Comparative evaluation of viability qPCR, against traditional culture (ISO 11731) for detection and quantification of *Legionella* in field samples.

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Abstract

Aims: To evaluate the capability of a modern, multiplex, qPCR method, to detect, enumerate, and characterize *Legionella* spp. in various premise water systems; as well as to determine the role qPCR could play in evaluation of routine monitoring for *Legionella* spp. in Water Management Programs. This study constitutes Phase 2 of a multi-phase *Legionella* study – Phase 1 was reported on in 2021 (1).

Methods and Results: A total of 91 samples were submitted by water treatment companies, as well as facility owners encompassing a variety of premise water systems including cooling towers, sinks/showers, storage tanks, and water fountains. Each sample was processed concurrently both culturally via ISO 11731, and via a commercially available, multiplex qPCR kit employing viability reagents (referred to in Phase 1 as "Method C"). In summary, 15.4% of samples were positive for *Legionella* spp. (14.3% *Legionella pneumophila* serogroup 1) by culture, compared to 65.9% of samples positive for *Legionella* spp. (31.9% *L. pneumophila* serogroup 1) by qPCR. 32.9% of samples had no *Legionella* detected by either method.

Conclusions: Based on the field samples evaluated, qPCR with viability reagents detected *Legionella* spp. in 65.9% of samples, compared to only 15.4% of samples evaluated by culture. Likewise, 31.9% of samples evaluated by qPCR with viability reagents were positive for *L. pneumophila* serogroup 1, compared to only 14.3% of samples evaluated by culture. This suggests that culture may be underreporting the incidence of *Legionella* spp. in premise water systems.

Introduction

The cultural method (i.e., ISO 11731) is often purported to be the "Gold Standard" in *Legionella* testing. However, there are many known problems associated with this method – the most often noted being its long time to result which can be up to two weeks or more. This lengthy time to result puts the water system operator in a constantly reactive position, forcing the system to be used "at risk", or even taken out of service for weeks or months at a time, whenever a significant *Legionella* recovery is observed.

We reported to AWT at the 2021 Annual Convention on results from Phase I of a study we conducted evaluating three distinct modern, PCR/qPCR-based technologies that employ "live/dead" differentiation chemistries. Phase I of this study compared these methods to one another as well as to the cultural method for lab-inoculated water samples. All three rapid methods showed equivalent or better qualitative detection efficiency in one day, compared to the 7-14 days required for the cultural method (1). Phase II of the study compared the most promising of the three rapid methods to the cultural method, utilizing samples gathered in various end-use field applications. We observed an alarming trend of detection of *Legionella* spp. via qPCR in the same samples for which detection was missed by culture. This is consistent with data we collected in Phase I which showed that the cultural method had poor qualitative detection efficiency at low levels of *Legionella* contamination.

These data demonstrate that there are now multiple, accurate, rapid *Legionella* testing alternatives to the cultural method, and technical improvements in recent years make them an excellent choice for routine *Legionella* monitoring. From a practical perspective, it is easy to grasp how a faster time to result would mean that water treaters could be more agile in their approach to managing the health of their water systems. However, these new data suggest that rapid methods, in many cases, may be the optimal choice

for obtaining the most accurate assessment of *Legionella* risk in a facility's water system. The sensitivity, accuracy, and faster time to result of qPCR puts the water management team in a far more proactive position to efficiently detect *Legionella*, assess the risk, and take appropriate action.

Materials and Methods

Sample Collection

Between July, 2021 and August, 2022, ninety-one water samples were submitted to the laboratory from sinks, showers, ice machines, decorative fountains and cooling towers. Water samples were collected in sterile polyethylene bottles containing sodium thiosulfate (Microtech Scientific) and hand-delivered or shipped to the laboratory overnight.

Sample Analysis

Heterotrophic Plate Count

Upon sample arrival a Heterotrophic Plate Count (HPC) was conducted following SMEWW 9215 B (2). Samples were homogenized, diluted accordingly in Butterfield's Phosphate Buffer and plated in duplicate with Plate Count Agar (PCA). Agar plates were incubated at 35 °C for 48 hours. After incubation, colonies were enumerated to determine heterotrophic bacterial concentrations.

