ND
Median	ND	ND	ND	ND	ND	ND

ND=not detected

BQL=detected, but below quantification limit

Table 5. Summary of observed inorganics in private wells in Texas (n=38), Florida (n=40), and Louisiana (n=38).

Inorganic parameter	Standard	Florida (n=40)				Texas (n=38)				Louisiana (n=38)			
		Median	90 th percentile	Max.	% Exceeding	Median	90 th percentile	Max.	% Exceeding	Median	90 th percentile	Max.	% Exceeding
Arsenic, µg/L	10	<0.5	0.8	4	0.0	1.9	6.6	9.6	0.0	<0.5	2.3	27.4	2.6
Cadmium, µg/L	5	<1.0	<1.0	<1.0	0.0	<1.0	<1.0	<1.0	0.0	<1.0	<1.0	<1.0	0.0
Chromium, µg/L	MCL 100	<1.0	1.7	16.7	0.0	<1.0	2.2	81.1	0.0	<1.0	<1.0	1.9	0.0
Nitrate, mg/L as N	10	0.1	1.8	31.8	2.5	0.1	1.8	5	0.0	NA	NA	NA	NA
Copper, µg/L	Action level 1300	3	24.5	176.1	0.0	3.8	36.6	152.5	0.0	1.4	13.9	208.4	0.0
Lead, µg/L	15	<1.0	<1.0	1.2	0.0	<1.0	1.3	6	0.0	<1.0	2.3	5.9	0.0
Chloride, mg/L ^a	250	24.3	218.9	777.5	10.0	87.8	223.6	749.6	10.5	5.8	26.8	109.2	0.0
Iron, µg/L	300	14.5	278.4	720	7.5	50.7	629.2	2029	28.9	132	613.7	1872	28.9
Manganese, µg/L	50	1	8.9	746.4	2.5	9.6	102.4	296.3	31.6	67.4	166.4	221	55.3
Silver, µg/L	SMCL 100	<1.0	<1.0	<1.0	0.0	<1.0	<1.0	<1.0	0.0	<1.0	<1.0	<1.0	0.0
Sulfate, mg/L ^a	250	5.5	162.7	448.7	5.0	14.2	47	170.6	0.0	2.5	4.8	6.2	0.0
Zinc, µg/L	5000	15.3	163.6	868.6	0.0	14.4	161.3	1508	0.0	29.6	374.9	2485	0.0
Sodium, mg/L	DWEL 20	18.9	178.1	645.6	50.0	58.3	184.8	430.5	86.8	56.7	86.4	129.2	92.1
Hardness, mg/L as CaCO ₃	No standard -	103.2	296.1	535	-	120.9	333	401.1	-	28	46.3	124.8	-

NA: parameter not analyzed.

MCL: maximum contaminant level

SMCL: secondary maximum contaminant level

DWEL: drinking water equivalent level

^aICP reported for Texas and Florida, IC reported for Louisiana

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Chapter 4: Conclusions and Future Work

Conclusions

Approximately 13 million households in the United States rely on private wells as their primary source of drinking water, but microbial risks in private wells have been understudied compared to microbial risks in municipal drinking water systems. While monitoring in private wells has traditionally focused on detecting total coliform and *E. coli* bacteria as indicator organics for contamination and human health risk, this work focused on detecting opportunistic pathogens (OPs) and serves as a foundation for knowledge surrounding background incidence of opportunistic pathogens in private wells, users of which have been underserved by scientific research.

Indicators and disinfection for private well users.

Private wells and municipal systems differ in terms of treatment practices and microbial communities. The results presented confirmed previous reports regarding the lack of association between indicator bacteria and OPs in private wells. However, the findings in this work also suggest that conditions that support the growth of total bacterial (as measured by 16S rRNA genes) might also support the growth of OPs in private wells. Unlike municipal systems, private wells do not generally use continuous disinfection, and have much higher “water age,” potentially creating environments where naturally occurring, oligotrophic OPs grow in conjunction with other bacteria, relatively to the hostile and rapidly changing environment associated with municipal water with high levels of secondary disinfectants. Furthermore, recent surveys of municipals systems with secondary disinfectants showed a strong association between *Legionella* spp. and

temperature that was not present in private wells in this study, though sample size and other factors such as variation in sample collection by untrained well-users, sample transportation and processing may limit how universally this conclusion can be applied to private wells. Shock chlorination may not be adequate in treating private well water against OPs contamination, particularly if there is a high background incidence of OPs in private well water supplies. Identifying key differences between municipal systems and private wells that may impact the occurrence of OPs is necessary to minimize the risk of OP infections linked to private wells.

