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Water Impact Statement

Here we conducted a community-wide survey of opportunistic pathogens following the 2016 Louisiana Flood. We found substantial detection of *Legionella* spp. and *Naegleria fowleri* gene markers, in 77.5% and 20% of 40 homes, respectively. The findings indicate that microbial issues in private wells, which serve 15 million households in the U.S. but are not regulated, merit greater attention.

Molecular survey of *Legionella* and *Naegleria fowleri* in private well water and premise plumbing following the 2016 Louisiana flood

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Abstract

Private wells are a critical drinking water source and are susceptible to contamination from flooding. Opportunistic pathogens (OPs), such as *Legionella*, are an increasing source of drinking water-related outbreaks, but are poorly characterized in private wells. Here we conducted a molecular survey of OPs in private wells and plumbing systems shortly after the 2016 Louisiana flood. Detection frequency of fecal indicators was not notably high (total coliform 24.8% and *Escherichia coli* 3.5% in 113 private wells) ten weeks after flooding. Gene markers of *Legionella* spp., *L. pneumophila,* and *Naegleria fowleri* were detected in 77.5%, 15.0%, and 20.0% of a subset of 40 homes that were tested specifically for these OPs, respectively. *Legionella* spp. varied from 8.4 gc/mL to 1.8×10^4 gc/mL in first draw and flushed water. Positive detections and levels of *Legionella* spp., as well as positive detections of *L. pneumophila,* were correlated with total bacterial numbers (measured as 16S rRNA gene copy numbers), suggesting that total bacterial numbers could be an indicator of OP occurrence under the conditions of

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private wells, which usually do not have disinfection treatment installed. Further, *Legionella* spp. positivity in first draw water from cold and hot taps was associated with their detection in flushed water, suggesting that the well itself can be a source of OPs. OP detection was not predictable from total coliform, well characteristics, or observable well damage, but was associated with higher metals in flushed water resulting from plumbing corrosion. Given that the majority of Legionnaires' Disease cases are sporadic, private wells merit greater attention as a potential source of exposure.

Keywords

Opportunistic pathogens; *Legionella pneumophila*; drinking water; groundwater; water quality

1 Introduction

A slow-moving storm hit southern Louisiana, U.S. August 12-14, 2016, depositing 7.1 trillion gallons of rain in the areas surrounding Baton Rouge. This caused widespread flooding,[1, 2] with many areas experiencing a 1-in-1000-year flooding event.[3] As a result, a state of emergency was declared and a federal disaster was designated in 20 parishes.[4] The flooding resulted in 150,000 homes being damaged, 13 deaths, and a recovery cost of $10-15 billion, making it the worst natural disaster in the U.S. since Hurricane Sandy in 2012.[1, 5, 6]

It is well established that drinking water sources can be significantly compromised after flooding or heavy rainfall runoff.[7-9] In the aftermath of the Louisiana flood, several public water utilities issued boil advisories for their consumers,[6] but were able to restore

function quickly.[10] However, private wells are not regulated by federal or state agencies and thus it is the responsibility of homeowners to assess and remediate water quality following a potential contamination event. Nonetheless, state and local health departments do provide some guidance and support, such as well inspection, water testing and post-flooding disinfection, as was the case following the Louisiana flood.[11, 12]

Microbial contamination in private wells is traditionally evaluated by testing for the presence of total coliforms (TC) and *Escherichia coli* (EC), which are intended to indicate the presence of potentially harmful bacteria, especially fecal pathogens. While federal standard for public drinking water of zero TC detection may provide a point of reference,[13] TC/EC prevalence or levels are not presently regulated for private well water in the U.S. or by Louisiana state agencies. TC and EC can be elevated in private wells following natural disasters,[14, 15] but these indicators do not likely inform risks associated with important non fecal-sourced pathogens, such as *Legionella*, *Naegleria fowleri*, and other opportunistic pathogens (OPs).

Legionella has been relatively well-studied in municipal drinking water, with *L. pneumophila* in particular now widely cited as a primary tap water-related source of disease outbreak in the U.S. and many other developed countries.[16-18] However, there is very limited knowledge regarding the prevalence of *Legionella* in private wells,[19, 20] which provide drinking water to about 15% of the U.S. population.[21] *Legionella* has been detected in public groundwater wells and thus is also likely present in private wells, especially with the intrusion of floodwater.[20, 22-24] A survey of *Legionella* occurrence in

private wells post-flooding is needed to evaluate exposure risks for well users, especially considering that the majority of Legionnaires' Disease cases are sporadic and of unknown etiology.[25]

N. fowleri is of particular interest to the warm southern state of Louisiana, where it was recently isolated from public drinking water systems and patients' household tap water.[26, 27] The thermophilic "brain-eating" amoeba has a high fatality rate (97.5%) and is naturally found in warm lakes, rivers, soils, groundwater, and floodwater.[28, 29] Its presence in high-volume public wells has been reported.[30-32] It can also serve as a host for *L. pneumophila*.[33] Thus the occurrence of *N. fowleri* in private wells post-flooding is of great interest, but not yet reported.

A major challenge in combatting OPs is that they are typically capable of re-growth within distribution systems and premise plumbing.[34-36] Thus, it is critical to evaluate OPs after water has passed though and stagnated in premise plumbing before reaching distal taps, the relevant point of exposure. Compared to widely studied premise plumbing in municipal systems, premise plumbing served by private wells has many similar characteristics (e.g., long stagnation, elevated water temperature, various pipe materials, and high surface area) that can contribute to OP regrowth. One dissimilar characteristic that may affect OPs re-growth in private well systems is the lack of disinfection, where disinfectant is only rarely added to private wells on an "as-needed" basis and residuals are not maintained.[37] Portions of the plumbing system delivering hot water are of particular concern, where optimum growth temperatures for OPs (e.g., 25-42°C for *L.*

pneumophila) are common.[38] However, previous studies of private well water generally neglected the premise plumbing, typically collecting from the wellhead, outside spigot, or an inside faucet after flushing to obtain water representative of water column in the well.[39, 40]

Following the 2016 flooding in Louisiana, we conducted a rapid-response study to evaluate the drinking water quality in private wells located mainly in Ascension and Livingston Parishes (population sizes of 137,000 and 119,000, respectively), which were among the hardest-hit zones.[41] The purpose of this study was to 1) evaluate the prevalence of indicator organisms (TC and EC) in private wells located in flood-impacted parishes; 2) survey the occurrence and levels of *Legionella* spp., *L. pneumophila,* and *N. fowleri* gene markers in flushed private well water as well as in stagnated water in premise plumbing; 3) and examine relationships amongst microbial targets, water chemistry parameters, and well characteristics. The quantification of microbial contamination in private wells post-flooding can provide valuable information for assessing potential health risks and guiding remedial action.