Using a paired study design, water samples were analyzed for the presence of *Legionella* species following traditional, culture methodology (ISO 11731:2017), and a modern, commercially available viability qPCR assay. A 100 mL volume of water was filter concentrated for non-potable samples (i.e., those originating from cooling towers, and decorative fountains). For potable water samples, either 250 mL or 1000 mL of water was filter concentrated. The volume filtered was determined by the amount of water sample submitted to the laboratory.

Regardless of water source, all samples were vacuum-filtered through 0.2 µm membrane filters. Each membrane filter was aseptically transferred using sterile forceps to a 5 mL tube containing 5 mL of sterile, diluted, ¼-strength Ringers Solution. Tubes were inserted onto a vortex mixer equipped with a horizontal tube adapter for 1 min. at full speed. After 1 min. elapsed, tubes were vertically rotated 180 degrees and vortex mixed for an additional minute. Finally, sample tubes were centrifuged at 1,000 x *g* for 1 min.

***Legionella* Culture Method (ISO 11731:2017)**

A 100 µL aliquot of the eluent was spread plated using sterile plating beads onto the surface of one Buffered Charcoal Yeast Extract (BCYE) agar plate and one GPCV agar plate (BCYE supplemented with Glycine, Polymyxin B Sulfate, Cycloheximide and Vancomycin). After the inoculum was absorbed into the agar surface the plating beads were disposed of and the plates incubated at 36 ± 2 °C under humidified conditions. Plates were examined after 72-96 hours for evidence of suspect *Legionella* colonies. Presumptive colonies were identified at the species level using MALDI-TOF technology. Regardless of identification, all plates were placed back into the incubator for the remainder of the incubation period. Agar plates were again examined for presumptive colonies after incubating for 7 days. Colonies that identified as a *Legionella* species, such as *L. feelei*, were considered confirmed. Colonies that identified as *L. pneumophila* were further subcultured onto BCYE with and without cysteine (BCYE-). Growth on BCYE with cysteine and no growth on BCYE- is indicative of most Legionella growth characteristics; therefore, the *L. pneumophila* serogroup was then determined using a commercially available Legionella latex agglutination kit.

Viability qPCR

The commercially available qPCR DNA extraction procedure began with 500 µL from the same eluent used in the cultural method being transferred to 500 µL of the included rinse buffer in a microcentrifuge tube. To this, 400 µL of a cell viability reagent was added and the suspension mixed. After incubating at room temperature for 10 minutes in the dark, the tubes were exposed to a light source for 5 minutes. Centrifugation was conducted at 8,000 x *g* for 5 minutes to pellet the bacterial cells. The resulting supernatant was removed using a sterile micropipette and the pellet resuspended in 150 µL of lysis buffer. Mechanical disruption of the cells was achieved by placing the tubes on a Disruptor Genie (Scientific Industries, Inc.) for 8 minutes. The final steps in DNA extraction involved heating the microcentrifuge tubes at 95 °C for 5 minutes followed by centrifugation at 13,000 x *g* for 1 minute.

The commercially available qPCR assay includes ready-to-use positive/negative controls and quantification standards, as well as PCR reaction tubes prefilled with lyophilized reagents. A 25 µL volume of sample DNA extract was analyzed for each qPCR reaction using the Bio-Rad CFX96 Touch Real-Time PCR Detection System.

Results of the qPCR analysis are determined by entering the sample information (sample ID and filtration volume), sample Cq values, kit control and kit standard Cq values into the quantification template provided by the assay manufacturer. If detection of Legionella occurs, quantitative data is presented in Genomic Units (GU) for *Legionella* species (HEX channel), *L. pneumophila* (FAM channel) or *L. pneumophila* SG 1 (ROX channel), or a combination of the three channels.

Results

In total, 91 samples were submitted by volunteer water treatment companies. Companies were asked to indicate the source of the water, however for 42 of the samples, the source type was not indicated. These samples were still tested.

