

Detection of *Salmonella enterica* and *Listeria monocytogenes* in alternative irrigation water by culture and qPCR-based methods in the Mid-Atlantic U.S.

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ABSTRACT Alternative irrigation waters (rivers, ponds, and reclaimed water) can harbor bacterial foodborne pathogens like *Salmonella enterica* and *Listeria monocytogenes*, potentially contaminating fruit and vegetable commodities. Detecting foodborne pathogens using qPCR-based methods may accelerate testing methods and procedures compared to culture-based methods. This study compared detection of *S. enterica* and *L. monocytogenes* by qPCR (real-time PCR) and culture methods in irrigation waters to determine the influence of water type (river, pond, and reclaimed water), season (winter, spring, summer, and fall), or volume (0.1, 1, and 10 L) on sensitivity, accuracy, specificity, and positive (PPV), and negative (NPV) predictive values of these methods. Water samples were collected by filtration through modified Moore swabs (MMS) over a 2-year period at 11 sites in the Mid-Atlantic U.S. on a bi-weekly or monthly schedule. For qPCR, bacterial DNA from culture-enriched samples ($n = 1,990$) was analyzed by multiplex qPCR specific for *S. enterica* and *L. monocytogenes*. For culture detection, enriched samples were selectively enriched, isolated, and PCR confirmed. PPVs for qPCR detection of *S. enterica* and *L. monocytogenes* were 68% and 67%, respectively. The NPV were 87% (*S. enterica*) and 85% (*L. monocytogenes*). Higher levels of qPCR/culture agreement were observed in spring and summer compared to fall and winter for *S. enterica*; for *L. monocytogenes*, lower levels of agreement were observed in winter compared to spring, summer, and fall. Reclaimed and pond water supported higher levels of qPCR/culture agreement compared to river water for both *S. enterica* and *L. monocytogenes*, indicating that water type may influence the agreement of these results.

IMPORTANCE Detecting foodborne pathogens in irrigation water can inform interventions and management strategies to reduce risk of contamination and illness associated with fresh and fresh-cut fruits and vegetables. The use of non-culture methods like qPCR has the potential to accelerate the testing process. Results indicated that pond and reclaimed water showed higher levels of agreement between culture and qPCR methods than river water, perhaps due to specific physiochemical characteristics of the water. These findings also show that season and sample volume affect the agreement of qPCR and culture results. Overall, qPCR methods could be more confidently utilized to determine the absence of *Salmonella enterica* and *Listeria monocytogenes* in irrigation water samples examined in this study.

KEYWORDS water quality, irrigation, *Salmonella*, *Listeria monocytogenes*, qPCR, culture, methods, river, ponds, Mid-Atlantic, accuracy, detection, pathogens

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Alternative water sources (rivers, ponds, and reclaimed water) are used to irrigate fruit and vegetable crops in order to conserve groundwater, a critical resource. However, irrigation waters can harbor foodborne pathogens such as Shiga-toxin-producing *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes* (1–3). Fresh produce, like leafy greens, melons, tomatoes, and cucumbers, irrigated with contaminated water, can lead to outbreaks of foodborne illness. The development of rapid and accurate detection (PCR-based) methods for bacterial pathogens like *S. enterica* and *L. monocytogenes* could offer cost- and time-saving benefits for fruit and vegetable growers, as well as shippers and distributors of produce.

Currently, culture-based methods offer reliable and standardized procedures for pathogen detection in water or pre-harvest produce environments (4). However, culture methods are time-consuming, with non-selective and selective enrichment steps taking up to 7 days or more for final confirmation of a specific pathogen (5). In addition, these methods can be quite laborious, requiring extensive time for media preparation, numerous transfer steps from one media to another, and several incubation periods each lasting 24 h or longer depending on the specific pathogen. In some cases, real-time PCR (referred to as quantitative PCR, qPCR, in this work) may provide similar levels of sensitivity for detection of pathogens (6), and multiplex qPCR can simultaneously identify multiple pathogens in various food and other matrices (4). PCR-based detection methods offer faster results compared to culture-based methods, potentially eliminating the need for selective enrichment steps and biochemical confirmations in some cases. If a qPCR method was determined to be as sensitive as culture-based methods, then their use would represent a step-wise improvement to allow growers and regulators to make water-use decisions more quickly. Park et al. (7) developed a multiplex qPCR assay for the simultaneous detection of *Salmonella*, *Campylobacter*, and *E. coli* O157:H7 in water samples. Ding et al. (8) developed a multiplex qPCR technique combined with a primary enrichment step suitable for the detection of *Staphylococcus aureus*, *L. monocytogenes*, and *S. enterica* in raw milk and the dairy farm environment (feces, soil, feed, and water).

Our current study modified a multiplex qPCR assay developed for the detection of *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 in pork samples which included a prior primary, non-selective enrichment step (9). The objective of the study presented here was to compare the detection of *S. enterica* and *L. monocytogenes* by culture-based methods and qPCR-based methods from non-selective enrichments to expedite the detection of pathogens in irrigation waters. Alternative irrigation water sources in the Mid-Atlantic U.S. were analyzed and the influence of sample volume, water type (source), and season on the agreements of qPCR- and culture-based results were investigated. For work presented here, the qPCR method refers to the technique formerly known as real-time PCR and PCR results here are reported in a positive /negative context without a quantitative component.

