Molecular detection of opportunistic pathogens and insights into microbial diversity in private well water and premise plumbing

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ABSTRACT

Private well water systems in rural areas that are improperly maintained will result in poor drinking water quality, loss of water supply, and pose human health risk. The purpose of this study was to investigate the occurrence of fecal indicator bacteria (FIB) and opportunistic pathogens in private well water in rural areas surrounding New Orleans, Louisiana. Our results confirmed the ubiquitous nature of *Legionella* (86.7%) and mycobacteria (68.1%) in private well water in the study area, with gene concentration ranged from 0.60 to 5.53 and 0.67 to 5.95 Log_{10} of GC/100 mL, respectively. *Naegleria fowleri* target sequence was detected in 16.8% and *Escherichia coli* was detected in 43.4% of the water samples. Total coliform, as well as *Legionella* and mycobacteria genetic markers' concentrations were significantly reduced by 3-minute flushing. Next-generation sequencing (NGS) data indicated that the abundance of bacterial species was significantly increased in water collected in kitchens compared with samples from wells directly. This study provided integrated knowledge on the persistence of pathogenic organisms in private well water. Further study is needed to explore the presence of clinical species of those opportunistic pathogens in private well water systems to elucidate the health risk.

Key words | *Legionella*, *Mycobacterium*, *Naegleria fowleri*, next-generation sequencing, qPCR

HIGHLIGHTS

- Legionella spp. was found in 86.7% (98/113) followed by mycobacteria in 68.1% (77/113).
- Total coliform, *Legionella* and mycobacteria DNA genetic markers were significantly reduced by 3-minute flushing.
- Proteobacteria was the most dominant phylum followed by Chlorobi, Actinobacteria, Chloroflexi, Cyanobacteria, and Bacteroidetes.

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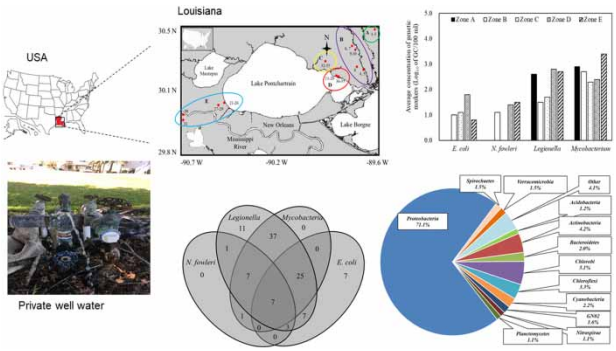
GRAPHICAL ABSTRACT



INTRODUCTION

In the United States, approximately 15.8 million occupied households (15% of the population) rely on a private well as drinking water source due to its advantages such as being cheap, more convenient, and less vulnerable to surface water pollution (DeSimone *et al.* 2009; Won *et al.* 2013). In Louisiana, 13% of the population (~588,000 residents) use groundwater from privately owned wells for their domestic water use. The U.S. Environmental Protection Agency (EPA) regulations that protect public drinking water systems do not apply to individual water systems. Private well users are solely responsible for the maintenance of their own water supply system. Various contaminants in groundwater may be found in private wells, such as bacteria, viruses, parasites, chemicals, and unknown etiology (Craun *et al.* 2010; Kirk *et al.* 2011). Many private well owners in Louisiana live in rural regions where there is limited access to the financial resources necessary to address water quality issues (Allevi *et al.* 2013).

The microbiological water quality in private wells is generally assessed by monitoring fecal indicator bacteria (FIB) such as total coliform and *Escherichia coli* (*E. coli*) (Allevi *et al.* 2013; Won *et al.* 2013; Smith *et al.* 2014). The most important limitation of FIB arises from their poor correlation with pathogens in water (Ahmed *et al.* 2009). Culture-based methods for identification of pathogens are often time-consuming and may fail to detect some organisms due to unsuccessful cultivation, i.e., the viable but not-culturable state (VBNC) (Evangelista & Coburn 2010; Ramamurthy *et al.* 2014). Opportunistic bacterial pathogens such as *Legionella* spp. and mycobacteria species have attracted worldwide attention as they are now the primary source of drinking water-related disease outbreaks all over the world (Edge *et al.* 2013; Wu *et al.* 2014; Beer *et al.* 2015; Benedict *et al.* 2017; Wang *et al.* 2017). *Legionella* continues to be the most frequently reported (over 55% of outbreaks) etiology among drinking water-associated outbreaks in the USA for several decades (Craun *et al.* 2010; Centers for Disease Control and Prevention (CDC) 2013; Beer *et al.* 2015; Benedict *et al.* 2017). The presence of *Legionella* in drinking water systems is the cause of both Legionnaires' disease and Pontiac fever (Nazarian *et al.* 2008; Hamilton *et al.* 2017; Wang *et al.* 2017), whereas *Mycobacterium* spp., such as *Mycobacterium tuberculosis* complex (MTC), are responsible for more than two million human deaths every year (García-Quintanilla *et al.* 2002). Nontuberculous mycobacteria (NTM), on the other hand, are a major cause of opportunistic infections in immunocompromised individuals, such as AIDS patients (Falkinham 1996). In addition, primary amoebic meningoencephalitis (neurological illness) caused by *Naegleria fowleri* (*N. fowleri*) poses a risk in human health (Craun *et al.* 2010; Xue *et al.* 2017). These microorganisms are natural inhabitants of drinking water systems and can survive in temperatures over 50 °C (Leoni *et al.* 2001; Falkinham 2009). Moreover, free-living amoeba have been considered as hosts for *Legionella* and mycobacterial

species, facilitating their transmission and replication (Delafont *et al.* 2014).