A total of 14 samples (15.4%) were positive for *Legionella* spp. when analyzed by culture (ISO 11731), compared to 60 (65.9%) when analyzed by qPCR. Furthermore, 13 samples (14.3%) were determined to be *Legionella pneumophila* serogroup 1 when analyzed by culture, compared to 29 (31.9%) when analyzed by qPCR. Interestingly, only 2 samples (2.2%) were positive for *Legionella* spp. by culture, that were determined to be something other than *L. pneumophila* serogroup 1 (one instance of *L. pneumophila* serogroup 2-15, and one instance of *Legionella feeleii*). Thirty samples (32.9%) had no detection by either method.

Notably, there were no instances of *Legionella* detection by culture, that was not detected by qPCR. However, there were 46 samples (51.1%) that were positive by qPCR, but negative by culture. It is important to note that the method's prescribed viability reagent, which has been previously evaluated for efficacy, was employed when analyzing the samples by qPCR (1). As such, there is strong evidence to suggest that the positive samples likely do not represent significant detection of DNA from dead cells. Rather, these data suggest that culture is significantly underreporting the incidence of *Legionella*.

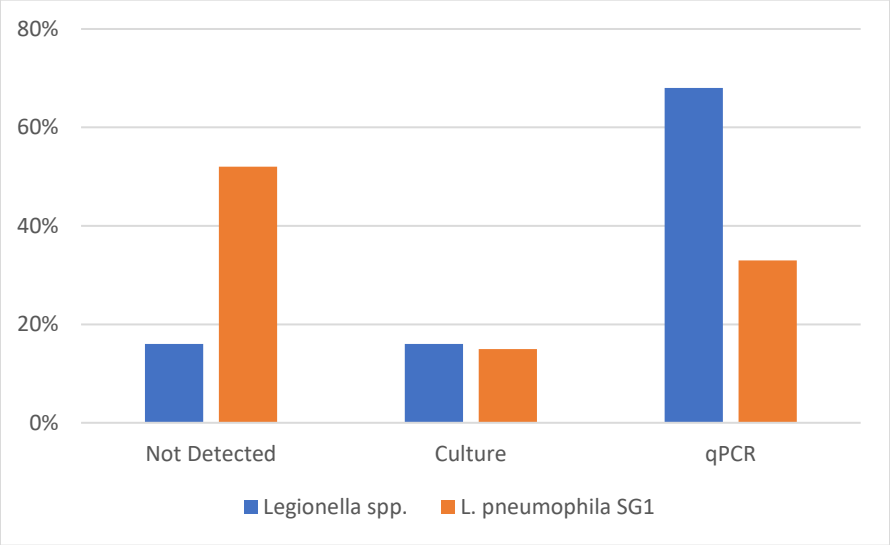


Figure 1 – Comparison of *Legionella* detection by culture and viability qPCR.

As can be seen in Table 1, the largest category of source identifiable samples determined to be positive for *Legionella* was Sinks/Showers with 38.5% of samples being positive. However, this should be considered skeptically as the largest category of positive samples overall was where sample source information was not provided.

Table 1 – Incidence of Legionella spp., and L. pneumophila in various sample types.

Sample Source[*,†]	**No. of Samples (%)**	**No. of Samples + for *Legionella* spp. (%)**	**No. of Samples + for *L. pneumophila* (%)**
Cooling Tower	13 (14.3)	8 (61.5)	5 (38.5)
Sink/Shower	35 (38.5)	11 (31.4)	9 (25.7)
Ice Machine	1 (1.1)	1 (1.1)	- (-)
Not Provided[†]	42 (46.2)	25 (59.5)	27 (64.3)

Of the samples positive by culture, strong qualitative concordance was observed with qPCR with 100% of samples positive by culture, also being positive by qPCR. When samples were positive by culture, a fairly strong relationship was observed when comparing Log CFU and Log GU as was previously reported on in Phase 1. In Phase 1, a Log difference of 1.16 was reported for the HEX channel which represents *Legionella* spp. Channels FAM and ROX were not reported, because Phase 1 was comparing to two other (q)PCR methods that could only report *Legionella* spp. In Phase 2, when evaluating the Log difference for the FAM and ROX channels (representing *L. pneumophila* and *L. pneumophila* SG 1, respectively), an even smaller Log difference was observed with a mean Log difference of 0.89 for FAM, and 0.80 for ROX (ref. Table 3). It should be noted that only those samples that were positive by culture could be included in the comparison. Likewise, only those samples positive by qPCR with detection at or above the LOQ for the assay could be included in the comparison. This reduced the data points for comparison to the 11 samples outlined in Table 2.