Flooding impacts of private wells.

The results presented in Chapter 3 are the first comparison of OPs occurrence and the potential impact of flooding in private wells across multiple states. The results suggest that wellhead submersion may introduce *N. fowleri* in private wells. This may be a particular concern for private well users who reside in warmer parts of the United States, since *N. fowleri* is a warm freshwater organism and since the study area (Counties in Texas, Florida, and Louisiana) was located in the warmer region of the United States, and are more vulnerable to hurricanes in the late summer months. However, baseline levels for all OPs investigated in this work were not measured in the sampled areas, thus quantifying the change in OPs levels in private wells as a result of flooding is a suggested future research direction.

Wide variance in private wells.

Private well water quality can vary from home to home even if their private wells are drawing water from the same aquifer due to the heterogeneity of groundwater and the creation of localized environments which may promote the growth or spread of certain microorganisms. Furthermore, there is wide variation in individual private well household practices, including design, operation, and well stewardship practices such as how often the well is shock chlorinated, how often the septic tank is emptied, how comfortable private well users are with managing their system, and which treatment options are employed. This adds complexity to determining recommended private well stewardship practices.

Future work

Additional state and storm for post-flood comparison

Samples were collected in North Carolina private wells following Hurricane Florence in 2018 and will be subject to the same molecular analyses for *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *N. fowleri*, and shiga toxin-producing *E. coli* for added to the multi-state comparison covered in Chapter 3. These private wells were sampled 6-8 weeks following the storm and could inform differences in OPs detection as a function of time between the storm and sampling.

Antibiotic resistance

A microbial contamination concern that was not addressed in this thesis but also of importance is the spread of antibiotic resistance. While *E. coli* is accepted as an indicator

for fecal contamination in wells, studies have surveyed and reported their resistance to one or more antibiotics. Following Hurricanes Harvey and Irma, *E. coli* was isolated from IDEXX packets for *E. coli*-positive samples and were preserved for antibiotic resistance analysis using the Kirby Bauer disk assay. The DNA extracts from samples that were collected from these could also be subject to qPCR for different antibiotic resistance genes (ARGs). This would add to the body of knowledge surrounding the spread of antibiotic resistance in private wells, since current knowledge is mostly relates to antibiotic resistant indicator bacteria occurrence in private wells, but there is limited reporting of ARG occurrence in private wells, let alone following a flood.

Viability tests

To better assess potential health impacts related to OPs for private well users following floods, methods assessing the viability of OPs could be employed in conjunction with molecular methods as viable OPs are capable of causing infections in individuals. This is also necessary to assess what conditions in private wells and their plumbing systems may enhance the growth of certain OPs.

Minimizing risk of OP exposure and infection in private wells

There is a need for improved understanding for how to minimize exposure to OPs in private well systems. Managing the risk of OP exposure in private well systems may require a more individualized approach since private well systems typically serve one house instead of many and will vary based on geology and background water quality, as well as individual well characteristics. Risk of infection by OPs will also depend on the

health of the well users within the house, and if their health status puts them at risk for infection by certain OPs, extra precautions should be undertaken to minimize the risk of infection. Best practices for treatment options for those who are a risk for OPs infections are a research area that need to be explored.

Appendix A: Supplemental Information for Chapter 2

Section SI-1. Confirmation of identity of the 5 samples which were positive for *L.*

pneumophila by IDEXX and negative by qPCR

Initial Results

Fifty-seven samples were tested for *L. pneumophila* using both IDEXX Legiolert and qPCR. Of these, 18 samples tested positive for *L. pneumophila* DNA via qPCR and 6 samples tested positive for culturable *L. pneumophila* via IDEXX Legiolert. Only one sample tested positive for *L. pneumophila* via both methods. Five samples tested positive using IDEXX Legiolert, but negative using qPCR. Further testing was conducted as described below to better understand these discrepancies.

Follow Up Testing

qPCR at higher dilutions to check for inhibition.

The DNA extracts for the five samples that were negative for *L. pneumophila* using qPCR but positive using IDEXX we reanalyzed using qPCR for *L. pneumophila* at higher dilutions (up to 1:100 from raw DNA extract) with and without positive matrix spikes to check for inhibition. All no-spike samples returned negative, and all spike samples returned positive, suggesting that PCR inhibition was not likely the cause of positive IDEXX/negative qPCR samples. Thus, the level of *L. pneumophila* genes may have been below the detection limit of qPCR assay.

BCYE plate culture confirmation.

