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Occurrence of opportunistic pathogens in private wells after major flooding events: A four state molecular survey

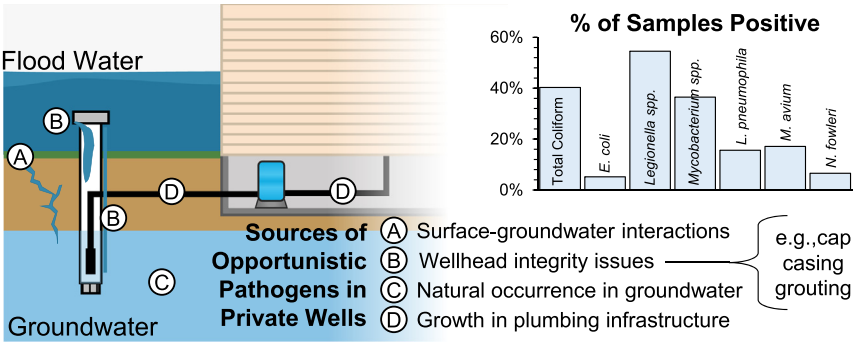
Kris Mapili [a], William J. Rhoads [a,b,*], Mary Coughter [a], Kelsey J. Pieper [c,**], Marc A. Edwards [a], Amy Pruden [a]

[a] Virginia Tech, Civil and Environmental Engineering, 418 Durham Hall, Blacksburg, VA 24061, United States of America
[b] Eawag – Swiss Federal Institute of Aquatic Science and Technology, Department of Environmental Microbiology, Überlandstrasse 133, 8600 Dübendorf, Switzerland
[c] Northeastern University, Civil and Environmental Engineering, 360 Huntington Ave., Boston, MA 02115, United States of America

HIGHLIGHTS

- Opportunistic pathogen (OP) occurrence in flood-impacted private wells was commonplace.
- Lack of baseline data constrained ability to assess OP contamination due to flooding.
- Timely sample collection is a major barrier in assessing post-flood well water quality.
- Future work should prioritize developing a deeper understanding of OP occurrence in and interventions for private wells.

GRAPHICAL ABSTRACT



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ABSTRACT

Private wells can become contaminated with waterborne pathogens during flooding events; however, testing efforts focus almost exclusively on fecal indicator bacteria. Opportunistic pathogens (OPs), which are the leading cause of identified waterborne disease in the United States, are understudied in private wells. We conducted a quantitative polymerase chain reaction survey of *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *Naegleria fowleri,* and shiga toxin-producing *Escherichia coli* gene markers and total coliform and *E. coli* in drinking water supplied by private wells following the Louisiana Floods (2016), Hurricane Harvey (2017), Hurricane Irma (2017), and Hurricane Florence (2018). Self-reported well characteristics and recovery status were collected via questionnaires. Of the 211 water samples collected, 40.3% and 5.2% were positive for total coliform and *E. coli*, which were slightly elevated positivity rates compared to prior work in coastal aquifers. DNA markers for *Legionella* and *Mycobacterium* were detected in 54.5% and 36.5% of samples, with L. *pneumophila* and *M. avium* detected in 15.6% and 17.1%, which was a similar positivity rate relative to municipal system surveys. Total bacterial 16S rRNA gene copies were positively associated with *Legionella* and *Mycobacterium*, indicating that conditions that favor occurrence of general bacteria can also favor OPs. *N. fowleri* DNA was detected in 6.6% of samples and was the only OP that was more prevalent in submerged wells compared to non-submerged wells. Self-reported well characteristics were not associated with OP occurrence. This study exposes the value of routine baseline monitoring and timely sampling after flooding events in order to effectively assess well water contamination risks.

* Correspondence to: William J. Rhoads, Virginia Tech, Civil and Environmental Engineering, 418 Durham Hall, Blacksburg, VA 24061, United States of America.
** Corresponding author.
E-mail addresses: william.rhoads@eawag.ch (W.J. Rhoads), k.pieper@northeastern.edu (K.J. Pieper).

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1. Introduction

The extent to which opportunistic pathogens (OPs), the leading cause of reported waterborne disease in the United States, are found in drinking water supplied by private wells is largely unknown. *Legionella* (specifically L. *pneumophila*) and nontuberculous mycobacteria (specifically the *Mycobacterium avium* complex [MAC]) can cause severe pneumonia, especially in immunocompromised individuals, via inhalation or aspiration of aerosol-entrained bacteria (Falkinham et al., 2015). *Naegleria fowleri* can cause primary amoebic meningoencephalitis (PAM), a rare but highly lethal brain disease, via nasal aspiration (Bartrand et al., 2014). *Legionella* spp., *Mycobacterium* spp., and other OPs are known to survive and proliferate in biofilms (Lehtola et al., 2007) and are common inhabitants of drinking water systems. *N. fowleri* is commonly detected in warm freshwater and has been found in surface water sourced drinking water distribution systems with low chlorine residuals (Miller et al., 2017). *Legionella, Mycobacterium,* and *N. fowleri* have also been observed to occur naturally in groundwater, as studies report that 7.7–83% samples detected at least one of these OPs (Riffard et al., 2001; Costa et al., 2005; Richards et al., 2018; Blair et al., 2008; Marciano-Cabral et al., 2003; Laseke et al., 2010). However, research directly monitoring OPs in drinking water supplied by private wells is lacking.

After flooding events, drinking water supplied by private wells can become contaminated with floodwater, increasing microbial loading to private well supplies, including waterborne pathogens (Van Biersel et al., 2007; Eccles et al., 2017; Dai et al., 2019; Pieper et al., 2022). Contamination is typically assessed by the presence of coliform bacteria (i.e., total coliform and *E. coli*), which are indicators of surface water and fecal contamination. However, these bacteria do not adequately predict sources of microbial risks beyond those originating from fecal contamination and are inconsistently associated with OP occurrence (Dai et al., 2019). As with surface water and fecal contamination, OPs may be introduced into private wells directly (e.g. through the well casing) or indirectly (e.g., through the aquifer) during flooding. However, because OPs are common inhabitants of aquatic environments, nutrients and host organisms introduced during flooding may also facilitate proliferation of OPs already existing in the system at low numbers. Prior work suggests that when OPs occur in well water, the levels detected in the water collected from within the home plumbing tend to be higher than in water directly collected from the well, potentially due to favorable growth conditions in the home plumbing, including elevated water temperatures and high surface area of small diameter home plumbing pipes (Dai et al., 2019).

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 buildings supplied by municipal water distributed with a secondary disinfectant residual, well water studies have reported contradictory findings, suggesting that municipal water knowledge cannot always be translated to well water. For example, it is widely recognized that there is no relationship in municipal water between the occurrence of *Legionella* and aerobic heterotrophic plate count bacteria (an indicator for total bacterial growth) (Duda et al., 2015). However, a correlation between *Legionella* spp. and the total bacterial 16S rRNA gene markers has been documented in private wells, suggesting that conditions that favor occurrence of bacteria in general are capable of supporting occurrence of some OPs as well (Dai et al., 2019). In addition, water temperature has been documented to strongly correlate with the incidence and levels of *Legionella* markers in home plumbing served by municipal systems (W.J. Rhoads, et al., Impact of Residential Water Heater Cleaning on Sediment Composition, Inorganic Loading, and Legionella Gene Markers in the Aftermath of Widespread Distribution System Corrosion in Flint, MI, In preparation), but temperature has not been found to be correlated with *Legionella* in home plumbing from private wells (Mapili et al., 2020). We speculate these differences could be limited to OPs with ecological advantages for surviving in oligotrophic environments. For instance, *Legionella* can develop a host-pathogen relationship with amoeba (Fields et al., 1989), and their growth is controlled by physiochemical factors (e.g., water temperature profiles) in individual systems. Additionally, differences in nutrient loading, water age, and disinfection practices between private wells and municipal systems may impact variation in survival and growth of OPs.

Given that background knowledge about OP occurrence in private wells is limited and studies suggest that flooding events increase microbial contamination, we conducted a quantitative polymerase chain reaction (qPCR) survey of *Legionella*, *L. pneumophila*, *Mycobacterium, M. avium,* and *N. fowleri* gene markers in well water across four US states subject to flooding events. Drinking water samples from private wells and corresponding participant questionnaires were collected in flood-impacted areas following four flooding disasters from 2016 to 2018. The objectives of this study were to: (i) assess the prevalence of OPs in private wells after the disasters and (ii) identify associations between post-flood damage, water quality, well system characteristics, and detection of OPs in private wells.