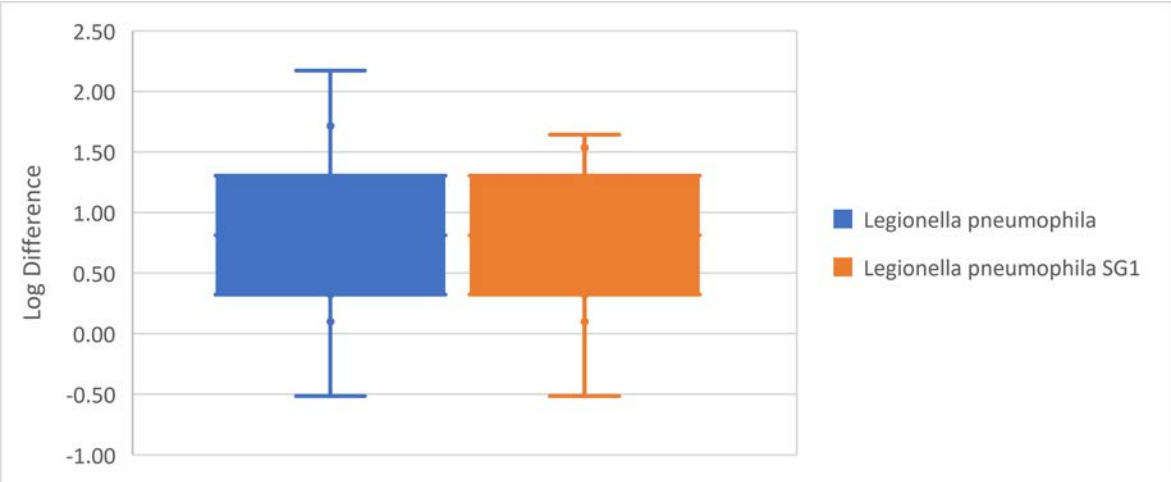
Table 2 – Log Difference between culture concentrations and GU. NB: only culture positive *Legionella* samples could be used in the Log comparison, and all but one *Legionella* positive sample were identified as *L. pneumophila* SG 1.

Log GU/mL FAM	Log GU/mL ROX	Log CFU/mL	Log Difference FAM	Log Difference ROX
3.41	3.34	1.70	1.72	1.64
4.57	3.93	2.40	2.17	1.54
5.00	5.00	3.76	1.24	1.24
3.45	3.45	2.70	0.75	0.75
5.41	5.11	4.61	0.80	0.50
5.40	5.40	4.26	1.14	1.14
3.59	3.59	2.78	0.81	0.81
5.87	5.87	4.57	1.30	1.30
5.52	5.52	5.42	0.10	0.10
3.62	3.62	3.30	0.32	0.32
4.66	4.66	5.18	-0.52	-0.52