Although bacterial community diversity has been previously studied extensively in groundwater used for drinking in various locations with or without contamination (Cho & Kim 2000; Chang *et al.* 2001; Röling *et al.* 2001; Reardon *et al.* 2004; Won *et al.* 2013; Maran *et al.* 2016; Murray *et al.* 2018), as well as the occurrences of opportunistic pathogens in fresh water systems (Huang *et al.* 2010; Bargellini *et al.* 2011; Mansi *et al.* 2014; Hamilton *et al.* 2017, 2018), much less information is available on the bacterial diversity in salt-marsh groundwater samples. To the best of our knowledge, many studies have looked at the occurrence of *N. fowleri* in surface water, geothermally heated water (hot spring), and other thermally impacted waters such as power plant cooling reservoirs (Niyyati *et al.* 2012; Siddiqui *et al.* 2016; Bright & Gerba 2017; Xue *et al.* 2017), but very few have studied its occurrence in private well water, except one study conducted by Bright *et al.* (2009) in the Phoenix area in 2002. In addition, it is also unclear how and to what extent the populations of FIB would affect the occurrence of *Legionella* spp., *Mycobacterium* spp., and *N. fowleri* in private wells.

Advances in molecular methods and next-generation sequencing (NGS) offered a unique opportunity for water quality assessment through direct detection and enumeration of specific pathogens such as *Legionella*, mycobacteria, viruses (e.g., Norovirus) and parasites (Tan *et al.* 2015). Quantitative polymerase chain reaction (qPCR) targeting regions of the small subunit (SSU) rRNA gene and functional genes allow rapid and sensitive identification/quantification of pathogenic bacteria and protozoa before culture results. This study provides a comprehensive molecular survey of the occurrence of FIB, *Legionella* spp., *Mycobacterium* spp., and *N. fowleri* in groundwater in a subtropical area. The aims of this study were: (1) to reveal the occurrence pattern and identify correlations, if any, between pathogenic bacteria and *N. fowleri*; (2) to explore the effect of environmental factors on the occurrence of *Legionella* spp., *Mycobacterium* spp., and *N. fowleri*; (3) to determine whether FIB monitoring might reflect the presence/absence of these pathogenic bacteria; (4) to explore the microbial community diversity in private well water in Louisiana.

EXPERIMENTAL PROCEDURES

Sample collection

In total, 113 water samples were collected from 55 private wells (two samples from each well), and three wells (8, 23, and 34) have three samples collected (one more sample collected from the kitchen tap) in New Orleans, Louisiana (Figure 1). According to the location of each well, we have divided all our samples in five sampling zones: Zone A (1–3, northeast of Pearl River Wildlife Management Area), Zone B (4–10, southwest of Pearl River Wildlife Management Area), Zone C (32–35, northeast of Lake Pontchartrain in land), Zone D (11–20 and 36–55, northeast of Lake Pontchartrain on the beach), Zone E (21–31, southwest of Lake Pontchartrain). Two types of water samples were collected from each site: pre-flush sample is the water sample collected at the very beginning; then allowed to flush at maximum rate for 3 minutes before post-flush water samples were collected. Two liters of pre- and post-flush water samples were collected in sterile plastic containers and placed on ice immediately. A YSI Pro2030 meter (YSI Incorporated, OH, USA) was used to measure *in-situ* dissolved oxygen (DO), temperature (Tm), salinity, specific conductivity, and pH. To prevent cross-contamination between each sample, the YSI meter probe was rinsed by using distilled water.

DNA extraction

On the day of sample arrival, each sample was filtered through a 0.45 μm pore size membrane filter (Thermo Fisher Scientific, Waltham, MA, USA) under vacuum. After filtration, sterile forceps were used to aseptically fold each of the membrane filters which were placed in separate Whirl-Pak™ bags and stored at −20 °C until DNA extraction. Genomic DNA was isolated from membrane filters using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. To maximize DNA extraction efficiency, membrane filters were cut into small pieces with sterile scissors and the DNA was quantified with a NanoDrop ND-2000 UV spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The DNA samples were stored at −20 °C prior to use.

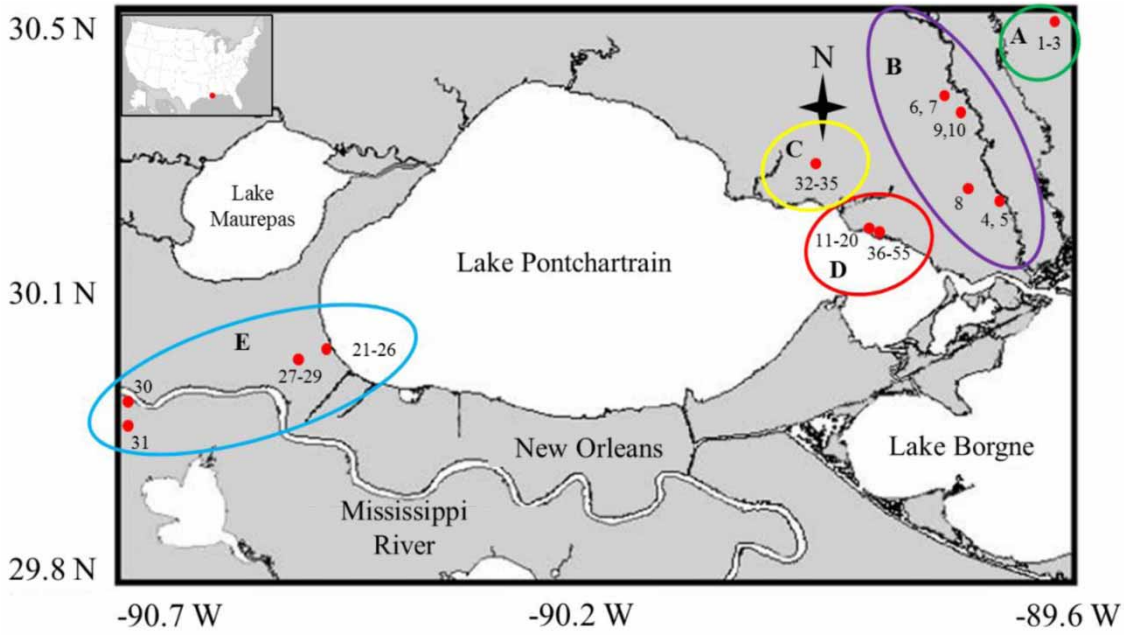


Figure 1 | Private well locations around Lake Pontchartrain. The distribution of private wells was divided into five sampling zones (A, B, C, D, and E) based on well location.