2. Methods and materials

2.1. Citizen science water sampling

Four citizen science well water testing campaigns were conducted in the aftermath of a severe rainfall event and three 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 collected sampling kits provided by our research team at specified pick-up locations. Each kit included: sampling instructions (Section SI-1-3), sampling bottles, and a questionnaire about the well characteristics and flood impacts (Dai et al., 2019; Pieper et al., 2022). Participants returned the sampling kits on predetermined mornings to specified drop-off locations. Sample kits were collected by our research team or extension agents, packaged on ice in secondary containers, and delivered the next day 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). The primary focus of each sampling campaign was to determine the rates of microbial contamination in the aftermath of major flooding events (Dai et al., 2019; Pieper et al., 2022). In each campaign, there were two types of sample kits provided: (Falkinham et al., 2015) “basic” kits assessed coliform bacteria and inorganic and anion concentrations and (Bartrand et al., 2014) “advanced” kits assessed coliform bacteria and inorganic and anion concentrations as well as analyzed for OP DNA. This study focuses exclusively on analysis from the advanced kits. 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 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. A total of 50 advanced kits were randomly distributed among participating residents (38 were returned). In the advanced kit, sequential 250 mL and 1 L samples were collected after 5 min 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 counties in Florida. Sample collection in Texas occurred on 7 different dates between September 18 and October 11, 2017, resulting in 61 returned samples. Sample collection in Florida occurred on 6 different dates between October 9 and October 24, 2017, resulting in 40 returned samples. 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 min 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.

Sample collection in North Carolina occurred on 7 different dates between October 22 and November 29, 2018, resulting in 72 returned samples. Kits only included the advanced kit, with 1 L collected after 5 min of flushing, split for separate analyses as before. Participation in all campaigns was voluntary and all procedures were approved by Virginia Tech Institutional Review Board (#16-918).

2.2. Water quality analysis

Aliquots or the 250 mL samples were acidified with 2% nitric acid and digested for a minimum of 16 h prior to analysis using inductively coupled plasma-mass spectrometry (ICP-MS) per methods 3030D and 3125 B (American Public Health Association, 1998). 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 18 method (Westbrook, MN), with a detection limit of 1 MPN/100 mL.

2.3. qPCR analysis

The remainder of 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). A negative DNA extraction control, consisting of an un-used filter and extraction tube was included each time DNA extraction was performed. DNA extracts for each sampling campaign were diluted 1:5 or 1:10 with nuclease-free water for qPCR to minimize PCR inhibition based on results of a dilution curve (no dilution, 1:5, 1:10, 1:20; 1:50) of six samples per campaign, where the lowest dilution with no evidence of inhibition was used for all samples. Filters, DNA extracts, and diluted extracts 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 using previously published and validated assays (Wang et al., 2012; Garner et al., 2018) on Bio-Rad CFX98 real-time systems. Detection of shiga toxin-producing *E. coli* (*stx*1 and *stx2* genes) were determined using PCR. Primers, reagents, standards, and thermocycling settings are described in detail in the supplementary information (Table SI-1) (Nazarian et al., 2008; Radomski et al., 2010; Wilton and Cousins, 1992; Mull et al., 2013; Fagan et al., 1999; Suzuki et al., 2000). Serially-diluted genomic DNA 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 OPs) were included in each qPCR run. Comparison of recovery efficiency for qPCR assays are presented elsewhere (Wang et al., 2012; Garner et al., 2018). The limit of quantification (LOQ) was defined as the lowest standard concentration that amplified resulting in $R^2 > 0.98$ and efficiency ranging 80–110%, resulting in 100 gc/reaction for total bacteria, 10 gc/reaction for *Legionella* spp. and *L. pneumophila*, and 10 or 50 gc/reaction for *Mycobacterium* spp., *M. avium*, and *N. fowleri*. The LOQ was applied for each qPCR run. qPCR reactions for each sample, standards, and a non-template control (NTC; molecular-grade water) 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 the LOQ were considered quantifiable and the average of the two or three wells reported. Samples with positive amplification, but not meeting the above quantifiable criteria, were considered detectable, but <LOQ. These samples were treated as half of LOQ in non-parametric analyses, while samples with no positive amplification were considered as non-detectable and treated as zero. All DNA extraction negative controls and NTCs were non-detectable.

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 was 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.

2.4. Data analysis

Data analysis was performed in RStudio using R (version 3.4.3). Non-parametric analyses were performed on inorganic concentrations and gene copy numbers. Inorganic concentrations below the minimum reporting level (MRL) were treated as half of the MRL to establish the same rank for nonparametric analysis. The Wilcoxon and Kruskal-Wallis Tests were used to determine differences in water quality between two or more groups. Where Kruskal-Wallis indicated a difference, the Dunn's test with Bonferroni correction was used to determine differences among groups. Spearman 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.

3. Results

Post-flooding private well water quality, system characteristics, and system recovery were documented after four natural disasters occurring in four US states: 1) Great Louisiana Flood of 2016 (August 2016); 2) Hurricane Harvey in Texas (August 2017); 3) Hurricane Irma in Florida (September 2017); and 4) Hurricane Florence in North Carolina (September 2018) (Table 1). The percent of private wells in flood-impacted counties was 16–66% of the population across the four states and the number of well users was 315,000-1,331,192 (assuming 2–4 people per well). Private wells sampled in Texas, North Carolina, and Louisiana were likely drawing groundwater from the Coastal Plain aquifer systems, which generally contains layers of clay, silt, sand, and gravel, but varies locally (USGS, n.d.). Private wells sampled in Florida were either drawing from the Surficial aquifer system which is an unconsolidated sand aquifer or Floridian aquifer system which is a carbonate bedrock aquifer (USGS, n.d.).

3.1. Impacts of flooding on private wells surveyed

A questionnaire was used to document well characteristics and flood impacts (Table 2). Of the private wells sampled, owners reported that 51.4–80.3% were drilled, with a median well depth of 41–400 ft (12.5–122 m) and median year of construction of 1995–2002 in each state. Wellhead submersion was a suspected primary route for floodwater contamination, and was highest in Texas (41.0%) compared to Florida (22.5%), North Carolina (19.4%), and Louisiana (7.9%). Interestingly, wellhead submersion reported by owners was not associated with increased system damage. Similar fractions of residents reported system damage in Louisiana (31.6%) and Texas (26.2%), despite reporting much lower levels of wellhead submersion in Louisiana. The most common type of system damage reported 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 for our sample population was re-instating the ability to supply well water to the home and not physical damage to the wellhead or piping system. Shock chlorination (i.e., dosing and recirculating high concentrations of chlorine in the entire drinking water system to disinfect the system) is the most common remediation for private wells 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, 10.0% in Florida, and 2.8% in North Carolina, which generally aligns with reported wellhead submersion rates suggesting that shock chlorination practices correspond to extent of flooding.

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

Storm characteristics	Florida	Louisiana	Texas	North Carolina
Name of natural hazard	Hurricane Irma	Louisiana Floods	Hurricane Harvey	Hurricanes Florence and Michael
Date of landfall	September 10, 2017	August 12, 2016	August 25, 2017	September 14, 2018
Natural hazard type	Category 4 hurricane	Prolonged rainfall	Category 4 hurricane	Category 1 hurricane
Counties disaster declaration for individual assistance	49[a]	22[b]	41[c]	34[d]
# of potentially impacted	306,382 private wells[e] (612,764-1,225,528 users)[i]	78,750–157,500 private wells[f] (315,000 users)	215,906 private wells[g] (431,812–863,624 users)	332,798 private wells[h] (665,596-1,331,192 users)
% of private well population potentially impacted[j]	25–50%	16%	33–66%	28–55%
Aquifer systems[k]	Surficial aquifer system Unconsolidated; sand Floridan aquifer system Bedrock with solution channels; carbonate rocks	Coastal Lowlands aquifer system Poorly consolidated to unconsolidated; layers of clay, silt, sand, and gravel		

Sources and footnotes:
[a] https://www.fema.gov/disaster/4337/designated-areas & https://www.flgov.com/wp-content/uploads/orders/2019/EO_19-34.pdf.
[b] https://www.fema.gov/disaster/4277/designated-areas.
[c] *https://www.fema.gov/disaster/4332*.
[d] https://www.fema.gov/disaster/4332; 4 https://www.fema.gov/disaster/4393.
[e] https://waterwelljournal.com/potentially-750000-private-water-wells-affected-recent-hurricanes/.
[f] Ref. (Gilliland et al., 2021).
[g] https://waterwelljournal.com/potentially-750000-private-water-wells-affected-recent-hurricanes/.
[h] https://www.newsobserver.com/news/business/article220561095.html.
[i] Assumes 2–4 people per well.
[j] https://www.newsobserver.com/news/business/article220561095.html.
[k] https://www.usgs.gov/mission-areas/water-resources/science/principal-aquifers-united-states?qt-science_center_objects=0#qt-science_center_objects