2 Experimental

2.1 Neighborhood-scale sampling campaign

On August 27, 2016 (one week after floodwater receded), 5 well users in Livingston Parish were recruited via door-to-door campaign to assess the immediate impact of flooding on private wells. After 6+ hours stagnation, we collected five 1-L water samples (Table S1) from their kitchen taps. On September 3, 2016 (two weeks after floodwater

receded), sampling was repeated in four of the five homes. Water samples were immediately refrigerated, transported to the Virginia Tech laboratory on ice, and processed within 30 hours of sampling.

2.2 Community-wide sampling campaign

Residents were recruited via local media outlets to participate in free private well water testing between October 27-30, 2016 (around 10 weeks after floodwater receded, which differed slightly within the sampled area). Residents received a water sampling kit from our research team. Each kit included sampling instructions, sampling bottles, and a questionnaire. All water samples were collected from kitchen taps. Two types of sampling kits (100 basic and 50 advanced) were semi-randomly distributed at two locations by distributing each kit type to roughly every other resident in the order of their arrival and based on their willingness to collect extra samples until the limit of advanced kits was achieved and continuing with the basic kit. Deployment of the two kit types served to reach as many private wells as possible to support statistical analysis of traditional water quality measurements (inorganics and indicator organisms), with the more costly advanced kit distributed to the extent possible to enable testing of OPs and multiple sampling locations throughout a sub-set of residences. For the basic kit, residents collected a 250 mL first draw cold water, as well as one 250 mL and one 125 mL cold water sample after 5-min flush. Five-minute flushed samples are hereafter referred to as "*well water*" samples, because the flushing serves to bypass the impact of premise plumbing and obtain a representative well water column sample. By contrast, we use the term "private well water" to more generally refer to all water sourced from

private wells. For the advanced kit, three additional 1 L samples were collected for molecular analysis of OPs: a first draw cold water immediately after the 250 mL sample (*first draw*), a 5-min flushed cold water (*well water*), and a first draw hot water (*hot draw*) (refer to electronic supplementary information section ESI.1-2 for sampling protocols; and Table S2 for a summary of sampled wells, water samples, and water quality measurement). Residents also completed a questionnaire (ESI.3) about the characteristics, maintenance history, and flood-induced damages of their private wells. Residents collected and returned water samples to the research team on the same morning. Samples were transported and processed as described in Section 2.1. Participation in this campaign was voluntary and all procedures were approved by Virginia Tech Institutional Review Board (#16-918).

2.3 Water quality analysis

Water pH and conductivity were performed onsite by our research team when water samples were returned, using methods 4500-H^+ and 2510.[42] Inorganics were analyzed using inductively coupled plasma-mass spectrometry per methods 3030 D and 3125 B from an aliquot of the 250 mL samples.[42] TC and EC in the 125 mL well water samples were quantified using the IDEXX Colilert 2000 method (Westbrook, MN), with a detection limit of 1.01 MPN/100 mL. Trip and field blanks (i.e., sterile deionized water in sampling containers, with the trip blank remaining closed and field blank being opened during sampling) were included in both sampling campaigns along with laboratory controls. *Legionella* culture was conducted during the neighborhood-scale sampling campaign following the ISO 11731 protocol.[43]

159

160 ## 2.4 Molecular analysis

161 All 1-L water samples were filtered through mixed-cellulose ester membranes (0.22μm,
162 Millipore, Billerica MA), with DNA extracted directly from filters using a FastDNA
163 SPIN kit (MP Biomedicals, Solon OH). DNA extracts were diluted 10-fold with
164 nuclease-free water for quantitative polymerase chain reaction (qPCR) to minimize
165 potential inhibition. Filters, DNA extracts, and diluted samples were stored at -20 °C
166 until processed or analyzed. Gene copy numbers of total bacteria (targeting 16S rRNA
167 gene), *Legionella* spp. (targeting 23S rRNA gene), *L. pneumophila* (targeting *mip* gene),
168 and *N. fowleri* (targeting an intergenic spacer region) were determined by qPCR on a
169 CFX96 Realtime System (Bio-Rad, Hercules CA). Primers, reagents, qPCR protocols and
170 the specificity of qPCR assays are described in detail elsewhere.[44-47] DNA extracts from
171 pure cultures of *L. pneumophila* and *N. fowleri* were used as positive controls. Serially
172 diluted standards (from 10^8 to 10^1 gene copies (gc) per reaction) were included in each
173 qPCR run. The limit of quantification (LOQ) was 95 gc/mL water sample for total
174 bacteria, 2.7 gc/mL water sample for *Legionella* spp. and *L. pneumophila*, and 9.5 gc/mL
175 water sample for *N. fowleri* (i.e., 100 gc/reaction for total bacteria and 10 gc/reaction for
176 OPs). qPCR reactions for each sample, standards, and a no-template control were run in
177 triplicate on each qPCR plate. Samples with positive amplifications in at least two of the
178 three replicate reactions and with gene copy values above LOQ were considered
179 quantifiable. Samples with one or more positive amplification, but not meeting the above
180 quantifiable criteria, were considered detectable, but below 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.

2.5 Data analysis

Data analysis was performed using JMP Pro 12 (Cary, NC) for results from the community-wide sampling. Gene copy numbers were $\log_{10}$-transformed for plotting, normality test, and were also used in nonparametric analyses (Wilcoxon signed rank test, Wilcoxon test, Spearman correlation). Contingency tables and Chi-square tests were used to compare prevalence of OPs among different groups. Odds ratios were calculated from the contingency table. Spearman's correlation analyses were performed using the Fit Y by X module or multivariate module (α=0.05).