Enumeration of total coliform and *E. coli*

Fecal indicator bacteria (FIB) were measured by IDDEX method within the required time frame following sample collection. Undiluted water samples were each mixed with reagent and placed in a Quantitray/2000 according to the manufacturer's instructions (Colilert product insert; IDEXX Laboratories). The Colilert test kit was used for measuring total coliform bacteria and *E. coli*. Quantitray/2000 was sealed using Quanti-Tray Sealer and incubated at 35 °C for 24 hours. After incubation, the wells having a bright yellow color were quantified as positive for total coliforms for the Colilert test. The wells that fluoresced under UV light at 366 nm were quantified as positive for *E. coli*. The number of positive wells was compared to the manufacturer-provided MPN table to enumerate fecal coliform and *E. coli* in terms of MPN/100 mL.

Quantitative PCR assays and Illumina MiSeq high-throughput sequencing

Quantitative polymerase chain reaction (qPCR) assays targeting *E. coli*, *N. fowleri*, *Legionella*, and mycobacteria were performed using the Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, NY, USA) (see Supplementary materials). A summary of qPCR target organisms, primer/probe name, and sequences are detailed in Supplementary materials, Table S1. The amplification efficiencies (AE) were calculated based on the equation: $AE = 10^{(-1/slope)} - 1$.

Extracted DNA was transported on ice to Dr Zabaleta's laboratories at the Louisiana Cancer Research Center for 16S ribosomal RNA gene sequencing using Illumina MiSeq System (Illumina, Inc., San Diego, CA, USA) (Supplementary materials). The Illumina sequencing raw data obtained from this study were deposited in the NCBI Sequence Read Archive with accession no. PRJNA511469.

Data analysis

All statistical analyses in this study were conducted using R 3.4.1 (http://www.r-project.org/). Illumina MiSeq sequencing procedures are detailed in the Supplementary materials. Since qPCR data were not normally distributed, the non-parametric Kruskal–Wallis one-way ANOVA was used to compare the numbers of target organisms in different groups. Nonparametric Spearman rank order correlation coefficient (r) was calculated to illustrate the correlations between FIB, *Legionella* spp., *Mycobacterium* spp., and *N. fowleri*. These groups

were used in correlation analyses between each other as well as with environmental variables. The paired t-test was used to analyze differences of physical-chemical parameters (Tm, DO, pH, conductivity, and salinity) and biological genetic markers between pre- and post-flush groundwater samples. Tukey's honest significance test was used to compare the means of *Legionella* and mycobacteria to find means that were significantly different from each sampling zone. The *P*-value and *r* value for each regression model was used to provide an indication of significance and goodness of fit. Sample richness was measured with Shannon's Diversity Index.

RESULTS

Water quality characteristics

In total, 113 water samples were analyzed for chemical and physical parameters *in situ*. Two types of water sample were collected from each well: pre- and post-flush samples. The average water temperature (Tm) in pre-flush water samples (26.3 °C) was statistically significantly higher (*P*-value < 0.01) than that of post-flush water samples (24.2 °C) (see Supplementary Material, Figure S1). Paired t-test analysis of electrical conductivity (EC), dissolved oxygen, pH, and salinity indicated no significant differences were found between pre- and post-flush samples. The highest average value of water EC and salinity were from sampling zone E which is located on the west shore of Lake Pontchartrain (Table 1), and they both peaked at private well 29 (PW29). Based on the information we collected from the owner of each private well, the depth of the well ranged from 200 ft to 1,900 ft (Supplementary Material, Table S2), with an average depth of 536 ft.

Occurrence of fecal indicator bacteria (FIB) in water samples by IDEXX method

The biological water quality of those samples was assessed by using fecal indicator bacteria (FIB) such as total coliform and *E. coli*. Total coliform was present in 33.6% (38/113) of water samples from 20 private wells at levels ranging from 1 to 2,450 MPN/100 mL. Sampling zones B (77.8%) and E (59.1%) had higher detected frequency compared with other sampling zones (A 20.0%, C 22.2%, and D 13.3%) (Table 1). Water samples collected from PW8, 9, 10, and 30 had higher counts of total coliform in both pre- and post-flush water samples (data not shown). Numbers of total coliform were higher in pre-flush water samples compared to post-flush samples in the study (Supplementary Material, Figure S2, Table 2, *P*-value < 0.05), suggesting total coliform might multiply in water hoses other than in well water. *E. coli* was only detected in three water samples by culture-based method at value levels ranging between 1 and 2 MPN/100 mL. It was found in both pre- and post-flush water samples collected from PW28 which was located on the west shore of Lake Pontchartrain (Zone E).