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

Parameter	Florida	Texas	Louisiana	North Carolina
Number of days after storm samples were collected	32–38	24–34	73–76	20–70
Number of samples analyzed for this study	40	61	38	72
Well type, n, % of total samples				
Drilled	32 (80%)	49 (80.3%)	25 (65.8%)	37 (51.4%)
Dug or bored	1 (2.5%)	0 (0%)	2 (5.3%)	11 (15.3%)
Don't know or not reported	7 (17.5%)	12 (19.7%)	11 (28.9%)	24 (33.3%)
Well depth, feet				
n reported, % of total samples	18 (45%)	43 (70.5%)	21 (55.3%)	40 (55.6%)
Median	147.5	200	400	41.3
Range	35–300	30–650	25–2300	16–185
Year constructed				
n reported, % of total samples	25 (62.5%)	41 (67.2%)	21 (55.2%)	46 (63.9%)
Median	2002	2000	1995	1995
Range	1955–2015	1965–2017	1951–2015	1972–2018
Submerged, n, % of total samples				
Yes	9 (22.5%)[a]	25 (41.0%)[a]	3 (7.9%)[b]	14 (19.4%)
No	24 (60%)	26 (42.6%)	12 (31.6%)	44 (61.1%)
Don't know or not reported	7 (17.5%)	10 (16.4%)	23 (60.5%)	14 (19.4%)
Damaged, n, % of total samples				
Yes	5 (12.5%)	16 (26.2%)	12 (31.6%)	5 (6.9%)
Electrical damage	5 (12.5%)	8 (13.1%)	2 (5.3%)	NA[c]
Pump damage	2 (5.0%)	4 (6.6%)	5 (13.2%)	NA[c]
Pipe damage	2 (5.0%)	3 (4.9%)	1 (2.6%)	NA[c]
Casing damage	0 (0%)	2 (3.3%)	NA	NA[c]
Cover damage	0 (0%)	1 (1.6%)	NA	NA[c]
No	30 (75%)	42 (68.8%)	21 (55.3%)	55 (76.4%)
Don't know or not reported	5 (12.5%)	3 (4.9%)	5 (13.2%)	12 (16.7%)
Shock chlorinated, n, % of total samples				
Yes	4 (10%)	22 (36.1%)	3 (7.9%)	2 (2.8%)
No	28 (70%)	33 (54.1%)	35 (92.1%)	66 (91.7%)
Don't know or not reported	8 (20%)	6 (9.8%)	0 (0%)	4 (5.6%)

NA: Not Applicable. This question was not included in the Louisiana and North Carolina questionnaires.
[a] Checkbox survey question (yes, no, or don't know).
[b] Free text survey question.
[c] Damage specification not asked.

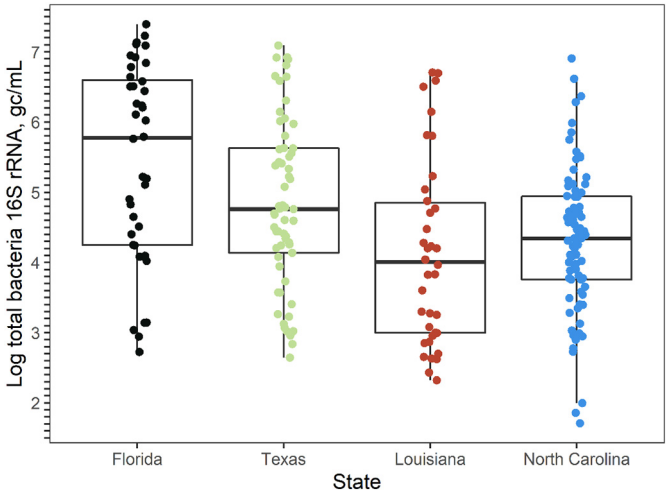

Fig. 1. Boxplots of total bacteria (16S rRNA) for all samples in Florida (n = 40), Texas (n = 61), Louisiana (n = 38), and North Carolina (n = 79). Boxplots represent the interquartile range (IQR), whiskers extend to median ± 1.5*IQR. 16S rRNA genes in private wells were significantly different among the four sampled states (Kruskal-Wallis, p = 0.00014).

3.2. Occurrence of indicator bacteria, total bacterial 16S rRNA genes, and OP gene markers

A total of 211 water samples were collected across private wells in Texas (n = 61), Florida (n = 40), Louisiana (n = 38), and North Carolina (n = 72). More than a third of wells tested (40.3%) were positive for total coliforms, with quantifiable samples ranging from 1.00 to more than 2429 MPN/100 mL. *E. coli* were detected in 11 of the 85 samples (12.9%) that were positive for total coliform and 5.2% of all samples, with quantifiable samples ranging from 1.00 MPN/100 mL to 77.6 MPN/100 mL. There were no differences in total coliform (Kruskal p = 0.49) or *E. coli* (Kruskal p = 0.11) MPNs by state. Enumerated targets were not elevated in comparison to contamination rates reported in other states, as prior studies report total coliform positivity rates of 14.6–46% and *E. coli* rates of 1.5–14% (Dai et al., 2019; USGS, n.d.; Pieper et al., 2015).

Total bacterial gene copy numbers varied among the four states (Fig. 1; Kruskal-Wallis, p = 0.00014), ranging from 2.1 × 10^2 to 2.47 × 10^7 gc/mL (Table 3, Appendix B Fig. 1). The highest level of total bacterial gene numbers were found in Florida, with a median level of 5.99 × 10^5 gc/mL, which was approximately one order of magnitude higher than Texas, Louisiana, and North Carolina. Compared to non-flooding scenarios in private wells in North Carolina (Mapili et al., 2020), total bacterial gene copies were approximately two orders of magnitude higher in this study. The

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

Total bacteria (16S rRNA)	Florida	Texas	Louisiana	North Carolina
	n = 40	*n* = 61	n = 38	n = 72
Detectable	40 (100%	61 (100%)	38 (100%)	72 (100%)
BQL	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Quantifiable	40 (100%)	61 (100%)	38 (100%)	72 (100%)
Below Detection	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Range (gc/mL)	5.30 × 10^2–2.47 × 10^7	4.42 × 10^2–1.23 × 10^7	2.1 × 10^2–3.90 × 10^6	5.01 × 10–8.10 × 10^6
Median (gc/mL)	5.99 × 10^5	5.77 × 10^4	3.86 × 10^4	2.21 × 10^4
Legionella spp. (23 s RNA)	n = 40	n = 61	n = 38	n = 72
Detectable	25 (62.5%)	32 (52.4%)	19 (50.0%)	39 (54.2%)
BQL	7 (17.5%)	7 (11.5%)	8 (21.0%)	12 (16.7%)
Quantifiable	18 (45.0%)	25 (41.0%)	11 (28.9%)	27 (37.5%)
Below Detection	15 (37.5%)	29 (47.5%)	19 (50.0%)	33 (45.8)
Range (gc/mL)	ND – 1.28 × 10^4	ND – 1.62 × 10^4	ND - 9.10 × 10^3	ND-2.3× 10^3
Median (gc/mL)	BQL	BQL	BQL	BQL
L. pneumophila (mip)	n = 40	n = 61	n = 38	n = 72
Detectable	7 (17.5%)	8 (13.1%)	3 (7.9%)	2 (2.8%)
BQL	6 (15.0%)	3 (4.9%)	3 (7.9%)	13 (18.0%)
Quantifiable	1 (2.5%)	5 (8.2%)	0 (0.0%)	1 (1.3%)
Below Detection	33 (82.5%)	53 (86.9%)	35 (92.1%)	57 (79.1%)
Range (gc/mL)	ND – 50.8	ND – 1.08 × 10^2	ND - BQL	ND-1.4× 10^2
Median (gc/mL)	ND	ND	ND	ND
Mycobacterium spp. (16S rRNA)	n = 40	n = 61	n = 38	n = 72
Detectable	18 (45.0%)	20 (31.7%)	5 (13.2%)	34 (47.2%)
BQL	14 (35.0%)	5 (8.2%)	1 (2.6%)	14 (19.4%)
Quantifiable	4 (10.0%)	15 (24.6%)	4 (10.5%)	20 (27.8%)
Below Detection	22 (55.0%)	41 (67.2%)	33 (86.8%)	38 (52.7%)
Range (gc/mL)	ND – 1.32 × 10^2	ND – 3.03 × 10^3	ND - 5.62 × 10^2	ND-5.9 × 10^2
Median (gc/mL)	ND	ND	ND	ND
M. avium (16S rRNA)	n = 40	n = 61	n = 38	n = 72
Detectable	13 (32.5%)	11 (18.0%)	3 (7.9%)	9 (12.5%)
BQL	13 (32.5%)	11 (18.0%)	3 (7.9%)	9 (12.5%)
Quantifiable	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0%)
Below Detection	27 (67.5%)	50 (82.0%)	35 (92.1%)	63 (87.5%)
Range (gc/mL)	ND – BQL	ND – BQL	ND - BQL	ND-BQL
Median (gc/mL)	ND	ND	ND	ND
N. fowleri (ITS)	n = 40	n = 61	n = 38	n = 72
Detectable	2 (5.0%)	8 (12.7%)	2 (5.2%)	2 (2.8%)
BQL	1 (2.5%)	8 (12.7%)	1 (2.6%)	1 (1.4%)
Quantifiable	1 (2.5%)	0 (0.0%)	1 (2.6%)	1 (1.5%))
Below Detection	38 (95%)	53 (86.9%)	36 (94.7%)	70 (97.2%)
Range (gc/mL)	ND – BQL	ND – BQL	ND – 2.51 × 10^2	ND-1.9
Median (gc/mL)	ND	ND	ND	ND

ND = not detected.
BQL = detected, but below limit of quantification.

differences in measured total bacterial gene copies may be related to geological differences among the sampling locations.