From the original IDEXX samples, the four samples that were positive by IDEXX but negative by qPCR (70-3, 145-4, 138-3, and 106-4) were stored by extracting the liquid from the Legiolert packets and adding 15% glycerol. These aliquots were then stored at -80 degrees C. The aliquots were streaked onto BCYE agar with L-cysteine. For plates with multiple morphologies, one colony for each morphology was T-streaked onto a second set of plates to obtain single morphology strains. This resulted in eight unique colony morphologies after the second round of T-streaking. A colony from each of these plates was then spread onto plates with L-cysteine and without L-cysteine. *Legionella* isolates will not grow on BYCE agar plates without L-cysteine. All plated colonies grew on both L-cysteine(+) and L-cysteine(-) plates while the control strain (130B) only grew on the L-cysteine(+). Thus, these four samples returned negative for *L. pneumophila* via spreading on BCYE plates. This suggests that the storage method implemented for IDEXX positive samples was not adequate for obtaining an isolate. Isolates were not T-streaked and purified before storage and were prepared for storage after 7 days of incubation instead of as soon as when the well indicated the presence of *L. pneumophila*. Both of which are not recommended practices.

PCR on liquid from IDEXX-positive trays

As a final check, PCR with Lpneu primers [1] was performed with sample liquid from IDEXX-positive trays that were stored with 15% glycerol as the DNA template. This was done for the four samples (70-3, 145-4, 138-3, and 106-4) at various dilutions (raw, 1:10, 1:20, 1:50, and 1:100). Bands were observed around the same 180 bp (same as the positive control) for sample 138-3 with no dilution as well as sample 145-4 at dilutions 1:20 and 1:50, indicating that *L. pneumophila* DNA was present in the original IDEXX samples. Bands did not appear for the other two samples, indicating *L. pneumophila* DNA could not be detected.

Section SI-2. Questionnaire used to collect information about private wells and treatment characteristics

1. Please describe the following to the best of your knowledge:
- | | | | |
|------------------------------------|--|---------------------------------------|-------------------------------------|
| a. Type of private well: | <input type="checkbox"/> dug or bored well | <input type="checkbox"/> drilled well | <input type="checkbox"/> don't know |
| b. What is the depth of the well? | _____ feet | | <input type="checkbox"/> don't know |
| c. What year was well constructed? | _____ | | <input type="checkbox"/> don't know |
| d. How many people in your home? | _____ | | <input type="checkbox"/> don't know |
| e. How many homes are supplied? | _____ | | <input type="checkbox"/> don't know |
2. What water treatment devices are currently installed? *Check all that apply.*
- | | | |
|---|---|--|
| <input type="checkbox"/> none | <input type="checkbox"/> acid neutralizer | <input type="checkbox"/> sediment filter |
| <input type="checkbox"/> ultraviolet (UV) light | <input type="checkbox"/> water softener | <input type="checkbox"/> reverse osmosis |
| <input type="checkbox"/> iron removal | <input type="checkbox"/> activated carbon (charcoal) filter | <input type="checkbox"/> chlorinator |
| <input type="checkbox"/> pitcher filter | <input type="checkbox"/> don't know/unsure | <input type="checkbox"/> other: _____ |
- If you have treatment, is it:** faucet unit whole house filter mixture of both