Occurrence and distribution of *Legionella*, mycobacteria, *E. coli*, and *N. fowleri* in each sampling zone

Quantitative real-time PCR were performed on private well water samples targeting *Legionella* spp., *Mycobacterium*

Table 1 | Analysis of physicochemical and biological parameters of water samples in different sampling zones

Sampling zone	Tm (°C)	DO (mg/L)	pH	EC (S/m)	Salinity (ppt)	Total coliform (Positive/Total) (%)	*Legionella* (Positive/Total) (%)	Mycobacteria (Positive/Total) (%)	*N. fowleri* (Positive/Total) (%)	*E. coli* (Positive/Total) (%)
A	23.9	4.77	9.75	375.3	0.19	(1/5) 20.0%	(5/5) 100.0%	(5/5) 100.0%	(0/5) 0%	(0/5) 0%
B	22.4	4.17	9.27	521.2	0.27	(14/18) 77.8%	(18/18) 100.0%	(15/18) 83.3%	(3/18) 16.7%	(6/18) 33.3%
C	24.4	4.92	9.13	419.0	0.20	(2/9) 22.2%	(9/9) 100.0%	(8/9) 88.9%	(0/9) 0%	(5/9) 55.6%
D	27.2	3.16	7.91	555.0	0.27	(8/60) 13.3%	(47/60) 78.3%	(29/60) 48.3%	(15/60) 25.0%	(34/60) 56.7%
E	22.2	4.48	8.54	1,703.5	0.91	(13/22) 59.1%	(21/22) 95.5%	(21/22) 95.5%	(1/22) 4.5%	(4/22) 18.2%

Note: Tm indicates temperature, DO indicates dissolved oxygen, EC indicates electrical conductivity.

Table 2 | Paired t-test analysis of FIB and genetic markers between pre- and post-flush water samples

	Mean value		
	Pre-flush	Post-flush	Paired t-test (*P*-value)
Total coliform (Log_{10} of MPN/100 mL)	2.64	2.49	$P < 0.05$
Legionella (Log_{10} of GC/100 mL)	2.38	1.64	$P < 0.05$
Mycobacteria (Log_{10} of GC/100 mL)	2.47	2.27	$P < 0.05$
E. coli (Log_{10} of GC/100 mL)	0.94	0.95	$P = 0.4354$
N. fowleri (Log_{10} of GC/100 mL)	0.6	0.41	$P = 0.3610$

spp., *E. coli,* and *N. fowleri*. The frequency of detection of *Legionella* was 86.7% (98/113) and mycobacteria was 68.1% (77/113), ranging from 0.60 to 5.53 and 0.67 to 5.95 Log_{10} of GC/100 mL, respectively (Supplementary Material, Table S2). *E. coli* and *N. fowleri* were observed in 43.4% (49/113) and 16.8% (19/113), respectively, with concentration ranging from 0.53 to 3.16 (*E. coli*) and from 0.96 to 2.52 Log_{10} of GC/100 mL for *N. fowleri*. *Legionella* and mycobacteria gene markers were detected from all sampling zones. Numbers of *Legionella* genetic marker were lower in samples collected from Zones B and C (Figure 2), significant differences were found between samples collected from Zone D-B ($P < 0.01$), D-C ($P < 0.05$), and E-B ($P < 0.01$) based on Tukey's multiple comparison. Similarly, mycobacteria genetic marker was found to be lower in samples collected from Zones C and D (Figure 2). Significant differences were also observed between Zones D and E ($P < 0.05$) and C-E ($P < 0.05$). *N. fowleri* was not found in Zone A or C, and only one positive signal from Zone E. For *E. coli* genetic marker, it was found in all sampling zones except A. Although no significant difference was found between sampling zones, the average concentrations of *E. coli* were highest in Zone D (Figure 2). qPCR results showed most water samples were detected positive with multiple genetic markers (Figure 3). Seventy-six water samples were found positive for both *Legionella* and mycobacteria. There were only seven water samples that were negative with all four markers.

Genetic marker removal efficiency by flushing process

Paired t-test analysis of the mean concentration of FIB and genetic markers found differences between pre- and post-flush water samples (Table 2). By comparing post- and pre-flush samples, paired t-test results indicated *Legionella* and mycobacterial gene markers were significantly higher in pre-flush water samples compared to the post-flush water samples ($P < 0.05$). Although several sites showed higher *Legionella* and mycobacterial marker concentrations in post-flush water samples, flushing reduced both genetic markers in water samples under most circumstances (Supplementary

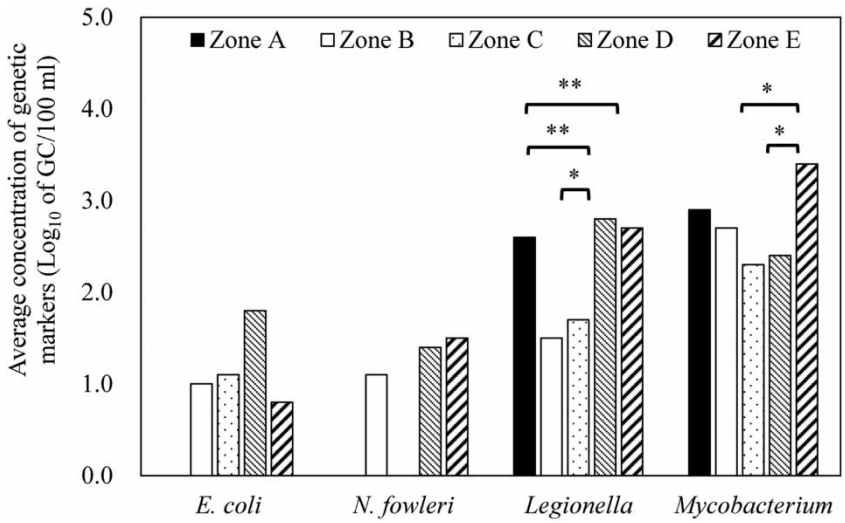


Figure 2 | The average concentration of genetic markers in groundwater samples collected from different sampling zones. Tukey's multiple comparisons of means were used to analyze target difference between each sampling zone: * indicates *P*-value < 0.05, whereas ** indicates *P*-value < 0.01.