Legionella spp. were detected in 115 of 211 samples (54.5%), with quantifiable samples (41.2%) ranging from 8.17 to 1.62×10^4 gene copies/mL. *L. pneumophila*, was detected in 33 of the 115 samples that were positive for *Legionella* spp. (28.7%) and 15.6% of all samples. Samples with quantifiable L. *pneumophila* (3.3%) 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, $p = 0.48$) when comparing among the states.

Mycobacterium spp. were detected in 85 of 211 samples (40.3%). Samples with quantifiable *Mycobacterium* spp. (20.4%) ranged from 13.3 to 3.03×10^3 gene copies/mL. *M. avium,* was detected in 36 of the 85 samples (42.4%) positive for *Mycobacterium* spp. and 17.1% of all samples. However, all *M. avium* samples were all below the LOQ. 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 incidence of *N. fowleri* was low, with detection in only 14 of 211 samples (6.6%). 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 by state (Test of proportions, $p = 0.25$).

3.3. Relationship between indicator organisms and OP occurrence

Total coliform and *E. coli* were not strong indicators of OP occurrence (Table SI-2). Median overall method agreement (i.e., both methods positive or both methods negative) was 59%, with a range 46–92%. The positive predictive agreement (PPA) for total coliform and E.*coli* bacteria was 10–16% and 0–36%, respectively, for each specific OP species (*L. pneumophila*, *M. avium*, and *N. fowleri*). The slightly higher PPA for *E. coli* is attributed to the low detection rate.

3.4. Impacts of wellhead submersion and well system damage

Private wells that were reported to have submerged wellheads during flooding events tended to have an increased detection of surface water-associated contamination. Higher incidence rates of total coliform (Test of Proportions, $p = 0.02$) and *N. fowleri* ($p = 0.01$) were detected in submerged compared to unsubmerged wells (Table 4). This trend appeared to be driven by the incidence of wellhead submersion in Texas, as total bacterial gene numbers and detection of total coliform and *N. fowleri* were higher in submerged compared to unsubmerged in Texas ($p_{\text{total bacteria}} = 0.01$; $p_{\text{total coliform}} = 0.01$; $p_{N.fowerli} = 0.046$), but not in Florida, Louisiana, or North Carolina ($p = 0.44$–1.0). This may be due to the lower rates of wellhead submersion as well as the longer intervals between flooding and sampling (Table 2) in the other three states.

The detection of *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., and *M. avium*, were not different across all wells reported to be submerged versus not submerged (Table 4; Kruskal Wallis, $p = 0.29$–0.66) or when examining this comparison within each state ($p = 0.48$–1.0). Likewise, there were no differences in the detection of *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., or *M. avium*, across well systems categorized as damaged versus undamaged, across all states ($p = 0.44$–1.0) or within each state ($p = 0.24$–1.0). In addition, total coliforms, which were associated with wellhead submersion and are used as an indicator for surface water and fecal contamination, were not associated with detection frequency of *Legionella* or *Mycobacterium* ($p = 0.57$–1.0).

Reported well damage was not associated with occurrence levels of any of the pathogen targets. The primary damage reported was associated with the ability of the system to deliver well water to the home (i.e. pump functionality), which would not be related to the introduction of surface water contamination in the well system. 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). Thus, reported wellhead submersion was a better indicator of surface water contamination than damage to the well system for this study population.

3.5. Impact of shock chlorination

Well users that had submerged wells were more likely to shock chlorinate their system than well users without a submerged well (29% vs 9%), but shock chlorination did not appear to impact the occurrence of OPs or total bacteria in this study. Accounting for all samples, 31 well users reported shock chlorinating their system after the flooding event. Total bacterial gene copy numbers were not significantly different between wells that reported shock chlorinating versus those that did not (Wilcoxon, $p = 0.17$). OP detection was not significantly different between shock chlorinated wells and non-shock chlorinated wells (Test of proportions, $p = 0.22$–1.0), likely attributed to baseline groundwater concentrations.

3.6. Well system characteristic relationship with OP genes

Various well characteristics did not appear to have influenced OP detection. Well depths were similar among Texas, Louisiana, and Florida locations, and were not linked to the incidence of individual OPs detected in private wells overall (Spearman's, $p = 0.51$–0.98) or within each state ($p = 0.15$–0.91; Table 2). North Carolina tended to have shallower wells, but was not associated with a higher frequency of detection of target organisms. *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *N. fowleri*, and total bacteria gene copy numbers were not correlated to well construction year overall ($p = 0.10$–0.65; *Legionella* spp. reported in Fig. 3a) or in submerged wells ($p = 0.32$–0.73). However, in unsubmerged wells, *Legionella* spp. gene copy numbers were correlated with well construction year across all states ($p = 0.0057$, $\rho = 0.41$, $n = 133$; Fig. 3b; Fig. SI-2), meaning that newer wells tended to have higher levels of *Legionella* spp., with no clear mechanistic explanation.

3.7. Association between total bacterial 16S rRNA genes and OP marker genes

Our prior work in Louisiana documented a correlation between total bacterial 16S rRNA and *Legionella* spp. gene copy numbers (Dai et al., 2019). Here, we confirmed this trend in flushed cold water samples collected from Texas, Florida, and North Carolina. Total bacterial 16S rRNA and *Legionella* spp. gene copy numbers were positively correlated, across all states (Spearman, $p < 2.2 \times 10^{-16}$, $\rho = 0.72$) and within each state ($\rho = 0.61$–0.77; Fig. 2a). Similarly, total bacterial 16S rRNA and *Mycobacterium* spp. genes were positively correlated across all states ($\rho = 0.41$) and within each state ($\rho = 0.32$–0.56; Fig. 2b). Lower detection rates of L. *pneumophila* and *M. avium* prevented similar statistical comparisons. However, total bacterial 16S rRNA gene numbers were elevated in both L. *pneumophila* positive (Wilcoxon, $p = 0.0018$; Fig. 2c) and *M. avium* positive samples ($p = 6.52 \times 10^{-8}$; Fig. 2d) in all states, except North Carolina. No correlation or trend was observed between total bacteria and *N. fowleri* ($p = 0.33$; data not shown).

3.8. Associations between OP marker gene occurrences and inorganic constituents

There were no convincing associations between inorganic constituents in the water and OPs in this study. The concentration of some inorganics (i.e., arsenic, iron, manganese, sodium, chloride) varied among the states (Kruskal-Wallis, $p = 3.02 \times 10^{-11}$ - 0.023; Table 5), with some varying specifically between the Texas and Louisiana sampling locations (i.e., hardness, manganese), likely due to local variation the Coastal Plain aquifer system (Kruskal Wallis, $p = 1.34 \times 10^{-6}$ and 4.72×10^{-3}) [30−32]. Within some states, associations were found between some OPs and inorganics. For example, in Florida samples, sulfate, copper, and nitrate were significantly higher when *Legionella* spp. were