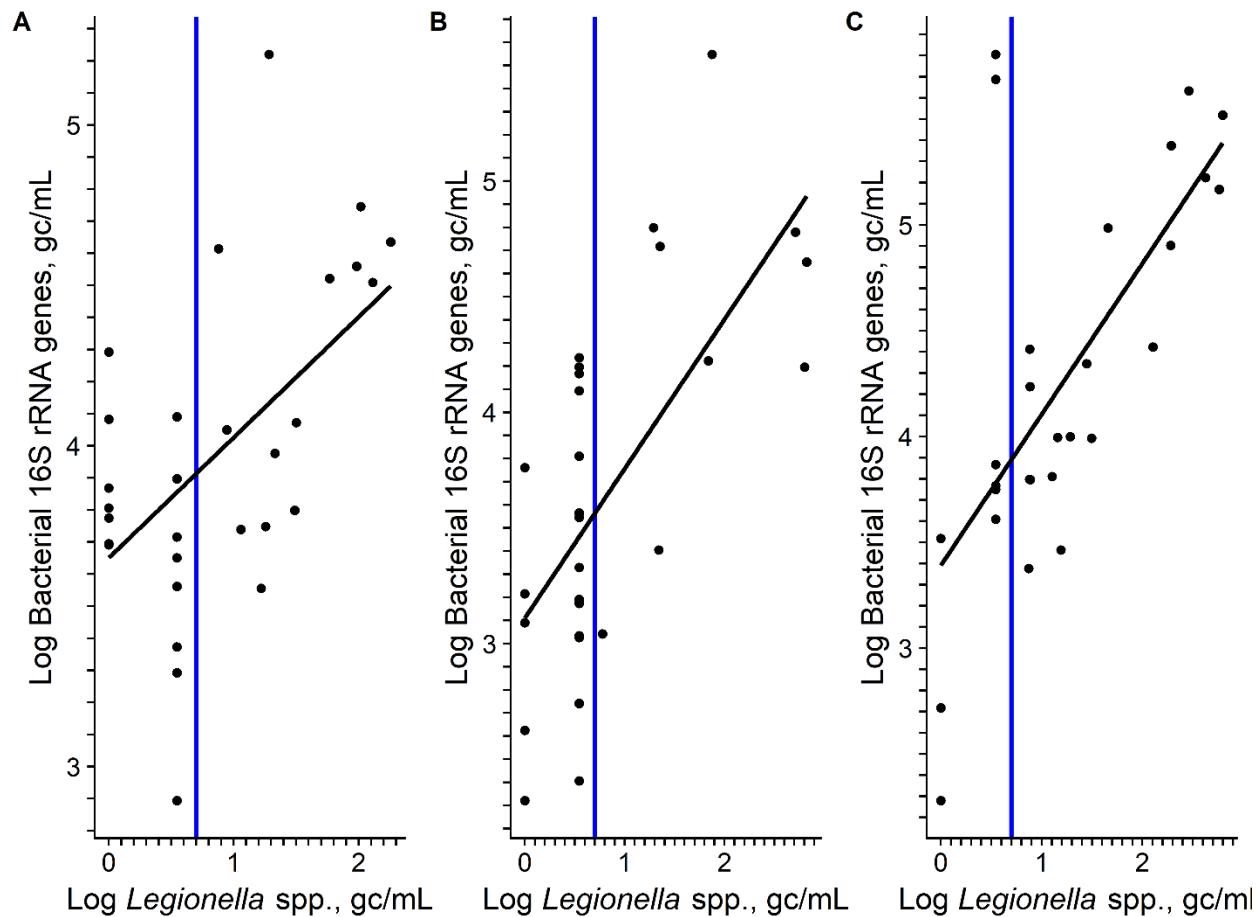


Figure SI-1. Positive correlations between *Legionella* spp. and total bacterial gene copy numbers in A) flushed cold B) first-draw hot and C) flushed hot water samples. Blue line represents limit of quantification (LOQ=5 gc/mL). Samples that were above detection, but below LOQ are plotted as half the LOQ (2.5 gc/mL). Samples below detection are plotted as zero.