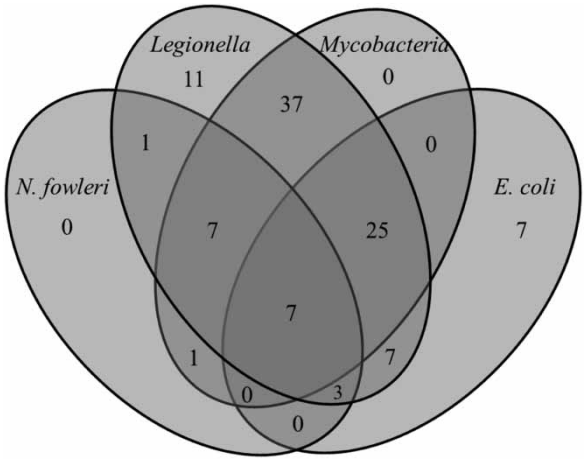


Figure 3 | *Legionella* spp., *Mycobacterium* spp., *E. coli*, and *N. fowleri* genetic markers detected in well water samples. *Note:* number indicates number of samples detected positive with certain genetic markers. Only seven samples were negative with all four markers.

Material, Figure S3). *N. fowleri* genetic marker was found to be lower in post-flush samples compared to pre-flush samples, the difference was not significant (Table 2). Similar results were observed for *E. coli* genetic marker.

Correlations between target organisms and FIB

Among all genetic markers tested, significant positive correlation between mycobacteria and *Legionella* was observed ($r = 0.60$, $P < 0.01$) (Figure 4(a)). Negative correlations were observed between *E. coli* and *N. fowleri* ($r = -0.57$, $P < 0.01$) (Figure 4(b)), and *Legionella* and pH ($r = -0.38$, $P < 0.01$) (Figure 4(c)). Correlation analysis was performed between target organisms and environmental factors, such as water temperature and salinity. Concentrations of *Legionella* ($r = 0.36$, $P < 0.01$) and *E. coli* ($r = 0.54$, $P < 0.01$) displayed significant positive association with temperature (Figure 4(d) and Figure S4(a)). A significant positive correlation between water salinity and the concentration of mycobacteria was observed ($r = 0.23$, $P < 0.05$) (Supplementary Material, Figure S4(b)). In addition, weak negative correlation between water salinity and *E. coli* ($r = -0.32$, $P < 0.05$) and *N. fowleri* ($r = -0.22$, $P < 0.05$) concentration was also observed (Supplementary Material, Figure S4(c), and S4(d)). We also observed weak negative correlation between *N. fowleri* and mycobacteria, *N. fowleri*

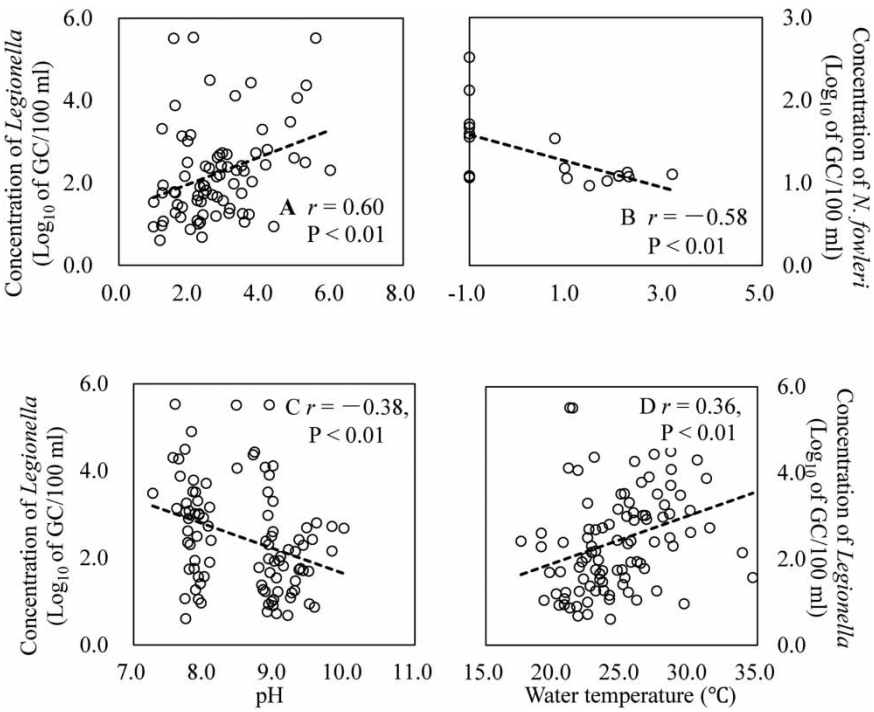


Figure 4 | Correlation analysis (Spearman rank order correlation coefficient) between (a) concentrations of *Legionella* and mycobacteria (Log_{10} of GC/100 mL); (b) concentrations of *E. coli* and *N. fowleri* (Log_{10} of GC/100 mL); (c) concentration of *Legionella* (Log_{10} of GC/100 mL) and pH; and (d) concentration of *Legionella* (Log_{10} of GC/100 mL) and water temperature (°C).

and *Legionella*, and temperature and mycobacteria (data not shown), but they were not statistically significant ($P > 0.05$).

Illumina sequencing of microbial communities in water samples

In total, 20 bacterial phyla were retrieved from 51 pre-flush water samples: Proteobacteria was the most dominant phylum in most samples, followed by Chlorobi, Actinobacteria, Chloroflexi, Cyanobacteria, and Bacteroidetes (Figure 5). At the genus level, five genera were abundant (>3%) in well water samples with *Methylomonas* (5.3%), *Methylosinus* (3.7%), *Mycobacterium* (3.4%), *Dechloromonas* (3.3%), and *Thiobacillus* (3.1%). The relative abundances of the class of *Gammaproteobacteria* and *Actinobacteria* were positively associated with qPCR results of *Legionella* spp. and mycobacteria (Supplementary Material, Figures S5, S6, S7), respectively. However, the regression analysis showed no significance ($P > 0.05$). Principal coordinates analysis (PCoA) of unweighted UniFrac indicates patterns of bacterial community composition in groundwater reflect sampling locations (Supplementary Material, Figure S8).