3 Results

3.1 Initial neighborhood-scale testing one week after floodwater receded

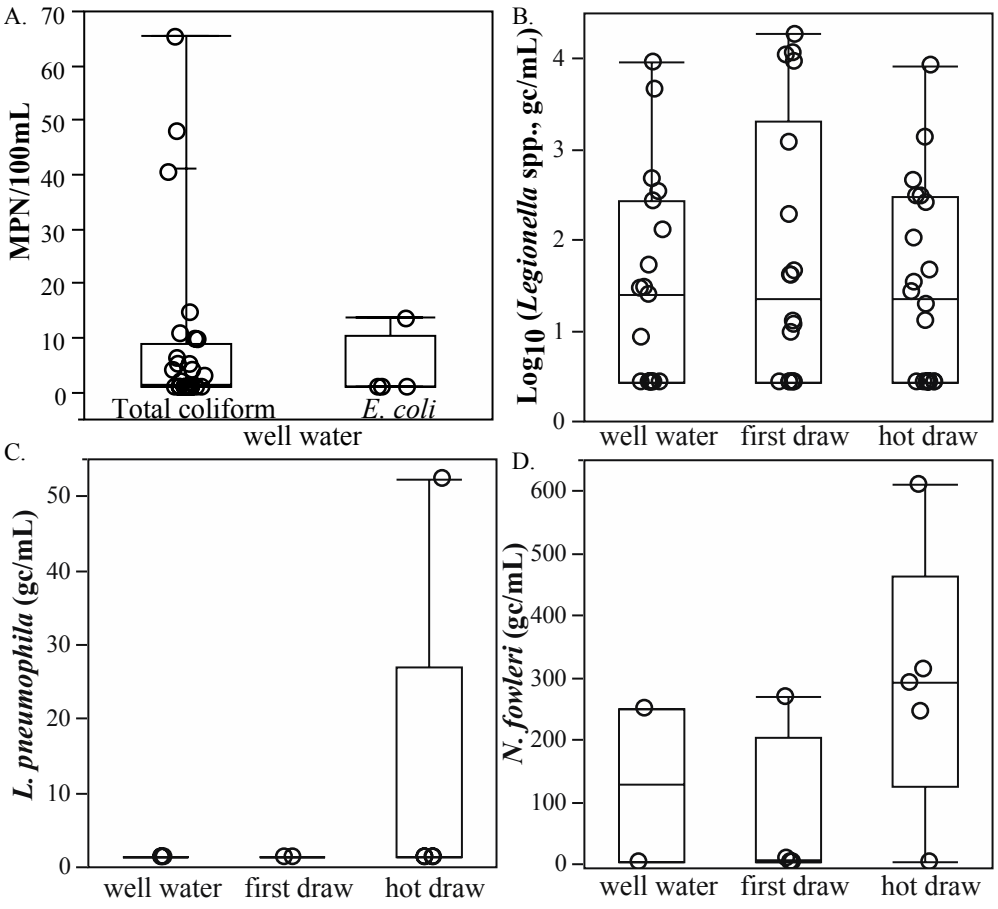
Five households were sampled to assess private well water quality shortly after flooding. One week after floodwater receded, three of the five well water samples tested positive for TC (1.01-22.66 MPN/100 mL), but none tested positive for EC (Table S1). Repeat testing was conducted one week later in four of the five households and one of the three wells initially positive for TC remained positive (9.7-9.8 MPN/100 mL). All homes were negative for culturable *Legionella* during both samplings, but *Legionella* spp. gene marker was detected at 4 of the 5 homes during the initial sampling and at all 4 homes during the follow-up sampling. *L. pneumophila* or *N. fowleri* gene markers were not detected in either sampling. Results from this initial assessment, in particular the

prevalence of TCs and gene marker levels of *Legionella* spp., suggested that water quality in private wells was deteriorated and also indicated greater variation among homes (private wells) than among multiple and sequential samples within the same home. Thus, wider-scale sampling of private wells was prioritized for further evaluation of private well water quality in the region.

3.2 Community-wide water quality ten weeks after floodwater receded

Considering substantial variation among homes observed above, kit deployment in the community-wide sampling focused on expanding to reach as many homes as possible, rather than replicated sampling of the same homes. Among the 150 test kits distributed, 113 (75.3%) were returned for analysis (73 basic and 40 advanced kits), with most sampled wells (87.6%, 99 out of 113 wells) located within the flood zone (Table S2). Overall, 24.8% (28 of 113) and 3.5% (4 of 113) of well water samples were positive for TC and EC, respectively (Table 1). Quantifiable TC and EC ranged from 1.00 to 65.35 MPN/100 mL and from 1.01 to 13.57 MPN/100 mL (Figure 1A). No difference was observed in the positivity of TC and EC between wells inside versus outside flood zones (TC 24.2% vs 28.6%, EC 4.0% vs 0.0%, n=99 vs. 14 wells, Chi-square tests, p=0.58 and 0.76) or between wells sampled with the basic versus advanced kits (TC 21.9% vs. 30.0%, EC 5.0% vs. 2.7%, n = 73 vs. 40, p= 0.34 and 0.54). Sampled wells were located mainly in two parishes (40% in Ascension, 55% in Livingston, Table S2) and no difference of TC or EC positivity was observed between parishes (p=0.17-0.75). Users of 38% of private wells reported some flood-induced damage to the well (e.g., broken pump, septic system backed up, submersion of the pump) and 52% reported no known

227 damage. The prevalence of TC and EC was not different between damaged and
228 undamaged wells or between wells with a damaged septic tank system and those with no
229 septic system damage (p=0.64-0.67, n= 43 vs 59, Table S2).



230

231 Figure 1. Quantification of microbial targets in water samples. The numbers of: A) total
232 coliform and *E. coli* in 113 well waters collected from taps after 5-min flush and gene
233 copies in well water, first draw cold water (first draw), and first draw hot water (hot
234 draw) samples from a subset of 40 homes representing B) *Legionella* spp., C) *L.*
235 *pneumophila*, and D) *N. fowleri*. Gene copy measurements detected but below LOQ in
236 panels B, C, and D are plotted at half of the corresponding LOQ value.

237

238 **3.3 Molecular survey of OPs in private wells and premise plumbing**

A survey of *Legionella* and *N. fowleri* gene markers in multiple samples from a subset of 40 homes was conducted to establish an understanding of their prevalence in water sampled from private well columns and premise plumbing post-flooding. *Legionella* spp. gene marker was detected in at least one sample in 77.5% homes (n=31 of 40) and in multiple samples in 40% of homes (n=16 of 40). For individual samples, *Legionella* spp. were detected in 48.6% of 37 first draw samples, 50.0% of 38 well water samples, and 54.1% of 37 hot draw samples (Table 1). There was no difference in detection frequency of *Legionella* spp. among the three sample types (Chi-square test, p=0.89). The abundance of *Legionella* spp. gene markers varied widely over four logs (from 8.4 gc/mL to 1.8×10^4 gc/mL) among sampled homes (Figure 1B), in agreement with the neighborhood-scale assessment (Table S1).

L. pneumophila gene markers were detected less frequently than those of *Legionella* spp., i.e., in at least one sample in 15% of 40 homes and in multiple water samples in 10% of 40 homes. Among positive detects, only one hot draw sample had quantifiable *L. pneumophila* (52.4 gc/mL), with the others below the LOQ (Figure 1C). As was the case with *Legionella* spp., there was no difference in the detection frequency of *L. pneumophila* among sample types (5.4% in first draw, 7.9% in well water, 13.5% in hot draw, p=0.46, n=37-38).

N. fowleri gene markers were detected in at least one sample in 20% of 40 homes and in multiple samples in 5% of 40 homes. *N. fowleri* detection among sample types was not

significantly different (10.8% in first draw, 5.3% in well water, and 13.5% in hot draw, p=0.47, n=37-38). Quantifiable *N. fowleri* varied from 11 to 610 gc/mL (Figure 1D).