Table 3

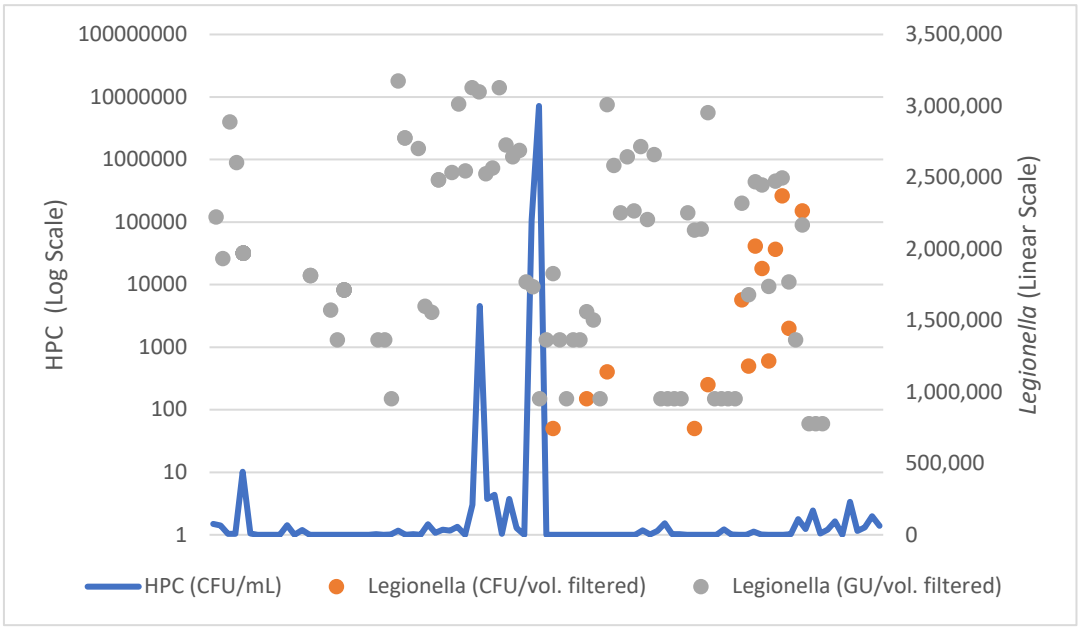
Analyte*	**Mean Log Difference**	**St. Dev.**
L. pneumophila[†]	0.89	0.75
L. pneumophila SG 1[†]	0.80	0.66

Figure 2 – Distribution of Log Differences (GU-CFU) for *L. pneumophila* and *L. pneumophila* SG 1.



Background HPC results ranged from <1 CFU/mL to 3.0E+6 CFU/mL. It is unclear what effect, if any, HPC background had on the detection of *Legionella* in building water systems. Recovery of *Legionella* via traditional culture was rare, and was observed even with background HPC results as high as 1.7E+5 CFU/mL. Detection of *Legionella* via qPCR was often high despite high HPC background. However, *Legionella* was detected at high concentrations by qPCR regardless of background HPC.

Figure 3 – *Legionella* detection by viability qPCR versus culture, with varying levels of HPC background.



Discussion

The results from Phase 2 of this study provide further evidence supporting the conclusions from Phase 1; that viability qPCR is a more sensitive technique for detecting *Legionella*, and as such, should be considered for use as a primary, routine, testing technique in water management programs. The data certainly suggest equivalent or better detection rates, with no instances of *Legionella* detection by culture that were not observed by qPCR, but the reverse was often true. Taking for granted the veracity of the qPCR results, then in the course of Phase 2 of this study, culture would have demonstrated an approximately 51% false negative rate. The majority of these culture "misses" were instances of low level contamination. We suggest a new risk model, that takes advantage of the sensitivity of qPCR, putting the responsible party in a more proactive position in terms of water management.

The United States Centers for Disease Control and Prevention released a multifactorial approach to performance indicator interpretation in routine *Legionella* testing (3). This model discusses four factors that should all be considered in a holistic manner when assessing risk of *Legionella* detection during routine testing as part of a robust water management plan. These factors are the concentration of *Legionella* in a discrete sample, the change in *Legionella* concentration over time, the extent to which *Legionella* is detected throughout a system, and the type of *Legionella* (species and serogroup) that may be detected. This model is based on culture, and gives suggested quantitative metrics in CFU/mL for both concentration, and change in concentration over time. We suggest that the same multifactorial approach can be applied to the GU/mL results from viability qPCR.