The profiles of the bacterial communities in each sampling zone were complex, and the results indicated a higher degree of variation between different sampling zones (Table 3). In general, water samples from Zones A and B had less bacterial communities compared with others. Zones C and D had similar bacterial communities. Samples collected on the southwest shore (Zone E) of Lake Pontchartrain tend to differentiate from samples collected on the northeast shore of the lake. Sequencing results for PW23 indicated filtering process could significantly reduce bacterial species in water (Supplementary Material, Figure S9). In PW34, both bacterial species and Shannon diversity index were significantly increased in water collected in kitchen compared with samples from well directly (Supplementary Material, Figure S9).

DISCUSSION

In the present study, private wells are in rural areas without municipal drinking water. The prevalence of *Legionella* and mycobacteria gene markers in our study confirmed that

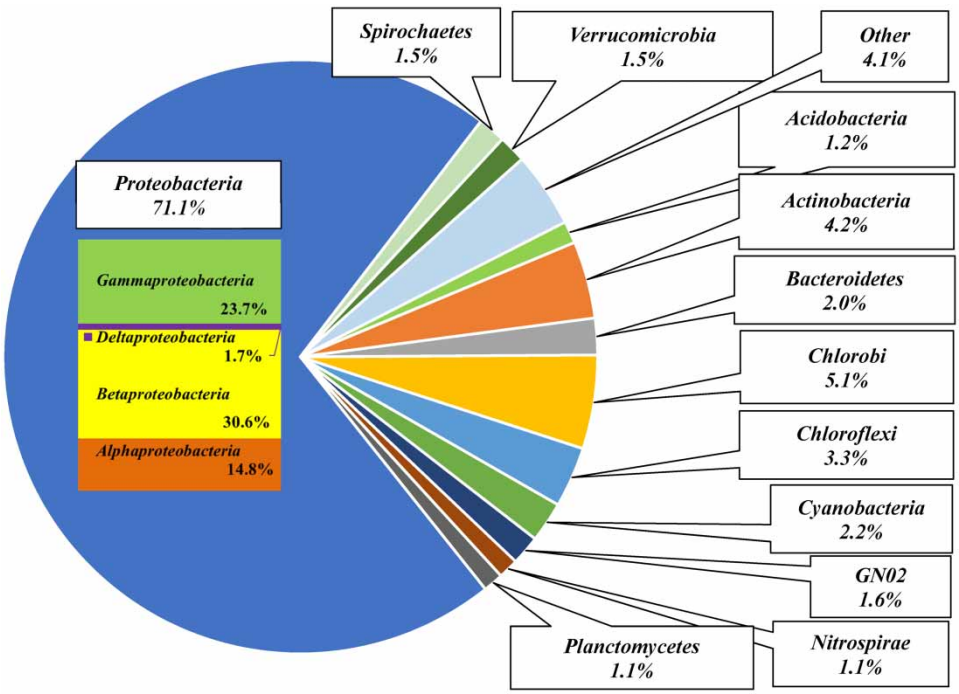


Figure 5 | Taxonomic classification of sequences from bacterial communities of the water samples. Relative abundance of the dominant phyla in private well water samples and relative abundance of the dominant class under phylum of *Proteobacteria*.

Table 3 | Relative abundance of the dominant phyla of water samples in each sampling zone

	Sampling zone				
Bacteria phylum	**A**	**B**	**C**	**D**	**E**
Acidobacteria	(0/3)	(0/3)	(0/4)	0.1–15.2% (17/30)	12.0% (1/11)
Actinobacteria	1.7–5.6% (3/3)	31.0–36.5% (2/3)	0.1–0.8% (2/4)	0.1–28.2% (15/30)	0.4–2.4% (4/11)
Bacteroidetes	(0/3)	8.0% (1/3)	0.1–3.2% (4/4)	0.1–16.0% (10/30)	0.1–35.6% (8/11)
Chlamydiae	1.0–8.4% (3/3)	(0/3)	(0/4)	25.4% (1/30)	(0/11)
Chlorobi	0.1–0.2% (2/3)	0.1–0.5% (3/3)	0.1–2.7% (4/4)	0.1–66.4% (29/30)	0.1–7.2% (7/11)
Chloroflexi	(0/3)	0.1–0.3% (2/3)	0.1–24.3% (3/4)	0.1–42.5% (18/30)	0.1–0.4% (4/11)
Cyanobacteria	(0/3)	(0/3)	0.2% (1/4)	0.1–11.2% (11/30)	0.1–77.3% (6/11)
Firmicutes	(0/3)	(0/3)	(0/4)	18.3% (1/30)	(0/11)
Gemmatimonadetes	3.5–11.4% (3/3)	(0/3)	(0/4)	(0/30)	(0/11)
Nitrospirae	1.3–6.7% (2/3)	(0/3)	0.1–6.8% (3/4)	0.1–12.2% (16/30)	0.1–10.0% (3/11)
Planctomycetes	6.5–8.8% (3/3)	(0/3)	0.1–0.4% (2/4)	0.1–17.1% (9/30)	(0/11)
Proteobacteria	55.3–84.6% (3/3)	60.7–99.8% (3/3)	62.7–98.0% (4/4)	25.7–98.1% (30/30)	10.3–97.6% (11/11)
Spirochaetes	(0/3)	(0/3)	0.1–10.1% (2/4)	0.1–22.0% (18/30)	0.1–1.7% (7/11)
TM6	1.1–10.5% (3/3)	0.2% (1/3)	0.2% (1/4)	(0/30)	0.2% (1/11)
Verrucomicrobia	(0/3)	(0/3)	(0/4)	0.2–24.6% (4/30)	0.1–21.3% (3/11)