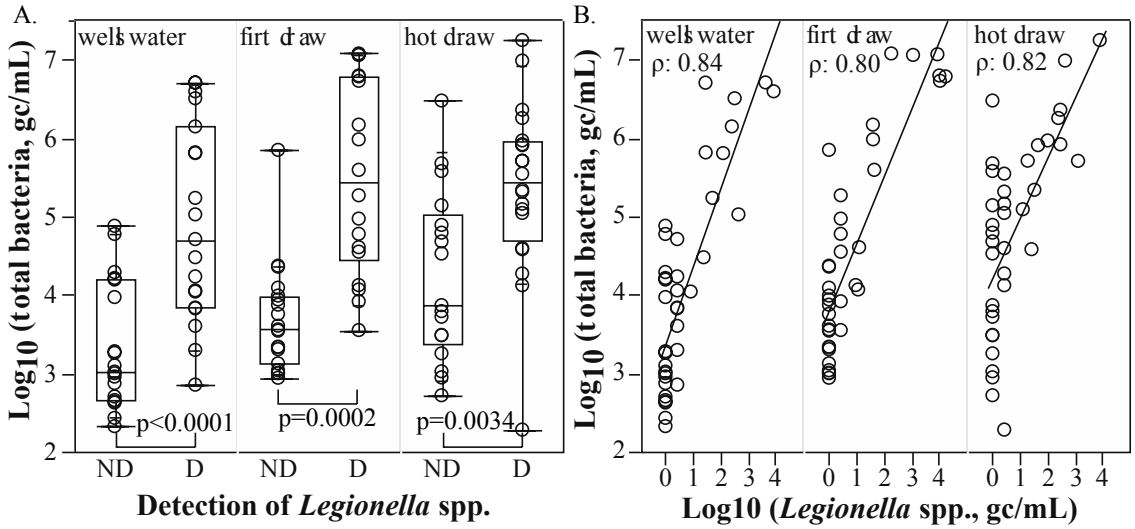
Among the 40 homes, 12.5% (5 of 40 homes) were positive for both *Legionella* spp. and *N. fowleri*, while 2.5% (1 of 40 homes) was positive for both *L. pneumophila* and *N. fowleri.* No correlation was found between positive detection of *L. pneumophila* and its potential host *N. fowleri* (p=0.31). The prevalence of OPs was not different between wells inside and outside flood zones (p=0.10-0.82, n = 34 vs. 6), or with and without reported flood-induced damage (p=0.43-0.62, n = 15 vs. 20). Moreover, there was no significant difference in the detection frequency of *Legionella* spp., *L. pneumophila,* or *N. fowleri* in well water samples that were TC positive (n= 12) compared to TC negative (n=28) (58% vs 42% for *Legionella* spp., 7.7% vs 8.3% for *L. pneumophila,* and 3.8% vs 8.3% for *N. fowleri,* n=38, p=0.16-0.94). There was also no correlation between the levels of *Legionella* spp. and TC when both were detected (Spearman ρ=0.27, n=8, p=0.27).

3.4 *Legionella* spp. in private well plumbing systems

Positive detection of *Legionella* spp. in tap water (first draw and hot draw) was dependent on its detection in well water (Chi-Square test, n=35, p=0.0002-0.03, Table 2). Specifically, *Legionella* spp. was 16.3 and 4.8 times (odds ratios) more likely to be detected in first draw and hot draw in homes with a positive detection in well water than homes with no detection. Furthermore, the quantifiable levels of *Legionella* spp. gene markers in first draw and hot draw were also both correlated with those in well water

283 (Spearman coefficients ρ=0.54 and 0.48, p values=0.011 and 0.02, n=21 and 23 for cold
284 and hot respectively, Figure S1).
285
286 *Legionella* spp. gene abundances in first draw trended higher than in well water of the
287 same home, but the p-value slightly above the selected significance cutoff (0.05) for this
288 study (matched pairs Wilcoxon signed rank test, p=0.053, n=21, average 0.41 log higher),
289 suggesting that some OPs regrowth could have been occurring in premise plumbing
290 served by well water. *Legionella* spp. gene abundances in hot draw, on the other hand,
291 were not higher than in well water within the same household (p=0.28, n=23). Lack of
292 increase in the hot draw water was likely influenced by a diverging pattern when
293 comparing the two sample types (Figure S2). Among the 23 homes included in the
294 comparison (excluding the other 17 homes with no detects), nearly half (11 homes)
295 contained more gene copies of *Legionella* spp. (0.37 to 2.41 log more) in hot draw than in
296 well water, while the other half (10 homes) contained less in hot draw (0.17 to 1.40 log
297 less, Figure S2).
298
299 **3.5 Relationship between total bacteria and OPs**
300 Here we approximated "total bacterial numbers" as gene copies of the universal bacterial
301 16S rRNA gene marker. Total bacterial numbers varied in all sample types from 10^2 to
302 10^7 gc/mL among homes (Figure S3). First draw and hot draw had significantly higher
303 total bacterial numbers than well water (0.6 and 0.8 log higher, matched pair Wilcoxon
304 signed rank test, n=35, p=0.0002 and <0.0001), indicating general regrowth of bacteria,
305 which is characteristic of premise plumbing. Total bacterial numbers in first draw and hot

306 draw were both positively correlated with corresponding numbers in well water
307 (Spearman ρ=0.70 and 0.58, p<0.0001, Figure S4).
308
309 In all sample types, total bacterial numbers were significantly higher when *Legionella*
310 spp. was detectable (1.2-1.8 log higher, p=<0.0001 to 0.0034, n=37-38, Figure 2A).
311 Further, total bacterial numbers were positively correlated with *Legionella* spp. levels
312 (Spearman ρ=0.80-0.82, p<0.001, n=21-23, Figure 2B). Sporadic detection of *L.*
313 *pneumophila* (n=2-4) prevented similar statistical comparison for individual sample type.
314 Combining all water samples together, total bacterial numbers were also higher in those
315 with detectable *L. pneumophila* (1.2 log higher, p=0.006, n=112, Figure 3). In contrast to
316 *Legionella* spp., the detection of *N. fowleri* gene markers or TC was not related with total
317 bacterial levels (Figure S5, p=0.21 and 0.06).



318

319 Figure 2. Relationship between gene copies of total bacteria (16S rRNA gene) and
320 *Legionella* spp. Total bacterial numbers were: A) significantly higher when *Legionella*
321 spp. gene marker was detectable (D) than when non-detectable (ND), and B) positively
322 correlated with gene copies of *Legionella* spp. in well water (5-min flushed cold water),

first draw (first draw cold water), and hot draw (first draw hot water). Wilcoxon tests p-values and Spearman correlation coefficients (ρ) are shown in panels A and B, respectively. Linear regression lines in panel B were added to assist visualization of the positive correlations.