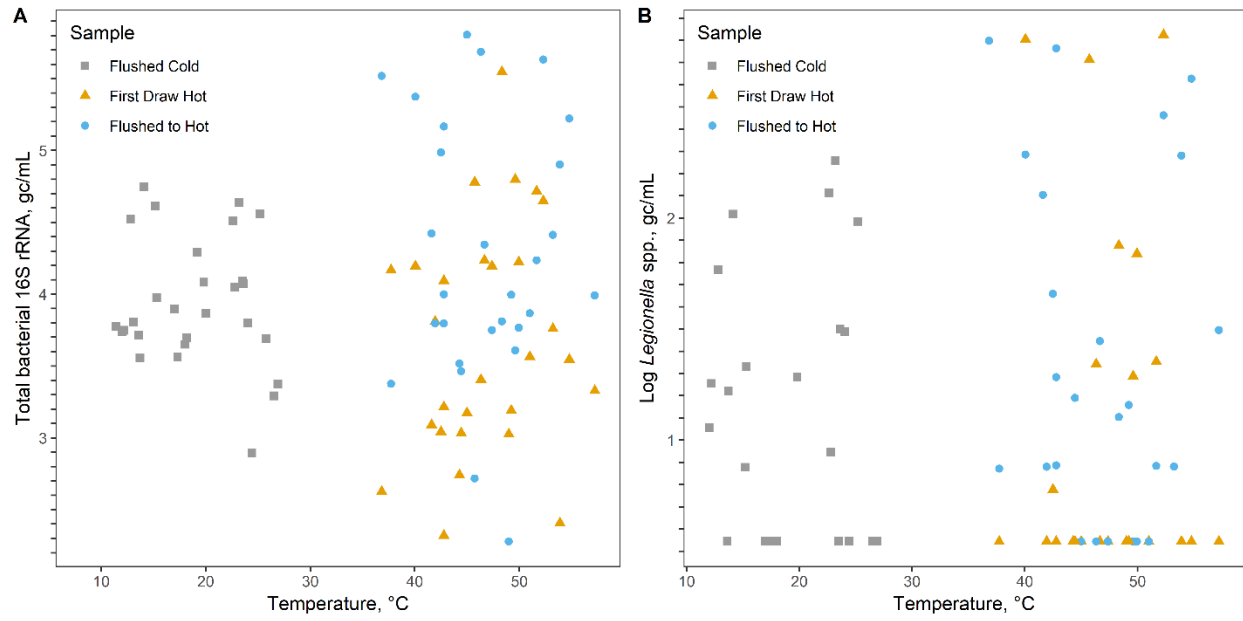


Figure SI-2. Enumeration of A) Total bacteria and B) quantifiable *Legionella* spp. gene copies against water temperatures measured and reported by residents

Table SI-1 Summary of sample kits distributed and analyses conducted

Sampling kit type	First draw cold water tap <i>n=44</i>	5-minute flush cold water tap <i>n=44</i>	5-minute flush cold water tap <i>n=44</i>	First draw hot water tap <i>n=28</i>	Flushed to hot hot water tap <i>n=28</i>
Basic	250 mL inorganic analysis <i>n=15</i>	250 mL inorganic analysis <i>n=15</i>	120 mL microbial culture analysis <i>n=15</i>	Not collected	Not collected
Advanced	250 mL inorganic analysis <i>n=29</i>	250 mL inorganic analysis <i>n=29</i>	1 L molecular and microbial culture analysis <i>n=29</i>	1 L molecular and microbial culture analysis <i>n=28</i>	1 L molecular analysis <i>n=28</i>

Table SI-2. Presence/absence of *L. pneumophila* in 57 private well samples analyzed by both IDEXX Legiolert and qPCR methods

IDEXX Legiolert result	qPCR result	
	Positive (n =18)	Negative (n=39)
Positive (n=6)	1	5
Negative (n=51)	17	34

IDEXX detection limit =0.1 MPN/mL

qPCR detection limit =1 gc/mL

Table SI-3. qPCR and PCR primers, probes, and assay conditions used in this study

Targeted organisms	Targeted genes	Sequences (5'-3')	Program		Amplicon (bp)	Reference
			Initial denaturation and enzyme activation	Denaturing / annealing / extension		
<i>Legionella</i> spp. (qPCR)	23S rRNA	Leg23SF: CCCATGAAGCCCGTTGAA Leg23SR: ACAATCAGCCAATTAGTACGAGTTAGC Probe: HEX-TCCACACCTCGCCTATCAACGTCGTAGT	95 °C for 2 min	40 cycles of 95 °C for 5 s and 58.5 °C for 10 s	92	[2]
<i>L. pneumophila</i> (qPCR)	mip	LmipF: AAAGGCATGCAAGACGCTATG LmipR: GAAACTTGTTAAGAACGTCTTTCATTTG Probe: FAM-TGGCGCTCAATTGGCTTTAACCGA	95 °C for 2 min	40 cycles of 95 °C for 5 s and 60 °C for 10 s	78	[2]
<i>L. pneumophila</i> (PCR)	mip	LpneuF: CCGATGCCACATCATTAGC LpneuR: CCAATTGAGCGCCACTCATAG	95 °C for 5 min	40 cycles of 95 °C for 1 min and 55 °C for 1 min	180	[1]
Total bacteria (qPCR)	16S rRNA	BACT1369F: CGGTGAATACGTTTCYCGG PROK: GGWTACCTTGTTACGACTT	98 °C for 2 min	40 cycles of 98 °C for 5 s and 55 °C for 5 s	124	[3]

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Appendix B: Supplemental Information for Chapter 3

Section SI-1. Sampling instructions for the Louisiana Sampling Campaign (Basic kit)

These sampling materials were developed based on the Virginia Household Water Quality Program's citizen science sampling for well users in Virginia (www.wellwater.bse.vt.edu).