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)		North Carolina (n = 72)	
	Submerged (n = 9)	Unsubmerged (n = 24)	Submerged (n = 25)	Unsubmerged (n = 26)	Submerged (n = 3)	Unsubmerged (n = 12)	Submerged (n = 14)	Unsubmerged (n = 44)
	(% of 9 samples)	(% of 24 samples)	(% of 25 samples)	(% of 26 samples)	(% of 3 samples)	(% of 12 samples)	n (% of 14 samples)	n (% of 50 samples)
Total bacteria								
Detectable	9 (100%)	24 (100%)	25 (100%)	26 (100%)	3 (100%)	12 (100%)	14 (100%)	44 (100%)
BQL	0 (0%)	0 (0%)	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%)	14 (100%)	44 (100%)
Below detection	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Range (gc/mL)	884–1.23 × 10^7	530–2.47 × 10^7	442–8.35 × 10^6	1.05 × 10^3–4.53 × 10^6	740–5.00 × 10^6	210–3.90 × 10^6	536–2.1 × 10^5	50–8.09 × 10^6
Median (gc/mL)	6.19 × 10^5	1.09 × 10^6	3.64 × 10^5	3.41 × 10^4	1.20 × 10^3	3.50 × 10^4	2.97 × 10^4	2.35 × 10^4
Legionella spp.								
Detectable	6 (66.7%)	14 (58.3%)	15 (60%)	10 (38.5%)	1 (33.3%)	7 (58.3%)	6 (42.9)	26 (59.1%)
BQL	3 (33.3%)	3 (12.5%)	1 (4.0%)	5 (19.2%)	0 (0%)	2 (16.7%)	2 (14.3)	6 (13.6%)
Quantifiable	3 (33.3%)	11 (45.8%)	14 (56%)	5 (19.2%)	1 (33.3%)	5 (41.7%)	4 (28.6)	20 (45.5%)
Below Detection	3 (33.3%)	10 (41.7%)	10 (40%)	16 (61.5%)	2 (66.7%)	5 (41.7%)	8 (57.1)	18 (40.9%)
Range (gc/mL)	ND - 2.88 × 10^2	ND - 1.28 × 10	ND - 1.62 × 10^4	ND - 6.26 × 10^2	ND - 29.1	BQL - 9.10 × 10^3	ND-920	0–2309
Median (gc/mL)	BQL	BQL	1.41 × 10^1	ND	ND	BQL	ND	BQL
L. pneumophila								
Detectable	0 (0%)	4 (16.7%)	2 (8.0%)	4 (15.4%)	0 (0%)	1 (8.3%)	3 (21.4)	10 (22.7%)
BQL	0 (0%)	3 (12.5%)	2 (8.0%)	1 (3.8%)	0 (0%)	1 (8.3%)	3 (21.4)	9 (18.2%)
Quantifiable	0 (0%)	1 (4.2%)	0 (0%)	3 (11.5%)	0 (0%)	0 (0%)	0 (0)	2 (4.5%)
Below Detection	9 (100%)	20 (83.3%)	23 (92%)	22 (84.6%)	3 (100%)	11 (91.7%)	11 (78.6)	34 (77.3%)
Range (gc/mL)	all ND	ND - 5.08 × 10^1	ND - BQL	ND - 1.08 × 10^2	all ND	ND - BQL	ND-BQL	ND-1.37
Median (gc/mL)	ND	ND	ND	ND	ND	ND	ND	ND
Mycobacterium spp.								
Detectable	6 (66.7%)	10 (41.7%)	9 (36%)	10 (38.5%)	1 (33.3%)	2 (16.7%)	6 (42.9)	22 (50%)
BQL	6 (66.7%)	6 (25%)	14 (56%)	4 (15.4%)	0 (0%)	1 (8.3%)	3 (21.4)	6 (13.6%)
Quantifiable	0 (0%)	4 (16.7%)	8 (32%)	6 (23.1%)	1 (33.3%)	1 (8.3%)	3 (21.4)	16 (36.4%)
Below Detection	3 (33.3%)	14 (58.3%)	16 (64%)	16 (61.5%)	2	10 (83.3%)	8 (57.1)	22 (50%)
Range (gc/mL)	ND - BQL	ND - 1.32 × 10^2	ND - 3.03 × 10^3	ND - 8.49 × 10^2	ND - 4.55 × 10^1	ND - 110	ND-79	ND-586
Median (gc/mL)	BQL	ND	ND	ND	ND	ND	ND	ND
M. avium								
Detectable	3 (33.3%)	8 (33.3%)	7 (28%)	3 (11.5%)	0 (0%)	2 (16.7%)	1 (7.1)	7 (15.9%)
BQL	3 (33.3%)	8 (33.3%)	7 (28%)	3 (11.5%)	0 (0%)	2 (16.7%)	1 (7.1%)	7 (5.9%)
Quantifiable	0 (0%)	0 (0%)	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%)	13 (92.9%)	37 (84.1%)
Range (gc/mL)	ND - BQL	ND - BQL	ND - BQL	ND - BQL	all ND	ND - BQL	ND-BQL	ND-BQL
Median (gc/mL)	ND	ND	ND	ND	ND	ND	ND	ND
N. fowleri								
Detectable	1 (11.1%)	0 (0%)	6 (24%)	1 (3.8%)	0 (0%)	1 (8.3%)	1 (7.1%)	1 (2.3%)
BQL	1 (11.1%)	0 (0%)	6 (24%)	1 (3.8%)	0 (0%)	0 (0%)	0 (0%)	1 (2.3%)
Quantifiable	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)	1 (7.1%)	0 (0%)
Below Detection	8 (88.9%)	24 (100%)	19 (76%)	25 (96.2%)	3 (100%)	11 (91.7%)	13 (92.9%)	43 (97.8%)
Range (gc/mL)	ND - BQL	all ND	ND - BQL	ND - BQL	all ND	ND - 251	ND-1.86	ND-BQL
Median (gc/mL)	ND	ND	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%)	8 (57.1%)	20 (45.5%)
Below Detection	5 (55.6%)	15 (62.5%)	10 (40.0%)	20 (76.9%)	3 (100%)	9 (75.0%)	6 (42.9%)	24 (54.5%)
Range (MPN/100 mL)	ND - 4.11 × 10^2	ND - 283	ND - 1000	ND - 416	all ND	ND - 14.7	ND- > 2419.6	ND-1203.3
Median (MPN/100 mL)	ND	ND	1.00	ND	ND	ND	7.8	ND
E. coli								
Detectable	1 (11.1%)	0 (0%)	6 (24.0%)	1 (3.8%)	0 (0%)	0 (0%)	1 (7.1%)	0 (0%)
Below Detection	8 (88.9%)	24 (100%)	19 (76.0%)	25 (96.2%)	3 (100%)	12 (100%)	13 (92.9%)	44 (100%)
Range	ND - 1.00	ND	ND - 77	ND - 2.00	ND	ND	ND-1.0	ND
Median	ND	ND	ND	ND	ND	ND	ND	ND

ND = not detected.
BQL = detected, but below limit of quantification.

detected than when not detected (Wilcoxon, p = 0.027–4.76 × 10^{-5}). Further, iron was significantly lower in samples where *Legionella* spp. were detected in samples without detected *Legionella* spp. (p = 0.029). However, the detection of OPs overall was sporadic, making it difficult to identify potential overarching patterns or associations between inorganics and OPs.

4. Discussion

4.1. Reported submersion as an indicator for pathogen occurrence

In this study, reported wellhead submersion served as a better indicator for potential surface water and fecal contamination than reported well

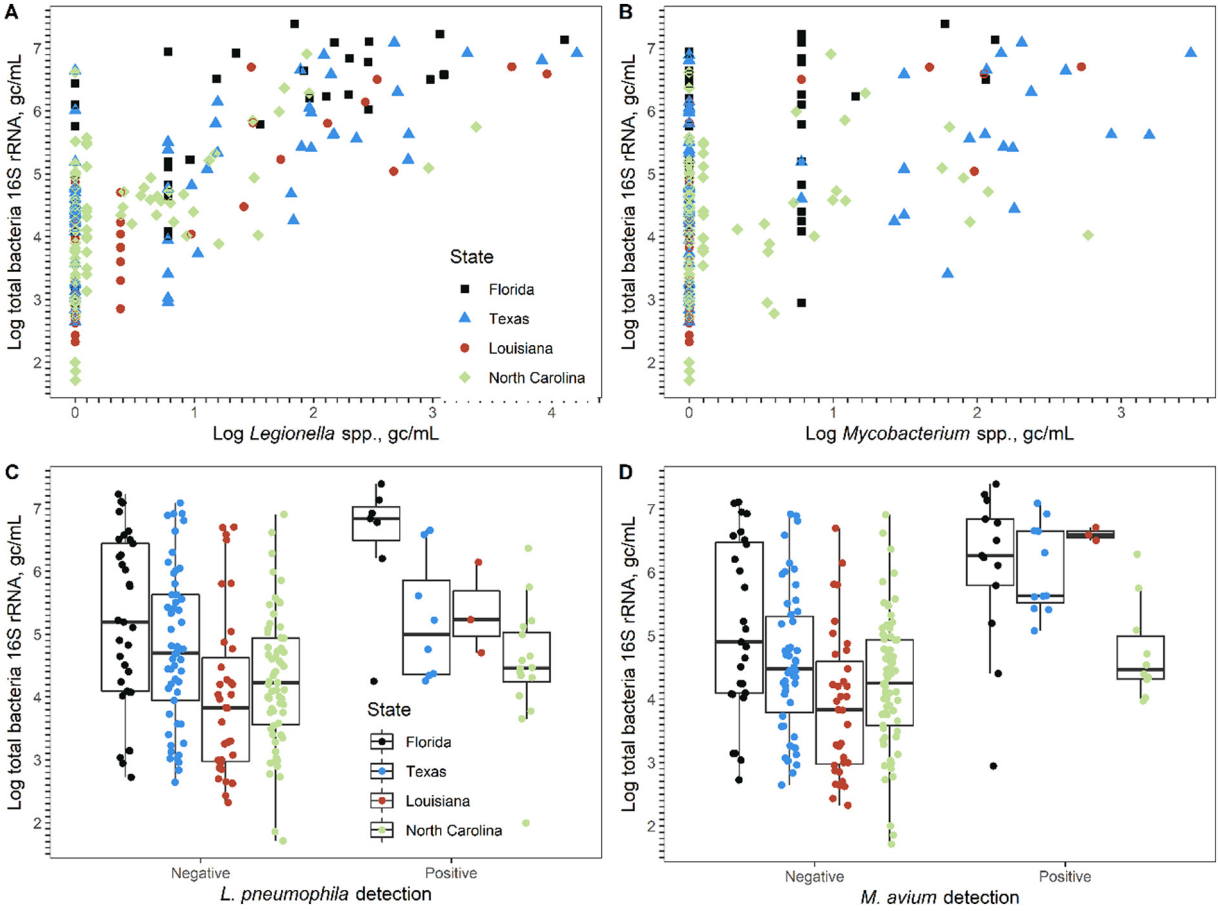

Fig. 2. Positive correlations between total bacteria and A) *Legionella* spp. and B) *Mycobacterium* spp., and boxplot of total bacteria within samples that were positive and negative for C) *L. pneumophila* and D) *M. avium*.