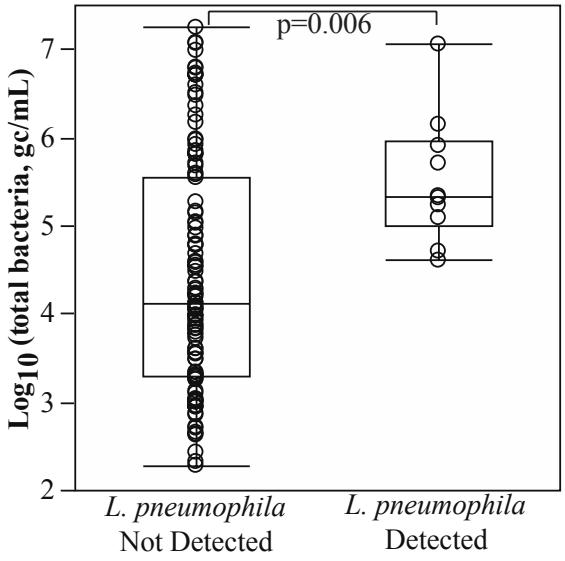


Figure 3. Higher total bacterial numbers (16S rRNA gene copies) as a function of the detection of *L. pneumophila* gene markers (*mip)* across all water samples. Total bacterial numbers were higher in water samples when *L. pneumophila* was detected than when not detected (Wilcoxon test, p=0.006). All well waters, first draw cold waters, and first draw hot waters from the subset of 40 homes were combined for this analysis (n=112).

3.6 Comparison of microbial and inorganic aspects of water quality

Inorganics in well water (5-min flushed cold water) were also characterized (ESI.4 & Table S3) and compared to microbial measures of water quality. None of the 16 inorganic parameters measured in well water were associated with TC positivity (Wilcoxon test, p=0.13 to 1.00, n=113). Among the 16 inorganics, only zinc levels were significantly

higher in homes with a positive detection of *Legionella* spp. than in homes with no detection (median 66.0 vs 15.0 µg/L, n=38, p=0.02, Figure S6). Naturally-occurring zinc in surface waters and groundwater are generally very low (10-40 µg/L),[48] but zinc and other metals such as copper and lead can be released from well pump and premise plumbing components receiving groundwater.[39, 49] None of the 40 homes had these metals exceeding corresponding standards (Table S3) in their well water. However, homes with relatively higher zinc (>200 µg/L) or copper (>10 µg/L) in their well water also had higher detection frequency of *Legionella* spp. (100% vs 71%, p=0.02, n=40) and more total bacteria in well water (median 5.4-log vs 3.7-log, p=0.01, n=38, Figure 4). No difference was found between relatively higher metals and positive detection of *L. pneumophila* or *N. fowleri* (p=0.50 and 0.63).

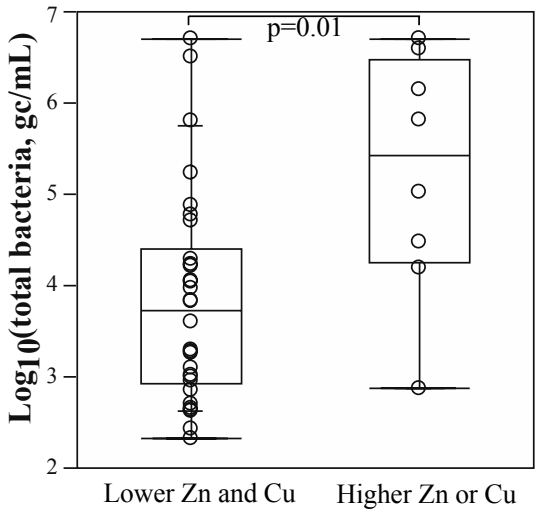


Figure 4. Higher total bacterial numbers (16S rRNA gene copies) in well water (5-min flushed cold water) with relatively higher levels of metals. The "higher Zn or Cu" category was defined as zinc >200 µg/L or copper >10 µg/L with the "lower Zn and Cu" category below these thresholds. The p value is based on a Wilcoxon test (n=38).

3.7 Effects of well characteristics

Our sampling campaign included different wells of various construction types (Out of the 113 private wells, 66% drilled, 8% dug/bored, 26% unknown, Table S2), ages (1 to 66 years) and depths (200 to 2300 ft), as well as presence/absence of a range of in-house treatment strategies, including softeners, filters, reverse osmosis, or chlorination (ESI.3)(20% of 113 homes incorporated treatment, 66% had no treatment, and 14% were unknown, Table S2). Since all water samples were collected from kitchen taps to represent a typical point of use, samples from a home with an in-house treatment were of "treated" water. There was no difference in the prevalence of TC (n=113), *Legionella* spp., *L. pneumophila,* or *N. fowleri* (n=40) in well water among wells with different characteristics (type, age, depth, or presence of an in-house treatment) or flooding-induced damages reported by well users (p=0.38-0.78).

4 Discussion

4.1 The need for rapid response and baseline monitoring

Spread of pathogens associated with flooding can pose health threats to well users, especially as private well water quality is not routinely monitored due to barriers such as lack of regulation, affordability, and limited access to water testing information.[50-52] Following a flooding event, public health agencies recommend that well users inspect wells, conduct emergency disinfection if wells are flooded, and test water quality to confirm the safety of water for drinking.[11, 12] However, only 4.4% of the 113 wells sampled in this study had been formally tested for water quality after the flooding prior to our sampling.

379

380 Rapid testing shortly after residents return home after a flood is thought to be critical for
381 accurate assessment of private well water contamination. Here we moved quickly to
382 sample five homes shortly (one week) after floodwater receded to evaluate immediate
383 impacts. Among the four homes that participated in repeat sampling, we observed that
384 TC positive detects reduced with time, from three wells positive one-week post
385 floodwater receding to one positive one week later (Table S1). We thus speculate that the
386 measured TC and EC prevalence (24.8% and 3.5%) in the community-wide sampling
387 (10-weeks post flooding) may be underestimated, given that TC and EC positivity tends
388 to decrease due to natural attenuation and home water use (Table S1).[53] While some
389 studies note that impacts to private well water quality are still detectable one year post
390 flooding (shown as high positivity of TC and EC),[54-56] we suspect based on this study that
391 rapid post-flooding sampling is key in order to accurately evaluating flooding impacts to
392 TC and EC prevalence.

393

394 Of course, as this flood was an unanticipated natural disaster, it was not possible to
395 sample prior to the flooding as part of the present study. Among the 113 wells sampled,
396 only 10% had ever been tested at some point during their operation and only 3.5% tested
397 for bacteria (TC and EC). The lack of baseline private well water quality made it
398 impossible to directly assess the effects of flooding on microbial water quality.
399 Promoting private well water testing and well user stewardship, recognizing that there are
400 risks even in absence of flooding, will greatly improve baseline monitoring and help to
401 more accurately estimate impacts of future natural disasters. An accurate evaluation of

flooding impacts is important to inform decision-making, including the allocation of limited resources and timely remediation to protect the health of well users. Several organizations and institutes have led programs to promote private well water testing under non-flooding scenarios [40, 57, 58] and many U.S. state departments of health have made testing services and information available. Legal enforcement may help to better promote baseline monitoring, e.g., more wells in New Jersey were tested after passing the New Jersey Private Well Testing Act.[59] It is noteworthy that these well water testing requirements currently focus on metals and coliform indicators. Our study and a previous groundwater study suggested that OPs also merit consideration in baseline monitoring for private wells, as they are a suspected source of sporadic OP infection and many households do not treat or disinfect their well water.[20]