1. After the water has not been used for at least 6 hours, unpack the plastic bag and remove the caps from the bottles. Set the caps on the counter upside down, trying not to touch the inside of the caps or bottles.
2. With bottle 1 held under the kitchen tap, open the cold water tap and fill the bottle completely at full flow (as if you were filling a glass of water). Once filled, leave the water running and set bottle 1 aside.
3. Immediately fill bottle 2 to the top. Continue to let the water run, and put the caps on bottles 1 and 2. Take care not to touch the inside of the caps or the bottles.
4. Allow water to run for 5 minutes at full flow.
5. At 5 minutes, fill bottles 3. Once all the bottles are filled, turn water off and place the caps back on the bottles. Take care not to touch the inside of the caps or the bottles.
6. Make sure the cap is securely tightened on all bottles so they do not leak during transport.
7. Place bottles 2 and 3 in the separate bags provided.
8. If you cannot drop the samples off immediately after collection, place the bottles into your refrigerator or on ice until you can drop them off. Do not freeze any samples.
9. Complete the two-page questionnaire.
10. Place bottles and questionnaire back into the plastic bag. Bring your sample bag to: [location].

Section SI-2. Sampling instructions for the Louisiana Sampling Campaign (Advanced kit)

These sampling materials were developed based on the Virginia Household Water Quality Program's citizen science sampling for well users in Virginia (www.wellwater.bse.vt.edu).

1. After the water has not been used for at least 6 hours, unpack the plastic bag and remove the caps from the bottles. Set the caps on the counter upside down, trying not to touch the inside of the caps or bottles.
2. With bottle 1 held under the kitchen tap, open the cold water tap and fill the bottle completely at full flow (as if you were filling a glass of water). Once filled, leave the water running and set bottle 1 aside.
3. Immediately fill bottle 2 to the top. Continue to let the water run, and put the caps on bottles 1 and 2. Take care not to touch the inside of the caps or the bottles.
4. Allow water to run for 5 minutes at full flow.
5. At 5 minutes, fill bottles 3, 4 and 5 back-to-back. Once all the bottles are filled, turn water off and place the caps back on the bottles. Take care not to touch the inside of the caps or the bottles.
6. With bottle 6 held under the kitchen tap, open the hot water tap and fill the bottle completely at full flow. Once filled, turn off the hot water and put the cap on the bottle.
7. Make sure the cap is securely tightened on all bottles so they do not leak during transport.
8. Place bottles 2, 4, and 6 in the separate bags provided.
9. If you cannot drop the samples off immediately after collection, place the bottles into your refrigerator or on ice until you can drop them off. Do not freeze any samples.
10. Complete the two-page questionnaire.
11. Place bottles and questionnaire back into the plastic bag. Bring your sample bag to: [location].

Section SI-3: Sampling instructions for the Texas and Florida Sampling Campaigns

Morning of collection

1. Turn on the cold water only at the highest flow rate fixture in your house (e.g., bath tub, shower head) and flush for 5 minutes.
2. After 5 minutes, turn off the water.
3. Go to your kitchen tap, unpack the sample bottle from the plastic bag. Remove the cap from the bottle.
4. Set the cap on the counter upside down, trying not to touch the inside of the cap or bottle.
5. Open the cold water tap and run the water for one minute.
6. At one minute, fill the bottle completely at full flow (as if you were filling a glass of water). Put the cap back on the bottle.
7. Turn off the water.
8. If you cannot drop the samples off immediately after collection, place the bottle into your refrigerator or on ice until you can drop them off.
9. Complete the two-page questionnaire.
10. Place bottle and questionnaire back into the plastic bag. Bring your sample bag to:
[LOCATION]

Table SI-1: qPCR and PCR primers, probes, and assay conditions used in this study