damage, which is consistent with our prior work exploring the incidence of coliform bacteria. Wellhead submersion can serve as a pathway for contaminated surface water to breach wells and at-risk systems could potentially be identified with remotely-sensed flooding map applications (e.g., Moderate Resolution Imaging Spectroradiometer [MODIS]). However, here, reported well damage was primarily related to pump

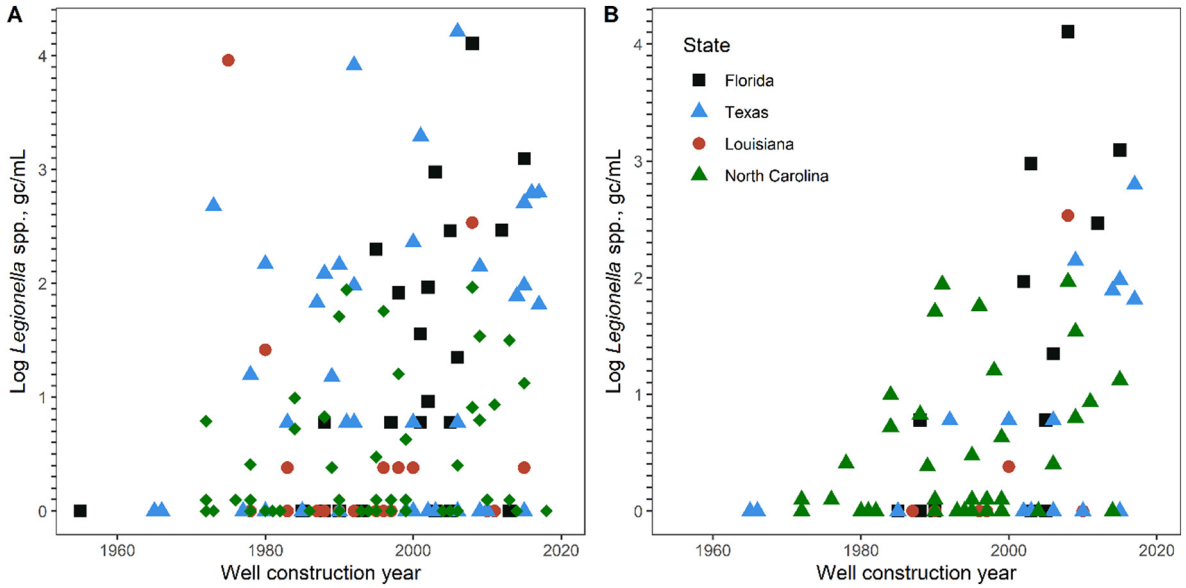

Fig. 3. Relationship between *Legionella* spp. and well construction year in A) all well samples and B) in only unsubmerged wells. No significant correlation was found between *Legionella* spp. and well construction year when all samples were included in the analysis. There was a significant positive correlation between *Legionella* spp. and well construction year in all unsubmerged wells as well as in unsubmerged wells in Texas and Florida.

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)				North Carolina (*n* = 79)			
			Med.	90th %ile	Max.	% > standard	Med.	90th %ile	Max.	% > standard	Med.	90th %ile	Max.	% > standard	Med.	90th %ile	Max.	% > standard
Arsenic, μg/L	MCL	10	<0.5	0.8	4	0.0	1.9	6.6	9.6	0.0	<0.5	2.3	27.4	2.6	<	0.6	5.4	0.0
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	<	<	0.5	0.0
Chromium, μg/L		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	0.3	1.0	9.0	0.0
Nitrate, mg N/L		10	0.1	1.8	31.8	2.5	0.1	1.8	5	0.0	NA	NA	NA	NA	<0.02	5.7	12.0	1.3
Copper, μg/L	Action	1300	3	24.5	176.1	0.0	3.8	36.6	152.5	0.0	1.4	13.9	208.4	0.0	8.4	63.0	192.7	0.0
Lead, μg/L	level	15	<1.0	<1.0	1.2	0.0	<1.0	1.3	6	0.0	<1.0	2.3	5.9	0.0	0.5	4.2	116.4	2.5
Chloride, mg/L[a]	SMCL	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	9.3	55.2	1526	1.3
Iron, μg/L		300	14.5	278.4	720	7.5	50.7	629.2	2029	28.9	132	613.7	1872	28.9	54.7	1702	14,115	32.9
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	10.9	46.7	195.9	8.8
Silver, μg/L		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	<	<	0.4	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	4.6	18.3	31.0	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	9.0	119	2715	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	7.9	131.4	614.6	31.6
Hardness, mg/L as $CaCO_3$	–	–	103.2	296.1	535	–	120.9	333	401.1	–	28	46.3	124.8	–	17.2	124.0	214.9	–

NA: parameter not analyzed.
MCL: maximum contaminant level.
SMCL: secondary maximum contaminant level.
DWEL: drinking water equivalent level.
[a] ICP reported for Texas, Florida, and North Carolina; IC reported for Louisiana.

functionality which would not have a direct impact on intrusion. This study observed that submersion of wellheads was associated with detection of *N. fowleri*, but the human health risk of infection following flooding events may still be low because exposure to this pathogen requires the organism to forcefully enter the nasal passages. However, if contamination of *N. fowleri* occurs during flooding, the amoeba might persist in the plumbing and later pose an opportunity to infect, further underscoring the need for long-term monitoring of OPs in well water.

Because wellhead submersion was not identified as a route of contamination for *Legionella* or *Mycobacterium* intrusion in private wells in this study, it is likely that, for these OPs, their elevated frequencies of detection and overall numbers is primarily a function of background occurrence in groundwater and specific conditions in individual homes stimulating their growth (e.g., water temperature and demand patterns) than flood impacts. Both *Legionella* and *Mycobacterium* are commonly detected in groundwater (Riffard et al., 2001; Richards et al., 2018; Stojek and Dutkiewicz, 2006; Stojek and Dutkiewicz, 2011; Brooks et al., 2004).

4.2. Limited benefit of shock chlorination for OP control

There have been numerous concerns raised regarding the reliability and efficacy of shock chlorination for post-flood pathogen control due to lack of standardized, science-based protocols (Pieper et al., 2020). There are additional concerns regarding the efficacy of shock chlorination to eliminate OPs in private wells. Prior research has indicated shock chlorination to be ineffective for OP control in large buildings water systems (Borella et al., 2016). Often, the same *Legionella* strains found before shock chlorination reemerge several weeks afterwards (e.g., see reference (Borella et al., 2016). *Mycobacterium* spp. are also known to be resistant to chlorine, which essentially acts to kill off competitors and enhance their ability to proliferate (Taylor et al., 2000). Therefore, it would not be expected that shock chlorination would be an effective strategy for mitigating potential introduction of OPs to private wells due to storm events, unless it can be confirmed that *Legionella* or *Mycobacterium* do not naturally occur in the groundwater or were not pre-existing in the system.

4.3. Contextualizing OP occurrence

While Legionnaires' disease has been linked to private wells in past research, the etiology of the 64% of Legionnaires' disease burden in the US is undetermined (Shah et al., 2018). Knowledge with respect to baseline incidence of *Legionella* in home plumbing systems is broadly lacking, particularly in private wells, and such knowledge is critically needed in order to begin to quantify potential disease burden related to home plumbing systems. The frequency of detection of *Legionella* spp. in this study was similar to positivity rates found in municipal water studies. For example, a survey of two chloraminated drinking water systems reported that 30–82% of samples were positive for *Legionella* spp. (Wang et al., 2012). Detection of L. *pneumophila,* however, was lower in this study (12.9%) than what was reported in a nationwide survey of municipal tap water, in which most of the sampling sites were large buildings (47% of 68 sites) (Donohue et al., 2014). The likelihood of *Legionella* proliferation in household plumbing systems supplied by private wells may be lower than in larger buildings due to the relatively simpler building structure (e.g., less surface area, fewer dead ends, smaller hot water storage and more system turnover). However, conditions characteristic of individual homes supplied by private wells may offset the potential benefits of smaller, simpler plumbing systems, particularly where *Legionella* are members of the background microbial community in the groundwater. Our 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 after passing through the premise plumbing and sampling at the corresponding taps (Dai et al., 2019).