4.2 Indicator organisms: TC and EC

Testing for TC and EC is routinely used to evaluate microbial water quality in private wells. TC and EC have been detected in some private wells located in areas regardless of a recent history of flooding.[14, 40, 60] Reported TC and EC prevalence in non-flooding scenarios vary widely among different U.S. states (Pennsylvania, Delaware, etc.) and other countries, such as Canada (TC 14.6%-46% and EC 1.5% to 14%).[15, 39, 58, 60-62] Among the many factors that may affect TC and EC detection (e.g., land use, well maintenance, climate, season and rainfall), the local geology has been demonstrated to be especially critical.[61-63] For example, wells in the Coastal Plain aquifer had lower baseline prevalence of TC and EC than Blue Ridge-Piedmont and Valley-Ridge aquifer wells in the same state (Virginia), according to a non-flooding survey.[61] Thus we attempted to

roughly estimate flooding impact by comparing our post-flooding TC and EC data to previous reports of private wells located in aquifers of the same type as our testing in the Louisiana - Coastal Plain.[64] The rough estimate suggested that TC and EC prevalence in sampled private wells 10-weeks after the flooding was not uncharacteristically high. The post-flooding TC and EC prevalence reported here was similar to that in non-flooded private wells in the Coastal Plain aquifer of Virginia (TC 30% and EC 4%, n=534),[61] and to some post-flooding reports (e.g., 16.7%-22% positivity of TC in 12,000 private wells) on private wells in Coastal Plain of North Carolina.[56, 65] Notably, TC prevalence used as a control also varied (30% in non-flooded Virginia vs. up to 22% in flooded North Carolina) among different states of the same aquifer type, making it even more challenging to use baseline data from other states to evaluate flood-attributed deterioration in private well water quality. This again emphasizes the need for local baseline monitoring.

4.3 Bacterial OPs: *Legionella* spp. and *L. pneumophila*

About 96% of Legionnaires' Disease cases are sporadic, or non-outbreak related.[17, 25] Among the various confirmed and suspected sources for sporadic cases, proximity to natural water sources, non-municipal household water, and groundwater have been implicated.[25, 66-68] However, there are limited *Legionella* data from private wells to enable the evaluation of its contribution to the overall incidence of Legionnaires' Disease. Notably, most recent reports indicate that Louisiana had a higher incidence of reported Legionnaires' Disease than 28 other states and regions in U.S..[69] The actual incidence may be even higher because many cases go unrecognized and thus unreported,

448 particularly in rural areas, where people are more likely to use private well water.[70] The
449 widespread (77.5%) detection of *Legionella* gene markers and some detection (15%) of
450 *L. pneumophila* gene markers in our survey indicate that OPs in private wells deserve
451 greater attention, particularly in terms of need for better understanding, communication,
452 and management of risks to well users.
453
454 In terms of what to monitor, there is some debate regarding whether to focus specifically
455 on *L. pneumophila* versus more generally on *Legionella* spp. By definition, legionellosis
456 is caused by any species of *Legionella*, of which twenty are known to contain human
457 pathogens.[71] While *L. pneumophila* accounts for 90% of reported Legionnaires' disease
458 in the U.S., the contribution of other *Legionella* spp. is likely to be underreported because
459 the widely-used urine antigen test for *Legionella* infections only detects *L.*
460 *pneumophila*.[20, 71] It is thought that other *Legionella* spp. are likely to contribute to the
461 many community-acquired pneumonia cases, but are not classified as legionellosis due to
462 lack of testing or lack of specificity of tests used.[72] In the European Union, recent water
463 monitoring guidelines recommend targeting *Legionella* spp.[73] Thus, we monitored both
464 *L. pneumophila* and *Legionella* spp. in the present study. We found that *Legionella* spp.
465 were more readily detectable and quantifiable and thus most of our statistical analyses,
466 findings, and discussion relate to *Legionella* spp., unless referring specifically to *L.*
467 *pneumophila*.
468
469 As was the case for TC and EC, we attempted to evaluate flooding impact on *Legionella*
470 spp. using literature data and found that flooding may have contributed to increased

occurrence of *Legionella* gene markers, given that post-flooding prevalence of *Legionella* spp. gene markers in our study (50.0% in 40 private wells) appeared higher than background levels in non-flooded wells. For example, *Legionella* spp. were detected in 28-29% of 114 groundwater samples collected from various regions representing a range of geologies in the U.S. and Canada, both by DNA-based and culture-based methods.[67] Another study reported a similarly low positive detection of *Legionella* spp. gene markers (25% of 12 wells in U.S.).[24] In contrast, an even higher positivity (73% of 11 wells) was reported from a known contaminated region in Nepal.[74]

Comparing prevalence of *Legionella* in homes served by private wells versus municipal systems is of particular interest. This comparison indicates that *Legionella* occurrence may be at least as problematic in our surveyed homes as it is for municipal water systems. For example, positivity of *Legionella* gene markers in our post-flooding survey (77.5% of 40 homes) was similar to that of a previous study of municipal water systems (69% of 29 sites).[44] The positivity of *L. pneumophila* gene markers in particular (15% of 40 homes) fell within the wide range of detection in municipal systems, from 0% (n=7) to 47% (n=68).[16, 44, 75] As would be expected, detection of *L. pneumophila* was less frequent than *Legionella* spp. that include dozens of other *Legionella* species. The relatively simple building structure and thus less complex configuration of premise plumbing in homes drinking from private wells may contribute to the lower colonization rate of *L. pneumophila* than that observed in a nationwide survey of municipal waters (47% of 68 sites) conducted by Donohue et al.[16] Specifically, a private well serving a single-family home (74% of 40 wells) to at most three homes (2.5%) in our survey, while the Donohue

et al. survey[29] sampled mostly from larger buildings (80%). More complex building structures and corresponding premise plumbing configuration have been implicated in higher prevalence of *Legionella.*[76]

While indicator organisms are widely used in private well water testing, this study further confirms the expectation that total coliform positivity is not appropriate for predicting the occurrence of *Legionella* spp. or *L. pneumophila*.[14, 77, 78] Similar observation of no correlations between fecal indicators and OPs have been noted.[30] On the other hand, total bacterial levels were significantly correlated with the positivity and abundance of *Legionella* spp. gene markers (Figure 2) and the positivity of *L. pneumophila* (Figure 3). While there is no direct health effect associated with total bacteria, or more traditionally targeted heterotrophic plate count (HPC) bacteria,[79] increases in total bacteria numbers are reflective of water conditions favorable to microbial growth and can be suggestive of deterioration of water quality during distribution and system maintenance.[80, 81] Interestingly, studies of municipal systems have indicated that detection of OPs is independent of total bacterial numbers or HPC.[82] This discrepancy may result from different disinfection practices in municipal versus private systems. Most public waters are treated with a primary disinfectant and distributed with a secondary disinfectant residual in the U.S., while private wells are usually free of disinfectant (76% of 113 wells had no history of disinfection). Since OPs (especially *Legionella*) and their amoeba hosts are often relatively tolerant to disinfectants, municipal systems with a disinfectant residual could limit heterotrophic growth while *Legionella* survives. In private wells with no disinfection, conditions in the aquifer, wells, and premise plumbing that are favorable