Targeted organisms	Targeted genes	Sequences (5'-3')	Program		Amplicon (bp)	Reference
			Initial denaturation and enzyme activation	Denaturing / annealing / extension		
<i>Legionella</i> spp. (qPCR)	23S rRNA	Leg23SF: CCCATGAAGCCCGTTGAA Leg23SR:ACAATCAGCCAATTAGTACGAGTTAGC Probe: HEX-TCCACACCTCGCCTATCAACGTCGTAGT	95 °C for 2 min	40 cycles of 95 °C for 5 s and 58.5 °C for 10 s	92	[2]
<i>L. pneumophila</i> (qPCR)	<i>mip</i>	LmipF:AAAGGCATGCAAGACGCTATG LmipR: GAAACTTGTTAAGAACGCTTTTCATTG Probe: FAM-TGGCGCTCAATTGGCTTTAACCGA	95 °C for 2 min	40 cycles of 95 °C for 5 s and 60 °C for 10 s	78	[2]
<i>Mycobacterium</i> spp. (qPCR)	16S rRNA	110F: CCTGGGAAACTGGGTCTAAT I571R: CGCACGCTCACAGTTA H19R: FAM-TTTCACGAACAACGCGACAAACT	95 °C for 2 min	45 cycles of 95 °C for 5 s and 55 °C for 15 s	462	[4]
<i>M. avium</i> (qPCR)	16S rRNA	MycavF F: AGAGTTTGATCCTGGCTCAG Mycav R: ACCAGAAGACATGCGTCTTG	98 °C for 2 min	40 cycles of 98 °C for 5 s and 68 °C for 18 s	180	[5]
<i>N. fowleri</i> (qPCR)	ITS	JBVF: AGGTACTTACGTTAGAGTGCTAGT JBVR: ATGGGACAATCCGGTTTTCTCA JBVP: FAM-ACGCCCTAGCTGGTTATGCCGGATT-BHQ1	95 °C for 2 min	45 cycles of 95 °C for 15 s and 63 °C for 33 s	123	[6]
Shiga toxin-producing <i>E. coli</i> (PCR)	<i>stx1</i>	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCTCCATTATG	95°C for 3 min	35 cycles of 95°C for 20 s, 58°C for 40 s	614	[7]
Shiga toxin-producing <i>E. coli</i> (PCR)	<i>stx2</i>	F: CCATGACAACGGACAGCAGTT R: CCTGTCAACTGAGCACTTTG	95°C for 3 min	35 cycles of 95°C for 20 s, 58°C for 40 s	779	[7]
Total bacteria (qPCR)	16S rRNA	BACT1369F: CGGTGAATACGTTTCYCGG PROK: GGWTACCTTGTTACGACTT	98 °C for 2 min	40 cycles of 98 °C for 5 s and 55 °C for 5 s	124	[3]