There has been extensive focus on L. *pneumophila,* but other species of *Legionella*, such as L. *longbeachae*, *L. micdadei*, *L. bozemanii*, and L. *dumoffii* are also known human pathogens (Muder and Yu, 2002). In this study, *L. pneumophila* was not the dominant species of *Legionella* detected, as L. *pneumophila* qPCR gene copy ratios represented less than 3% of the *Legionella* spp. gene copy numbers in 90% of all samples collected. This is also similar to studies conducted in municipal systems, where L. *pneumophila* accounted for 0.1–1.0% of the total *Legionella* spp. detected (Wullings and van der Kooij, 2006), though there are also examples where L. *pneumophila* was the dominant species (e.g., 33). 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 (Costa et al., 2005).

The risk for infections caused by the *M. avium* complex and other species of *Mycobacterium* associated with private wells following floods are unknown. To our knowledge, only one study 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. (Richards et al., 2018). However, the levels of *Mycobacterium* spp. detected in this study were similar to reported levels

in flushed samples collected from buildings in chloraminated municipal drinking water systems (Wang et al., 2012). In a study of municipal drinking water systems, *M. avium* numbers were correlated with turbidity in raw source waters cause by heavy rains (Falkinham et al., 2001), so it is possible that *Mycobacterium* occurrence increases in systems with submerged wellheads, but such associations were no identified in this study. According to qPCR gene copy ratios, *M. avium* dominated the *Mycobacterium* genus in approximately 20% of samples in this study. Although *M. avium* is documented to be the most common species associated with MAC infections in immunocompromised individuals, there are other known pathogenic nontuberculous mycobacteria, including as *M. intracellulare, M. kansasii, M. abscessus, and M. chelonae* (e.g., see reference (Shin et al., 2008).

N. fowleri has been linked to several public water utility supplies (Bartrand et al., 2014; Miller et al., 2017), and has been detected in wells used as a public drinking water supply (Blair et al., 2008; Gerba et al., 2009; Bright et al., 2009). Our comprehensive questionnaire of post-flood samples in Louisiana included additional samples taken from within the home plumbing systems and indicated that *N. fowleri* DNA was detected in 20% of homes supplied by private wells (Dai et al., 2019). As discussed above, assessing the health risk from *N. fowleri* is difficult because the exposure route requires nasal aspiration, but the fate of *N. fowleri* after being introduced to well systems may be important to document.

4.4. Deviations from conventional wisdom developed in municipal systems

Coliform and *E. coli* bacteria are used in municipal systems to meet the Total Coliform Rule requirements and in private wells as an indicator of surface water and fecal contamination. However, coliform bacteria are not effective indicators for OPs. OPs, in comparison to coliform and fecal pathogens, are naturally present in many aquatic environments and readily grow in many oligotrophic drinking water environments. In this study, as expected, total coliforms and *E. coli* did not serve as effective indicators of all OPs surveyed.

The positive correlations between total bacterial 16S rRNA and OP gene markers in this study concurs with our prior comprehensive survey of well water (Dai et al., 2019), but is contrary to observations from field work in buildings supplied by municipal drinking water. In municipal systems, *Legionella* often occurs independently of total bacterial numbers or heterotrophic plate counts (Duda et al., 2015). It is well-documented that *Legionella* can resist chemical disinfectants (Falkinham et al., 2015), while the majority of microbial members of the community may be more susceptible to disinfection. Since private wells rarely employ routine disinfection (Pieper et al., 2015; Swistock et al., 2013), conditions that support 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 (Dai et al., 2019). In experimental apparatuses and municipal systems that observe total biomass or heterotrophic plate count bacteria are associated with higher *Legionella* numbers, no residual disinfectant is detected (Bargellini et al., 2011; van der Kooij et al., 2005). However, high total bacterial numbers are not always associated with *Legionella* and are therefore not an effective screening metric, though excessive levels of total bacteria can indicate systemic water quality problems.

The finding that newer wells tended to harbor higher levels of *Legionella* spp. also conflicts with the conventional wisdom for municipal systems, wherein older buildings and homes are more frequently associated with the occurrence of *Legionella* (Alary and Joly, 1991). This discrepancy may also be related to municipal systems use of secondary disinfectants to prevent bacterial regrowth within the distribution system. Older areas of municipal systems tend to react with and deplete residual faster than newer areas with less reactive pipe materials, facilitating the growth of microorganisms. It is possible that better well construction practices in new wells, together with the lack of residual disinfectants, create a highly oligotrophic niche for *Legionella* without competition with other organisms that may be characteristic of older wells with more background organisms.

4.5. Need for well system sampling guidance

Timing of sampling after flooding events may impact the levels of waterborne pathogens measured. Based on availability of samples during an urgent time of emergency response, sampling occurred 20–76 days after the storms, depending on the state. While inactivation rate models have not yet been developed for the investigated OPs in private wells, one natural attenuation model predicts 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 were completely stagnant before measurement (Nevecherya et al., 2005). 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 were not available before the storms, rendering it impossible to differentiate the impact of the storm compared to normal conditions. To most accurately assess exposure risk of waterborne pathogens caused by flooding, sampling should occur as the well users begin using the water during the recovery process. Broader surveillance and reconnaissance efforts can also help to better define baselines and thus associated impacts of floods.

4.6. Study limitations

Sample size and participation in this study was largely dictated by accessibility, which was challenging during periods of post-storm emergency response due to disruptions in communication and transportation. In addition, sampling campaigns started at different periods after the respective storms, which was based on level of existing collaboration with community partners at the time of the study. These factors, combined with the lack of background incidence data, may have influenced our evaluation of the impact of flooding on OPs in private wells; however, it was possible to generally conduct a comprehensive survey of at risk wells to determine OP occurrence, something that is not previously documented, and to determine if there is evidence for elevated risk compared to what is known about OP occurrence in other tap water surveys. The questionnaire used in Louisiana was modified before application in Texas, Florida, and North Carolina, which resulted in different questions on the questionnaires (e.g., residents in Louisiana were not explicitly asked if their wells were submerged).

5. Conclusions: implications for private well stewardship practices

Overall, the contribution of private well systems as a potential source of OP infections in the US remains unclear. This study provides vital information about OP occurrences and levels for presumed worst-case conditions following major storms and potential breaching of systems. Given the general lack of association of OPs with private well characteristics, likely ineffectiveness of shock chlorination as an effective long-term remedial strategy, variability in pathogenic species of OPs, and multiple exposure routes, it may be difficult to generalize risk estimates for private well users. Such risks to human health might be best assessed on a site-by-site basis. While temperature of the water heater and water usage patterns are consistently successful interventions, they are not always successful. Thus, it may be more feasible to identify appropriate and effective preventative or remedial treatments that immunocompromised or concerned well users can implement.

CRediT authorship contribution statement

Kris Mapili: Data curation; Formal analysis; Writing - original draft. **William J. Rhoads:** Conceptualization; Funding acquisition; Supervision; Validation; Formal analysis; Writing - original draft, review & editing, Methodology. **Mary Coughter**: Data curation. **Kelsey Pieper:** Conceptualization; Funding acquisition; Supervision; Project administration; Writing - review & editing. **Amy Prduen**: Conceptualization; Funding acquisition; Writing - review & editing. **Marc A Edwards**: Conceptualization; Funding acquisition; Writing - review & editing.

Declaration of competing interest

The authors have no completing or conflicting interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153901.