Some have suggested converting GU/mL to CFU/mL. This is not simple as one GU does not equal one CFU. One GU more closely approximates the number of individual *Legionella* cells in a sample, and one CFU often arises from a clump of tens, hundreds, or perhaps thousands of individual cells. Ditommaso et al suggested an algorithm based on the results of their study whereby GU/mL result were to be multiplied by 28 to approximate CFU/mL (4). However, this is based on a specific, limited dataset, and requires a number of assumptions that may not always be applicable. Therefore, while a conversion algorithm of this type may be useful in some instances, we contend that it is not necessary. Moreover, such a conversion in practice may dilute the power of the superior sensitivity of qPCR.

In Phase 1 of this study, we reported that, on average, Log(GU/mL) results were approximately 1.16 Log higher than Log(CFU/mL) for *Legionella* spp. (HEX channel) (1). This is similar to Ditommaso et al who reported a 1.45 Log difference. In Phase 2, the *L. pneumophila* and *L. pneumophila* serogroup 1 differences were evaluated due to the characterization of culture recoveries, and found to be 0.89 and 0.80 respectively (ref. Table 3). However, as previously noted, these results are likely arbitrarily close given the limited sample size, that only those samples positive by culture could be considered in the comparison, and the fact that as previously shown in Phase 1, the culture method (ISO 11731:2017) shows significantly better recovery of *L. pneumophila* compared to other *Legionella* spp.

As one genomic unit more closely approximates the number of individual *Legionella* cells, not the number of colony forming units, asserting that one genomic unit equals one colony forming unit would be a very conservative approach to take. Given the experimental results of this study and others, we suggest that a 1 Log difference is reasonably conservative, easy to understand, and simple to implement in a new risk-based model for viability qPCR.

Figure 4 – Proposed approach for viability qPCR data interpretation in routine *Legionella* monitoring.

Concentration indicates that *Legionella* growth appears:					
Uncontrolled	**Poorly Controlled**	**Well Controlled**			
>100 GU/mL in potable water OR >1000 GU/mL in non-potable water	10-99 GU/mL in potable water OR 100-999 GU/mL in non-potable water	Detectable <LOQ – 9.9 GU/mL in a single round of testing in potable water OR Detectable <LOQ – 99 GU/mL in non-potable water	Detectable <LOQ in multiple rounds of testing	<LOD (no detection) in a single round of testing	<LOD (no detection) in multiple rounds of testing
Change in concentration over time indicates that *Legionella* growth appears:					
Uncontrolled	**Poorly Controlled**	**Well Controlled**			
100-fold or greater increase in concentration (e.g., 10 GU/mL to 1000 GU/mL)	10-fold or greater increase in concentration (e.g., 10 GU/mL to 100 GU/mL)	Legionella concentration steady (well controlled levels) for two consecutive sampling rounds	Detectable <LOQ in multiple rounds of testing	<LOD (no detection) in a single round of testing	<LOD (no detection) in multiple rounds of testing

The model outlined in Figure 4 applies a 1 Log increase to the values outlined in the CDC's multifactorial approach (3). The model also accounts for a scenario that was frequently observed during this study whereby a sample was positive, with detection below the Limit of Quantification (LOQ) of the qPCR method.

Table 4 – Comparison of data interpretation between existing CDC recommendation, and proposed qPCR model (Fig. 4).
*concentration defined level, but *L. pneumophila* detection may increase assigned risk

#	Sample Type	Culture Result (CFU/mL)	Interpretation Per CDC Culture Risk Model	qPCR Result (GU/mL)			Interpretation Per Proposed qPCR Risk Model	Discrepancy ✓ = none ○ = one level ● = two levels
				Legionella spp.	*L. pneumophila* SG 2-15	*L. pneumophila* SG 1		
1	Cooling tower	-	Well Controlled	1,200	-	-	Uncontrolled	●
2	Cooling tower	-	Well Controlled	260	-	-	Poorly Controlled	○
3	Cooling tower	-	Well Controlled	40,000	<LOQ	-	Uncontrolled	●
4	Cooling tower	-	Well Controlled	8,900	260	-	Uncontrolled	●
5	Cooling tower	-	Well Controlled	320	-	-	Poorly Controlled	○
6	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
7	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
8	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
9	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
10	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
11	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
12	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
13	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
14	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
15	Ice machine	-	Well Controlled	14	-	-	Well Controlled	✓
16	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
17	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
18	Potable POU	-	Well Controlled	3.9	-	-	Well Controlled	✓
19	Potable POU	-	Well Controlled	<LOQ	-	-	Well Controlled	✓
20	Potable POU	-	Well Controlled	8.2	-	-	Well Controlled	✓
21	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
22	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
23	Unknown	-	Well Controlled	-	-	-	Well Controlled	✓
24	Unknown	-	Well Controlled	-	-	-	Well Controlled	✓