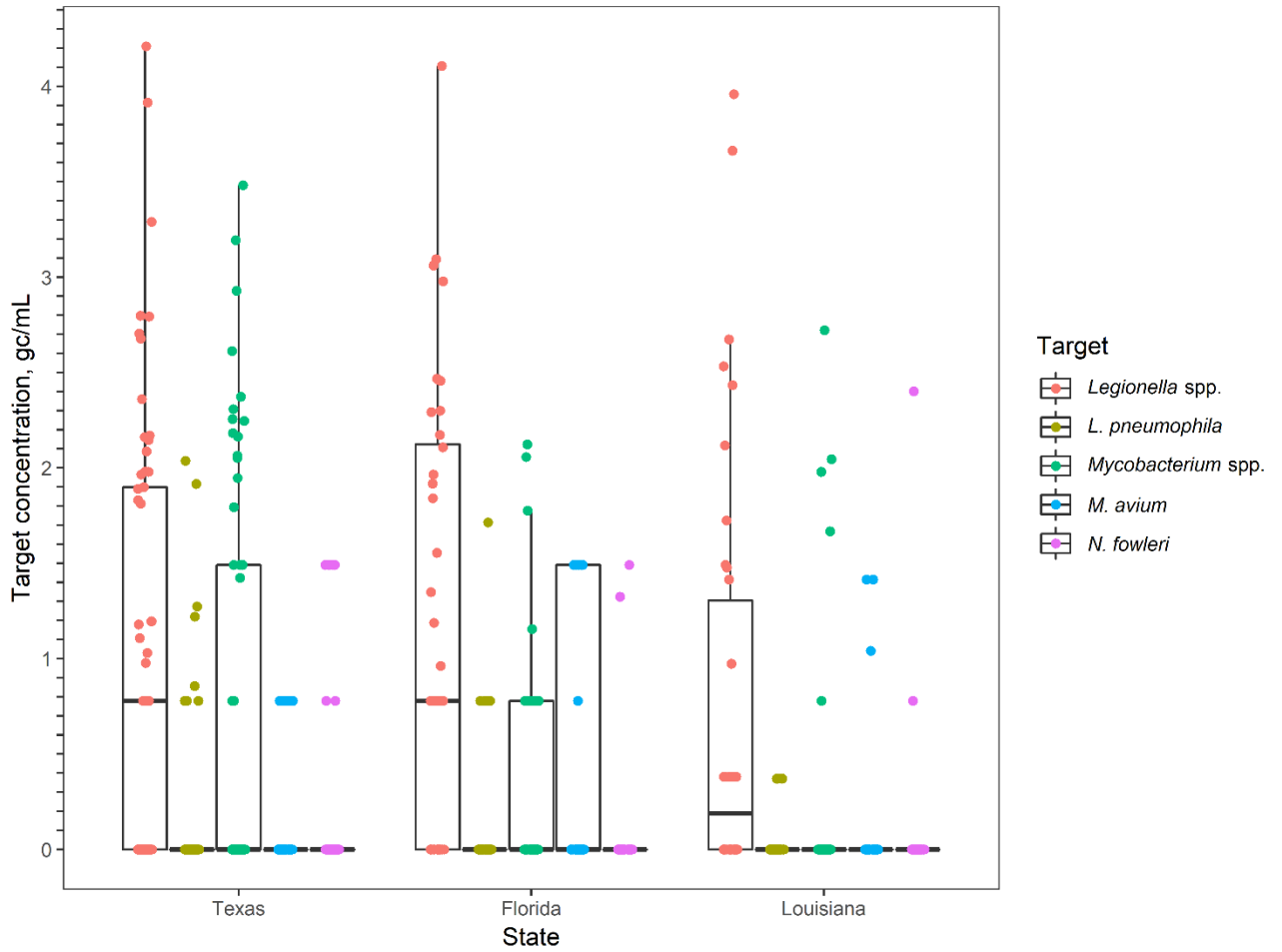


Figure SI-1: Enumeration of target pathogens in each state. Samples with gene copies below the quantification limit (BQL) are reported as half of the corresponding limit of quantification (LOQ); samples with no detection are reported as 0. Boxplot limits reflect data with the median \pm 1.5 time the inter-quartile range.

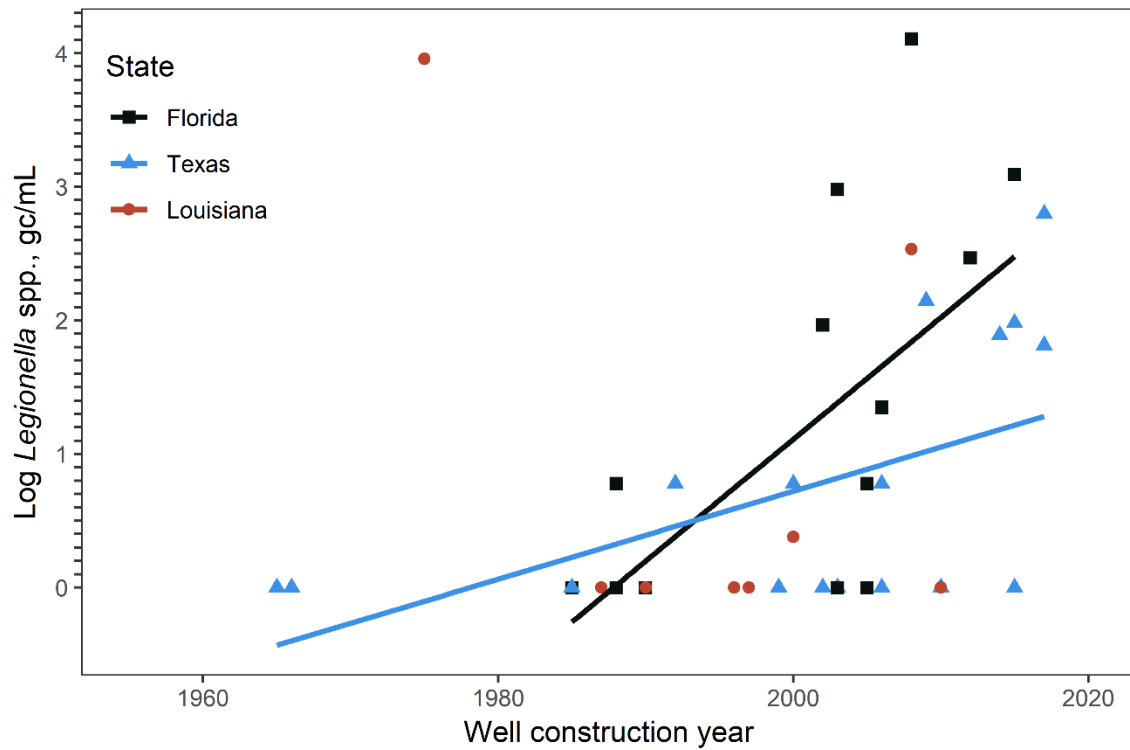


Figure SI-2: Positive correlation between *Legionella* spp. and well construction year in unsubmerged wells in Florida ($n = 15$, $p = 0.025$, $\rho = 0.45$; black line and points) and Texas ($n = 17$, $p = 0.014$, $\rho = 0.58$; light blue line and points). There were not enough data points for Louisiana to complete statistical correlation.

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