opportunistic pathogens can harbor in private well water systems. The similar effect of flushing process (Table 2) on the two opportunistic pathogens suggested the origin of contamination was less likely from groundwater aquifers. Instead, water hoses may harbor various bacteria due to the excellent bacterial growth environment (biofilm) inside the hose (Mansi *et al.* 2014; Proctor *et al.* 2018; Richards *et al.* 2018). Previous results have demonstrated the ability of these pathogens to colonize and persist within drinking water networks, such as garden hoses (Delafont *et al.* 2014; Thomas *et al.* 2014; Schwake *et al.* 2016). Due to direct exposure to sun light, water temperature in hoses are typically higher (Supplementary Material, Figure S1). Although we did not find correlation among mycobacteria and *N. fowleri* with temperature, a significant positive relationship between *Legionella* concentration and water temperature was observed in the present study (Figure 4(d)). It has been previously reported that increased water temperature can promote the growth of opportunistic pathogens and *N. fowleri* (Leoni *et al.* 2001; Falkinham 2009; Xue *et al.* 2017). The longer water retention time and lack of disinfectant residuals also promoted microbial growth and colonization of potential pathogens (Li *et al.* 2018). Meanwhile, the flexible polymeric materials of water hoses have been reported to leach significant amounts of biodegradable organic carbon which may exacerbate bacterial growth (Bucheli-Witschel *et al.* 2012; Proctor *et al.* 2016). Those bacteria that are released from biofilm in the water distribution system are especially considered as the major potential risk for drinking water biosafety (Liu *et al.* 2018).

Our results indicated 33.6% of private well water samples were contaminated with total coliform which is comparable to previous studies (Borchardt *et al.* 2003; Pieper *et al.* 2015; Murray *et al.* 2018). However, *E. coli* was only detected in three water samples from two wells, suggesting the origin of the coliforms was unlikely fecal contamination (Marrero-Ortiz *et al.* 2009). In addition, total coliform counts decreased significantly after 3-minute flushing (Table 2); however, *E. coli* was still found in one well after flushing but not the other. We also examined *E. coli* by qPCR method and *E. coli* genetic marker was found in 43.4% samples. It has been previously reported that qPCR-based measurements are more sensitive due to the much lower detection limit (Ferretti *et al.* 2011; Xue & Feng 2018; Xue *et al.* 2018). Previous studies have demonstrated that PCR assays are able to detect DNA from both culturable

and viable but non-culturable organisms as well as extracellular DNA which can persist in dead bacterial cells and as free molecules in natural waters (Rogers *et al.* 2011). In that case, the differences between culture- and qPCR-based methods reflect differences in the nature of analyses as well as differences in persistence of culturable *E. coli* versus DNA of *E. coli* in environmental waters.

Groundwater salinity was highest in samples collected on the southwest shore of the lake (Zone E) and the lowest average value was found in samples collected from the northeast part of the lake (Table 1). No significant effect of well depth on water quality parameters can be found due to difficulty in confirming the specific depths of each well, since the depth of private wells during the survey was usually not clear. Although groundwater is the only water source in rural areas in this region, only 15% of residents drink well water directly based on our survey. Approximately half of the US waterborne disease outbreaks documented every year are the result of contaminated groundwater (Borchardt *et al.* 2003; Fout *et al.* 2003), and human health is at great risk from exposure to contaminated groundwater supplies (Marrero-Ortiz *et al.* 2009).

Although many of the studies have explored the occurrence of *N. fowleri* in surface waters (Marciano-Cabral *et al.* 2003; Painter *et al.* 2013; Sifuentes *et al.* 2014; Xue *et al.* 2017), very few have investigated the presence of *N. fowleri* in groundwater, except for two studies from Arizona (US) and Turkey which have identified amoebas in well water (Bright *et al.* 2009; Kuk *et al.* 2013). However, neither of those studies have investigated the potential interaction between *N. fowleri* and *Legionella* or mycobacteria. It has been reported that amoeba can serve as hosts for *Legionella* and mycobacteria species, facilitating their replication and transmission (Thomas *et al.* 2006; Delafont *et al.* 2014). *N. fowleri* was found to coexist with *Legionella* and mycobacteria. For instance, *N. fowleri* and *Legionella* were both detected in 18 samples, *N. fowleri* and mycobacteria were both detected in 15 samples, and three of them were detected in 14 samples. Although no significant correlations were found between their concentrations, the coexistence of these microorganisms may also provide information that *N. fowleri* might provide protection for the survival of *Legionella* and mycobacteria. Not only were *Legionella* and mycobacteria genetic markers found to coexist in most samples (Figure 3), a significant positive correlation between their gene concentrations can be observed (Figure 4(a)). The occurrence pattern of both opportunistic pathogen gene markers in the present study was consistent with previous research, in which a household plumbing system was found to have a high frequency of detection of *Legionella* spp. and mycobacteria (Wang et al. 2012a, 2012b; Li *et al.* 2018). It was suggested that lower water usage in water supply systems might cause corrosion of pipes that will release nutrients, as well as constituents (e.g., Fe) which will promote the growth of *Legionella* (Rhoads *et al.* 2017a, 2017b).