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25	Unknown	-	Well Controlled	<LOQ	-	-	Well Controlled	✓
26	Potable POU	-	Well Controlled	<LOQ	-	-	Well Controlled	✓
27	Potable POU	-	Well Controlled	<LOQ	-	-	Well Controlled	✓
28	Unknown	-	Well Controlled	72,000	-	-	Uncontrolled	●
29	Unknown	-	Well Controlled	8,800	-	-	Uncontrolled	●
30	Unknown	-	Well Controlled	-	-	-	Well Controlled	✓
31	Cooling tower	-	Well Controlled	15,000	380	110	Uncontrolled	●
32	Cooling tower	-	Well Controlled	45	<LOQ	<LOQ	Well Controlled*	✓*
33	Cooling tower	-	Well Controlled	36	-	-	Well Controlled	✓
34	Cooling tower	-	Well Controlled	4,700	13	13	Uncontrolled	●
35	Unknown	-	Well Controlled	-	-	-	Well Controlled	✓
36	Unknown	-	Well Controlled	6,200	-	-	Uncontrolled	●
37	Unknown	-	Well Controlled	77,000	<LOQ	<LOQ	Uncontrolled	●
38	Unknown	-	Well Controlled	6,600	<LOQ	-	Uncontrolled	●
39	Unknown	-	Well Controlled	140,000	-	-	Uncontrolled	●
40	Unknown	-	Well Controlled	120,000	-	-	Uncontrolled	●
41	Unknown	-	Well Controlled	5,900	<LOQ-	-	Uncontrolled	●
42	Unknown	-	Well Controlled	7,300	<LOQ	<LOQ	Uncontrolled	●
43	Unknown	-	Well Controlled	14,000	<LOQ	<LOQ-	Uncontrolled	●
44	Unknown	-	Well Controlled	17,000	<LOQ	-	Uncontrolled	●
45	Unknown	-	Well Controlled	11,000	<LOQ	<LOQ	Uncontrolled	●
46	Unknown	-	Well Controlled	14,000	-<LOQ	<LOQ	Uncontrolled	●
47	Potable POU	-	Well Controlled	44	8		Poorly Controlled	○
48	Potable POU	-	Well Controlled	36.8	8	-	Poorly Controlled	○
49	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
50	Potable POU	-	Well Controlled	<LOQ	<LOQ	-	Well Controlled*	✓*
51	Cooling tower	0.2*	Well Controlled*	60	<LOQ	-	Well Controlled*	✓*
52	Cooling tower	-	Well Controlled	<LOQ	-	-	Well Controlled	✓
53	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
54	Potable POU	-	Well Controlled	5.2	<LOQ	<LOQ	Well Controlled	✓*
55	Potable POU	-	Well Controlled	<LOQ	-	-	Well Controlled	✓
56	Potable POU	0.6*	Well Controlled*	14.8	<LOQ	<LOQ	Poorly Controlled*	○
57	Potable POU	-	Well Controlled	10.8	<LOQ	<LOQ	Poorly Controlled	○
58	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
59	Cooling tower	4*	Well Controlled*	750,00	-	-	Uncontrolled	●
60	Unknown	-	Well Controlled	80,00	22	<LOQ	Uncontrolled	●
61	Unknown	-	Well Controlled	14,00	<LOQ	-	Uncontrolled	●
62	Unknown	-	Well Controlled	110,000	15	<LOQ	Uncontrolled	●
63	Cooling tower	-	Well Controlled	1,500	1,00		Uncontrolled	●
64	Unknown	-	Well Controlled	160,000	<LOQ	<LOQ	Uncontrolled	●
65	Unknown	-	Well Controlled	1,100	<LOQ	<LOQ	Uncontrolled	●
66	Unknown	-	Well Controlled	12,000	57	57	Uncontrolled	●
67	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
68	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
69	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
70	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
71	Unknown	-	Well Controlled	1,400	20	20	Uncontrolled	●
72	Unknown	0.5*	Well Controlled*	740	26	22	Uncontrolled	●
73	Unknown	-	Well Controlled	770	<LOQ	<LOQ	Uncontrolled	●
74	Unknown	5*	Poorly Controlled	560,000	370	86	Uncontrolled	○
75	Unknown	-	Well Controlled	-	-	-	Well Controlled	✓
76	Unknown	-	Well Controlled	-	-	-	Well Controlled	✓
77	Unknown	-	Well Controlled	-	-	-	Well Controlled	✓
78	Unknown	-	Well Controlled	<LOQ	<LOQ	-	Well Controlled*	✓*
79	Unknown	5.7*	Poorly Controlled	200	100	100	Uncontrolled	○
80	Unknown	0.5*	Well Controlled*	6.9	2.8	2.8	Well Controlled*	✓*
81	Unknown	41.2*	Uncontrolled†	440	260	130	Uncontrolled	✓*
82	Unknown	18*	Uncontrolled†	390	250	250	Uncontrolled	✓*
83	Unknown	0.6*	Well Controlled	9.3	3.9	3.9	Well Controlled*	✓*
84	Unknown	36.8*	Uncontrolled	450	740	740	Uncontrolled	✓
85	Unknown	262.2*	Uncontrolled	510	330	330	Uncontrolled	✓
86	Unknown	2*	Poorly Controlled	11	4.2	4.2	Poorly Controlled	✓
87	Unknown	-	Well Controlled	<LOQ	<LOQ	<LOQ	Well Controlled*	✓*
88	Unknown	15.6	Uncontrolled	90	46	46	Poorly Controlled*	✓*
89	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
90	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓
91	Potable POU	-	Well Controlled	-	-	-	Well Controlled	✓