517 for microbial growth could promote *Legionella* spp., *L. pneumophila,* and total bacteria
518 growth alike. Conditions that favored increased microbial growth rates were found to
519 result in elevated concentrations of *Legionella* in a simulating system supplied with un-
520 disinfected Dutch municipal water.[83] This suggests that high total bacterial numbers may
521 be an appropriate indicator of *Legionella* spp. and *L. pneumophila* in private water
522 systems. Thus, an initial but fast OPs screening in private well water may be achieved by
523 acquiring total bacterial numbers with more rapid and cost effective methods, such as
524 flow cytometry.[81]
525
526 Another important finding of this study is that it revealed the critical role of water quality
527 in well columns to potential consumer exposure and health risks at the point of use.
528 Positive detection and higher abundance of *Legionella* spp. gene markers in well columns
529 were more likely to yield detectable and higher levels of *Legionella* spp. at distal taps.
530 Similarly, higher total bacterial numbers in well columns leads to higher levels of total
531 bacteria at taps. This indicates that the source water quality in well columns was a critical
532 factor influencing water quality at the tap, i.e., point of exposure. This suggests the
533 importance of protecting wells from contamination via flooding or intrusion of debris or
534 soil through unsealed or insecurely sealed well caps.[84]
535
536 Unexpectedly, we did not observe a higher prevalence or abundance of *Legionella* spp.
537 gene markers in first draw hot water than in 5-min flushed cold water (section 3.4),
538 though total bacteria were higher (Figure S3). This lack of difference may due to the
539 observed diverging pattern in pair-wise comparisons, in which some homes had >1-log

greater *Legionella* spp. gene copies in their hot waters than well waters, while several others exhibited >1-log less (Figure S2). We speculate that the fate of *Legionella* spp. originating from the well water may have been stimulated in some homes and effectively controlled in others due to variable water heater settings and efficacy of hot water delivery. In other words, hot water temperature can either have a remedial effect (i.e., when >60°C to kill *Legionella* species) or a stimulating effect (i.e., when locate within the growth range of *Legionella* (25 to 42°C)). Though temperature tends to have an overarching effect,[38, 85] many other factors in the home, such as premise plumbing construction and materials, type of water heater, and water use patterns[76, 86-88] can influence OP propensity for re-growth and could have influenced *Legionella* spp. detection patterns in individual homes.

Metal plumbing components and associated heavy metals in water have been reported to be important factors in predicting *Legionella* occurrence in municipal water systems.[87, 89, 90] Similarly observed here in private well systems was that positive detection of *Legionella* spp. gene markers and higher total bacterial numbers in homes were associated with relatively higher zinc and copper in flushed cold water, though potential metal source and influencing factors such as pump and household plumbing materials were not determined in this study. Notably, elevated metal levels in water were from pump and plumbing corrosion and can further increase with extended stagnation and thus provide opportunity for OP regrowth.[39, 49, 91] In contrast, there did not appear to be any associations between indicators or OPs with well characteristics or reported well damage post flooding, agreeing with a previous report.[54]

563

564 ### 4.4 Occurrence of *N. fowleri* gene markers in private wells

565 *N. fowleri* has historically been of concern for recreational exposures in warm waters,
566 such as hot springs, lakes, and ponds in hot summer months.[92] However, recent deaths
567 have also been linked to municipal tap water exposure,[26, 27] notably occurring in the
568 southern states where "cold" tap water temperatures can be as high as 34°C.[26] While *N.*
569 *fowleri* is fairly sensitive to secondary disinfectants,[93, 94] disinfectants are rarely applied
570 or maintained in wells, as noted above. Here, we hypothesized that private well water in
571 this warm region of Louisiana could also be vulnerable to *N. fowleri* colonization,
572 particularly given potential contamination from the flooding, conducive growth
573 temperatures, and lack of disinfectant residual.

574

575 Combined DNA- and culture-based detection of *N. fowleri* was previously reported in
576 high-volume public drinking water wells[30-32] and in home water systems supplied with
577 municipal water.[26, 27] To our knowledge, this study is the first to report the detection of *N.*
578 *fowleri* gene markers in a community-wide sampling of private wells and premise
579 plumbing. We observed that *N. fowleri* gene markers were detectable in 20.0% of 40
580 homes drinking from private wells after the flood. The *N. fowleri* assay employed in this
581 study was selected based on its high sensitivity and specificity; thus, there is good
582 confidence that *N. fowleri* DNA was truly detected.[45] Further, the assay targets a low
583 copy number region (1-2 copies) of an *N. fowleri* ITS, suggesting that the gc/mL
584 measured in this study should be in the same order of magnitude of actual cells/mL.
585 However, as is the case with any molecular survey, gene detection does not provide direct

information about viability. A previous study showed that only one out of 11 DNA-based *N. fowleri* positives were culture confirmed.[31] However, detection of *N. fowleri* gene markers indicates that these organisms must have been alive at some point in the system, though the 20% of occurrence measured in this survey was likely an overestimate of viable and infectious *N. fowleri.*

Given the high mortality rate of *N. fowleri* infections, detection of its DNA in private wells merits further attention, as nearly 12.5% of the population (over 500,000 people) in Louisiana relying on private wells. Case studies following *N. fowleri* deaths from homes served by municipal water in Louisiana reported that the affected households had elevated cold water temperatures (>30 °C), had no or low disinfectant residual (<0.5 mg/L), and were located in regions historically flooded by Hurricane Katrina,[26] conditions consistent with the area in which the present survey was conducted. Future studies employing more rigorous methods to quantify viable and infectious *N. fowleri* cells in private well water are advisable to verify concerns for well owners. Regardless of water source, it is advisable to take appropriate precautions to avoid exposure of nasal canals with non-sterile water, e.g., irrigating sinuses.