References

Alary, M., Joly, J.R., 1991. Risk factors for contamination of domestic hot water systems by Legionellae. Appl. Environ. Microbiol. 57 (8), 2360–2367.
American Public Health Association, 1998. American Water Works Association; Water Environment Federation in Standard Methods for Examination of Water and Wastewater. 20th ed. American Public Health Association, Washington, D.C.
Bargellini, A., et al., 2011. Parameters predictive of Legionella contamination in hot water systems: association with trace elements and heterotrophic plate counts. Water Res. 45 (6), 2315–2321.
Bartrand, T.A., Causey, J.J., Clancy, J.L., 2014. Naegleria fowleri: an emerging drinking water pathogen. J. Am. Water Works Assoc. 106 (10), E418–E432.
Blair, B., et al., 2008. Naegleria fowleri in well water. Emerg. Infect. Dis. 14 (9), 1499–1501.
Borella, P., et al., 2016. Hospital-acquired Legionella infections: an update on the procedures for controlling environmental contamination. Ann. Ig. 28 (2), 98–108.
Bright, K.R., Merciano-Cabral, F., Gerba, C.P., 2009. Occurrence of Naegleria fowleri in Arizona drinking water supply wells. J. Am. Water Works Assoc. 101 (11), 43–50.
Brooks, T., Osicki, R.A., Springthorpe, V.S., Sattar, S.A., Filion, L., Abrial, D., Riffard, S., 2004. Detection and identification of Legionella species from groundwaters. J. Toxic. Environ. Health A 67 (20–22), 1845–1859.
Costa, J., et al., 2005. Presence and persistence of Legionella spp. in groundwater. Appl. Environ. Microbiol. 71 (2), 663–671.
Dai, D., Rhoads, W.J., Katner, A., Strom, L., Edwards, M.A., Pruden, A., Pieper, K.J., 2019. Molecular survey of Legionella and Naegleria fowleri in private well water and premise plumbing following the 2016 Louisiana flood. Environ. Sci.: Water Res. Technol. 5 (8), 1464–1477.
Donohue, M.J., et al., 2014. Widespread molecular detection of Legionella pneumophila Serogroup 1 in cold water taps across the United States. Environ. Sci. Technol. 48 (6), 3145–3152.
Duda, S., et al., 2015. Lack of correlation between Legionella colonization and microbial population quantification using heterotrophic plate count and adenosine triphosphate bioluminescence measurement. Environ. Monit. Assess. 187 (7), 393.
Eccles, K.M., et al., 2017. Lessons learned from the 2013 Calgary flood: assessing risk of drinking water well contamination. Appl. Geogr. 80, 78–85.
Fagan, P.K., et al., 1999. Detection of Shiga-like toxin (stx1 and stx2), intimin (eaeA), and enterohemorrhagic Escherichia coli (EHEC) hemolysin (EHEC hlyA) genes in animal feces by multiplex PCR. Appl. Environ. Microbiol. 65 (2), 868–872.
Falkinham III, J.O., Norton, C.D., LeChevallier, M.W., 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. Appl. Environ. Microbiol. 67 (3), 1225–1231.
Falkinham, J.O., Pruden, A., Edwards, M., 2015. Opportunistic premise plumbing pathogens: increasingly important pathogens in drinking water. Pathogens 4 (2), 373–386.
Fields, B.S., Sanden, G.N., Barbaree, J.M., Morrill, W.E., Wadowsky, R.M., White, E.H., Feeley, J.C., 1989. Intracellular multiplication of Legionella pneumophila in amoebae isolated from hospital hot water tanks. Curr. Microbiol. 18, 131–137.
Garner, E., McLain, J., Bowers, J., Engelthaler, D.M., Edwards, M.A., Pruden, A., 2018. Microbial ecology and water chemistry impact regrowth of opportunistic pathogens in full-scale reclaimed water distribution systems. Environ. Sci. Technol. 52 (16), 9056–9068.
Gerba, C.P., et al., 2009. Occurrence and control of Naegleria fowleri in drinking water wells. In: OrtegaPierres, G., et al. (Eds.), Giardia and Cryptosporidium: From Molecules to Disease. Cabi Publishing-C a B Int., Wallingford, pp. 238–247.
Gilliland, A.E., Pieper, K., Straif-Bourgeois, S., Rhoads, W.J., Dai, D., Edwards, M., Katner, A., 2021. Evaluation of preparedness and recovery needs of private well users after the Great Louisiana Flood of 2016. J. Public Health Manag. Pract. 27 (6), 577–587.
van der Kooij, D., Veenendaal, H.R., Scheffer, W.J., 2005. Biofilm formation and multiplication of Legionella in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res. 39 (13), 2789–2798.
Laseke, I., et al., 2010. Identification of Naegleria fowleri in warm ground water aquifers. J. Environ. Qual. 39 (1), 147–153.
Lehtola, M.J., et al., 2007. Survival of Mycobacterium avium, Legionella pneumophila, Escherichia coli, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. Appl. Environ. Microbiol. 73 (9), 2854–2859.
Mapili, K., Pieper, K.J., Dai, D., Pruden, A., Edwards, M.A., Tang, M., Rhoads, W.J., 2020. Legionella pneumophila occurrence in drinking water supplied by private wells. Lett. Appl. Microbiol. 70 (4), 232–240.
Marciano-Cabral, F., et al., 2003. Identification of Naegleria fowleri in domestic water sources by nested PCR. Appl. Environ. Microbiol. 69 (10), 5864–5869.
Miller, H.C., et al., 2017. Elimination of Naegleria fowleri from bulk water and biofilm in an operational drinking water distribution system. Water Res. 110, 15–26.
Muder, R.R., Yu, V.L., 2002. Infection due to Legionella species other than L. pneumophila. Emerg. Infect. 35, 990–998.
Mull, B.J., Narayanan, J., Hill, V.R., 2013. Improved method for the detection and quantification of Naegleria fowleri in water and sediment using immunomagnetic separation and real-time PCR. J. Parasitol. Res. 2013, 608367.
Nazarian, E.J., et al., 2008. Design and implementation of a protocol for the detection of Legionella in clinical and environmental samples. Diagn. Microbiol. Infect. Dis. 62 (2), 125–132.
Nevecherya, I.K., et al., 2005. Survival rate of pathogenic bacteria and viruses in groundwater. Water Resourc. 32 (2), 232–237.
Pieper, K.J., Krometis, L.A.H., Gallagher, D.L., Benham, B.L., Edwards, M., 2015. Incidence of waterborne lead in private drinking water systems in Virginia. J. Water Health 13 (3), 897–908.
Pieper, K.J., Rhoads, W.J., Saucier, L., Katner, A., Barrett, J.R., Edwards, M., 2020. Improving state-level emergency well disinfection strategies in the United States. Sci. Total Environ. 720, 137451.
Pieper, K.J., Jones, C.N., Rhoads, W.J., Rome, M., Gholson, D.M., Katner, A., Boellstorff, D.E., Beighley, R.E., 2022. Microbial contamination of drinking water supplied private wells after Hurricane Harvey. Environ. Sci. Technol. 55 (12), 8392-8392.
Radomski, N., et al., 2010. Development of a real-time qPCR method for detection and enumeration of Mycobacterium spp. in surface water. Appl. Environ. Microbiol. 76 (21), 7348–7351.
Richards, C.L., et al., 2018. Detection of pathogenic and non-pathogenic bacteria in drinking water and associated biofilms on the crow reservation, Montana, USA. Microb. Ecol. 76 (1), 52–63.
Riffard, S., et al., 2001. Occurrence of Legionella in groundwater: an ecological study. Water Sci. Technol. 43 (12), 99–102.
Shah, P., Shah, P.P., Barskey, A.E., Binder, A.M., Edens, C., Lee, S., Smith, J.C., Schrag, S., Whitney, C.A., Cooley, L.A., 2018. Legionnaires' Disease Surveillance Summary Report, United States: 2014-1015.
Shin, J.H., et al., 2008. Targeting the rpoB gene using nested PCR-restriction fragment length polymorphism for identification of nontuberculous mycobacteria in hospital tap water. J. Microbiol. 46 (6), 608–614.
Stojek, N.M., Dutkiewicz, J., 2006. Legionella and other gram-negative bacteria in potable water from various rural and urban sources. Ann. Agric. Environ. Med. 13, 323–335.
Stojek, N.M., Dutkiewicz, J., 2011. Co-existence of Legionella and other Gram-negative bacteria in potable water from various rural and urban sources. Ann. Agric. Environ. Med. 18 (2), 330–334.
Suzuki, M.T., Taylor, L.T., DeLong, E.F., 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl. Environ. Microbiol. 66 (11), 4605–4614.
Swistock, B.R., Clemens, S., Sharpe, W.E., Rummel, S., 2013. Water quality and management of private drinking water wells in Pennsylvania. J. Environ. Health 75, 60–66.
Taylor, R.H., Falkinham, J.O., Norton, C.D., LeChevallier, M.W., 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of Mycobacterium avium. Appl. Environ. Microbiol. 66 (4), 1702–1705.
USGS, n.d. USGS Ground Water Atlas of the United States, Hydrologic Atlas 730. 10.3133/ha730.
Van Biersel, T.P., Carlson, D.A., Milner, L.R., 2007. Impact of hurricanes storm surges on the groundwater resources. Environ. Geol. 53 (4), 813–826.
W.J. Rhoads et al, Impact of Residential Water Heater Cleaning on Sediment Composition, Inorganic Loading, and Legionella Gene Markers in the Aftermath of Widespread Distribution System Corrosion in Flint, MI. In preparation.
Wang, H., Edwards, M., Falkinham, J.O., Pruden, A., 2012. Molecular survey of the occurrence of Legionella spp., Mycobacterium spp., Pseudomonas aeruginosa, and amoeba hosts in two chloraminated drinking water distribution systems. Appl. Environ. Microbiol. 78 (17), 6285–6294.
Wilton, S., Cousins, D., 1992. Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube. Genome Res. 1, 269–273.
Wullings, B.A., van der Kooij, D., 2006. Occurrence and genetic diversity of uncultured Legionella spp. in drinking water treated at temperatures below 15 degrees C. Appl. Environ. Microbiol. 72 (1), 157–166.