All 91 samples were categorized based on the proposed quantitative data interpretation model outlined in Figure 4, and compared to the culture (ISO 11731) suggested quantitative data interpretation model from the CDC (see Table 4). In total, the overall "risk categorization" was the same in 54 (59.3%) of samples. This was significantly higher than expected given the much higher detection rates by qPCR compared to culture. 8 samples (8.8%) were one categorization level apart where the culture results would categorize the system as "well controlled", while qPCR would categorize the system as "poorly controlled". 29 samples (31.9%) were two categorization levels apart where the culture results would categorize the system as "well controlled", while qPCR would categorize the system as "uncontrolled". Notably, there was only one sample where the culture risk model was "more conservative" than qPCR (culture results would categorize the system as "uncontrolled", while qPCR would have categorized the system as "poorly controlled".

Perhaps most importantly, this proposed model demonstrates how the superior detection of viability qPCR does not need to necessarily have a significant impact on water management plan actions. Of the 54 samples where the overall risk categorization was the same, 17 (31.5%) had detection by qPCR, but not by culture. However, although there was detection by qPCR, the detection was low, and categorized as "well controlled". This information is incredibly important for a Water Treater to have in order to remain in a proactive position. It allows the Water Treater to be aware that *Legionella* exists in the water system at a very low level. Had the Water Treater relied on culture, they would not have been aware that *Legionella* was present at all. Given the relatively infrequent sampling for *Legionella*, by the time the next sampling event would occur, the system would be at significant risk of the *Legionella* concentration having risen to "poorly controlled" or "uncontrolled" levels.

These data from Phase 2 further demonstrate that viability qPCR is an excellent tool for routine water monitoring. We suggest that properly evaluated, and thoroughly validated viability qPCR methodologies are the optimal choice for routine sampling in a robust Water Management Program. These methodologies rapidly provide highly accurate, specific, information, that put the Water Treater in a proactive position to manage building water systems, and protect the user's health and safety.

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Conflicts of Interest

No conflicts of interest declared.

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