Studies have shown that *N. fowleri* grows better in the presence of *E. coli* and significant positive correlation was also found in surface water research (Marciano-Cabral *et al.* 2003; Ahmad *et al.* 2010; Xue *et al.* 2017). In contrast, a negative correlation between *N. fowleri* and *E. coli* was observed in the present study (Figure 4(b)). The occurrence of bacteria in groundwater may be affected by biofilms which form in water hoses, as well as groundwater aquifer (Thomas *et al.* 2014; Bright & Gerba 2017). This phenomenon reflects the difference in characteristics between groundwater and surface water environment, such as groundwater DO was significantly lower than surface water DO (data not shown), as well as water turbidity and pH. In addition, a negative effect of water salinity on *N. fowleri* concentration was observed in the present study and is consistent with previous studies (Supplementary Material, Figure S4(c)) (Xue *et al.* 2017), since *N. fowleri* was often found in fresh water (Rodriguezzaragoza 1994; Goudot *et al.* 2012). The negative correlation suggests water salinity has an adverse effect on the survival of *E. coli* and *N. fowleri* in well water. Although higher temperature was reported to promote the growth of microorganisms in water systems (LeChevallier *et al.* 1996), no correlations were identified between the presence of *N. fowleri* and water temperature, which is due partly to the limited sample numbers and relatively stable environmental conditions (Reardon *et al.* 2004; Griebler & Lueders 2009). We also hypothesize that the existence of biofilm might weaken the effect of water temperature on the growth of *N. fowleri*, since biofilms contain sufficient bacteria which may serve as food sources for *N. fowleri* (Battin *et al.* 2003; Tsvetanova & Dimitrov 2012).

Our analysis of the bacterial community diversity in each sampling zone outlines the distinct but temporally stable

microbial community in groundwater environment in New Orleans. It has been argued that a low level of biodiversity may indicate cleaner water aquifers (Cho & Kim 2000; Röling *et al.* 2001; Griebler & Lueders 2009). In our case, the relative abundance of the dominant phylum in each sampling zone indicates Zones A and B have cleaner water than other sampling zones (Table 3). Zone D, on the other hand, has most bacterial phyla detected. Furthermore, the unique bacteria phyla (Bacteroidetes and Cyanobacteria) found in Zone E confirmed that microbial community in groundwater environment may differ from other locations depending on physico-chemical condition (Griebler & Lueders 2009), as the water salinity from Zone E was significantly higher than other zones (Supplementary Material, Figure S1). Previous research suggested that the major environmental determinant of microbial community composition is salinity rather than extremes of temperature, pH, or other physical and chemical factors (Lozupone & Knight 2007). In addition, a distinct difference in community composition between filtered and unfiltered samples was observed (Supplementary Material, Figures S5 and S6). Analysis at phylum level revealed Bacteroidetes, Nitrospirae, SBR1093, Spirochaetes were removed, but unclassified bacteria phyla of GN02 and OD1 were increased. Members of phylum Proteobacteria, α-Proteobacteria and β-Proteobacteria, both decreased, γ-Proteobacteria was increased, and δ-Proteobacteria did not change. Further analysis on the genus level revealed more detail about bacteria community change, such as *Acidovorax* and *Sulfuritalea* were eliminated, but the relative abundance of genera *Methylocaldum*, *Methylomonas*, *Methylotenera*, and *Anaeromyxobacter* were increased. It is therefore not recommended that people who live in the researched area consume untreated groundwater; instead, flushing the water system will help to remove bacterial contamination. Interestingly, the relative abundance of members of phyla Bacteroidetes, Proteobacteria, and Nitrosperae were increased in one water sample collected from a kitchen (Supplementary Material, Figure S5). It has been suggested that more frequently used hoses had higher biofilm total cell concentration than water hoses only used water with high temperature in the tub (Proctor *et al.* 2016, 2018). In such cases, attention needs to be paid to these final meters of water distribution.

The composition of the identified core phyla in the present study complied with previous reported microbial community structures in groundwater. However, Firmicutes was not dominant at phylum level (only detected in one sample) compared to other research (Reardon *et al.* 2004; Griebler & Lueders 2009). Consistent with previous studies, Proteobacteria was the dominant community in our samples (Roesch *et al.* 2007; Hemme *et al.* 2010; Wang *et al.* 2012a, 2012b). Samples collected from Zones D and E had relative higher abundance of *Gammaproteobacteria* compared to other sampling zones, which is consistent with qPCR results (Figure 2). The higher abundance of *Gammaproteobacteria* may reflect similar characteristics of sampling location as they are all close to Lake Pontchartrain, and a number of scientifically important bacteria belong to this class, such as *Salmonella* spp., *Vibrio cholerae*, as well as *E. coli*, which are often found in environmental water samples. Compared to other sampling zones, samples collected from Zones D and E are most likely to be affected by lake water. Our results provided clear evidence that microbial communities can be largely affected by a specific environment and this has been confirmed by other studies (Thompson *et al.* 2017). For instance, *Chlorobi* was the second most dominant phylum in well water samples, bacteria of the phylum *Chlorobi* was strictly an anaerobic microbe (DO in well water samples was significantly lower than in surface water) and growing optimally at 47–48 °C (Podosokorskaya *et al.* 2013). *Mycobacterium* was among the top three genus levels in well water samples (Supplementary Material, Figure S6). The ubiquitous presence of mycobacteria in samples collected from 8, 13, 16, 42, and 51 was confirmed by both sequencing and qPCR results (data not shown). The mycobacterial species was frequently detected in drinking water systems in the United States, such as *M. avium* (Falkinham *et al.* 2001, 2008).

CONCLUSIONS

- Our results confirmed the prevalence of both *Legionella* spp. and mycobacteria in private well water and premise plumbing. Samples collected from Zones D and E tend to have higher concentrations of *Legionella* and mycobacteria.
- The occurrence of *N. fowleri* and *E. coli* in groundwater may be affected by sources other than water temperature.

The coexistence of *N. fowleri* with *Legionella* and mycobacteria may also provide information that *N. fowleri* might provide protection for the survival of *Legionella* and mycobacteria.

- Total coliform, as well as *Legionella* and mycobacteria genetic markers' concentrations were significantly reduced by 3-minute flushing, emphasizing the colonization of bacteria and opportunistic pathogens inside water hoses might pose a great health risk.
- NGS data demonstrated that the groundwater environmental characteristics differentiate microbial communities. Here, we found that the abundance of groundwater bacteria is greatly influenced by water filtration and attention needs to be paid to the final meters of water distribution.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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