5 Conclusions

Here we surveyed drinking water quality in homes reliant on private wells following the August 2016 flood in Louisiana. In addition to testing for traditional indicator organisms of fecal contamination (TC, EC*),* the occurrence of several OPs, including *Legionella* spp., *L. pneumophila*, and *N. fowleri* was surveyed by molecular methods. The wide

609 detection of *Legionella* spp. and sporadic detection of *L. pneumophila* and *N. fowleri*
610 gene markers raise general concerns in drinking water quality for private well users after
611 a flooding event, even though the detection frequency of TC and EC was not concerning
612 relative to pre- and post-flooding prevalence in regions with similar aquifer type. Total
613 bacterial measures, rather than TC positivity, may be a good indicator of OPs
614 colonization risk in home water systems supplied by private wells, which are not
615 regularly disinfected. Microbial levels, including OPs in well columns (sampled after 5-
616 min flushing), were critical for water quality at the consumption point (sampled as first
617 draw cold and hot water at taps). Both *Legionella* spp. detection and total bacterial
618 numbers were higher in homes with higher levels of zinc and copper in well columns,
619 indicating that water stagnation in premise plumbing may further stimulate OPs regrowth.
620 Overall, this study provided valuable information about prevalence of OPs in private well
621 water and potential microbial concerns following flooding events. Further testing to
622 confirm the viability of OPs would be worthwhile in future surveys to assess exposure
623 risks for well users. Insight gained into factors associated with elevated OP gene markers
624 can inform targeted monitoring and mitigation in the future.
625

626 ## 6 Conflicts of interest

627 There are no conflicts to declare.
628

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941 Table 1. Prevalence of target microorganisms in homes served by private wells ten weeks
942 after floodwater receded

Microorganisms	5-min flushed cold water (well water)	First draw cold water (first draw)	First draw hot water (hot draw)	Among homes*	All samples combined
Among all 113 homes sampled during the community-wide sampling					
Total coliform[a]	24.8%	Not tested	Not tested	/	/
E. coli[a]	3.5%	Not tested	Not tested	/	/
Among the subset of 40 homes receiving the advanced kits					
Total coliform[a]	30.0%	Not tested	Not tested	/	/
E. coli[a]	5.0%	Not tested	Not tested	/	/
Legionella spp.[b]	50.0%[n1]	48.6%[n2]	54.1%[n3]	77.5%	50.9%[n4]
L. pneumophila[b]	7.9%[n1]	5.4%[n2]	13.5%[n3]	15.0%	8.9%[n4]
N. fowleri[b]	5.3%[n1]	10.8%[n2]	13.5%[n3]	20.0%	9.8%[n4]

943 *Positive detection in a home if at least one water sample tested positive.

944 [a] IDEXX Colilert 2000 method; [b] qPCR targeting the 23S rRNA gene of *Legionella* spp., *mip*
945 gene of *L. pneumophila,* and an ITS region of *N. fowleri*.

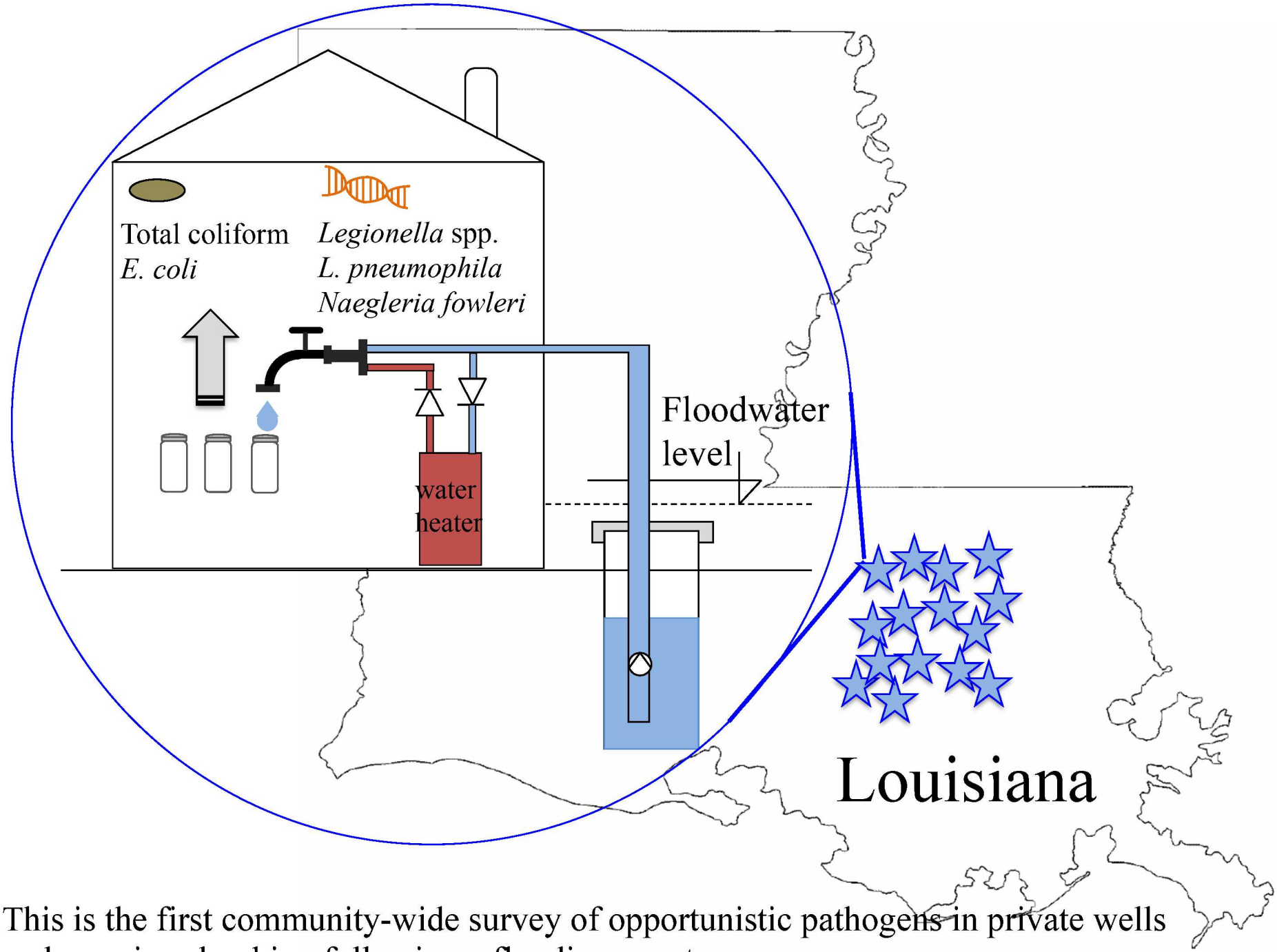
946 Sample numbers: n1=38, n2=37, n3=37 (a couple of samples were lost during sample
947 processing); n4=112 all water samples combined.

948

949 Table 2. Positive detection of *Legionella* spp. gene marker in first draw tap waters
950 significantly depended on their detection in flushed well water.

***Legionella* spp. in well water (n=35)[&]**	***Legionella* spp. in first draw**			***Legionella* spp. in hot draw**		
	Detected	Not detected	p[#]	Detected	Not detected	p[#]
Detected (n=17)	14 (82%)[*]	3 (18%)[*]	0.0002	12 (71%)[*]	5 (29%)[*]	0.03
Not detected (n=18)	4 (22%)[*]	14 (78%)[*]		6 (33%)[*]	12 (67%)[*]	

951 [*]The percentages were row % (e.g., 82% = 14/17); [&]Total sample number was 35, after
952 excluding homes with DNA sample lost during process; [#]The p values were from Chi-
953 Square tests; Well water: 5-min flushed cold water; first draw: first draw cold water; hot
954 draw: first draw hot water; all samples collected from kitchen taps.



This is the first community-wide survey of opportunistic pathogens in private wells and premise plumbing following a flooding event.