MATERIALS AND METHODS

Sample collection

Water samples were collected by filtration through modified Moore swabs (MMS) over a 2-year period from a total of 11 sites. These included three reclaimed water plants, two tidal/brackish rivers, four non-tidal freshwater creeks (classified as rivers), and two agricultural ponds, all located in the Mid-Atlantic region of the U.S. as previously described (2, 3). At each sampling event, 0.1, 1, and 10 L samples were taken, in triplicate, and filtered through a MMS. Previous work used these volumes to quantify *S. enterica* and *L. monocytogenes* levels using a Most Probable Number (MPN) assay (2, 3).

Water sample pre-enrichment

MMS swabs were pre-enriched in 100 mL of Universal Pre-enrichment Broth (UPB, Neogen, Lansing, MI) in Whirl-pak bags (Nasco, Fort Atkinson, WI). Sample bags

containing MMS were hand massaged for 1 min, followed by static incubation at 37°C for 18–24 h. Following incubation, sample bags were homogenized by hand massage for 1 min. Pre-enriched samples (40 mL) were transferred to a 50-mL conical tube (VWR, Radnor, PA) for retention and microbial analysis.

Pre-enrichment DNA extraction

For the qPCR-based method (hereafter referred to as qPCR method), DNA was extracted from UPB-enriched (pre-enriched) samples. Briefly, 1 mL of the pre-enriched samples ($n = 1,990$) was transferred to a 1.5-mL microcentrifuge tube and centrifuged (Eppendorf 5425, Hamburg, Germany) at 13,200 rpm ($16.1 \times 1,000 \times g$) for 5 min. The supernatant was discarded and pellets were frozen at -20°C until prepared for DNA extraction. DNA was extracted using the Isolate II Genomic DNA kit (Bioline, Memphis, TN) according to the manufacturer's instructions with the following modifications: (i) pellets were suspended in a user-made lysis buffer (supplemental material) and incubated at 37°C for 1 h; and (ii) a two-step elution process was used following the manufacturer's alternative directions. Extracted DNA was stored at -20°C until ready for qPCR assay.

Pathogen isolation from water samples

For isolation of pathogens from water samples, pre-enriched samples ($n = 1,990$) were subjected to pathogen-specific secondary enrichment, followed by plating on selective media. For *S. enterica* isolation, 1 mL and 100 μL of the pre-enriched sample were transferred to 9 mL of tetrathionate (TT, Accumedia) broth and 10 mL of Rappaport Vassiliadis (RV, Accumedia) broth, respectively, for selective enrichment. Samples were incubated at 42°C for 18–24 h. Selective enrichments were plated onto XLT4 agar and incubated at 37°C for 18–24 h. Three presumptive black *S. enterica* isolates per swab were selected, streaked on a new XLT4 plate, and incubated at 42°C for 18–24 h for isolation. Isolates were resuspended in 1 mL of TSB supplemented with 15% glycerol (vol/vol) and stored at -80°C for retention.

For *L. monocytogenes* isolation, 1 mL of the pre-enrichment was transferred to 9 mL of Buffered *Listeria* Enrichment Broth (BLEB; Neogen, Lansing, MI) containing 10 mg/L acriflavin, 50 mg/L cycloheximide, and 40 mg/L sodium nalidixic acid, and incubated at 37°C for 18–24 h. Ten microliters of the enriched broth were streaked onto RAPID[®]*L.mono* medium and incubated at 37°C for 48 h. Three presumptive turquoise *L. monocytogenes* isolates per dilution were transferred to a RAPID[®]*L.mono* plate and incubated at 37°C for 18–24 h for isolation. A single colony was transferred into 1 mL of TSB supplemented with 15% glycerol (vol/vol) and 0.6% yeast extract (wt/vol) and stored at -80°C .

Bacterial isolate DNA extraction

DNA extraction of these isolates from culture enrichment was conducted using the InstaGene Matrix DNA Kit (Bio-Rad, Hercules, CA), following the manufacturer's instructions with one modification. Instead of suspension in water and pelleting by centrifugation, a single colony was transferred directly to 200 μL of InstaGene matrix in a 1.5-mL microcentrifuge tube. Extracted DNA was stored at -20°C until ready for real-time PCR confirmation.

qPCR assay

qPCR was performed on DNA extracted from pre-enrichments and presumptive pathogen DNA extracted from culture isolation, using the methods and primers previously described by Kawasaki et al. (9). A multiplex real-time PCR assay for *S. enterica* and *L. monocytogenes* was conducted on a CFX96 Touch real-time PCR system (Bio-Rad) or an Mx 3005P QPCR system (Agilent, Santa Clara, CA, USA) using the SensiFAST Probe Lo-ROX kit (Bioline). PCR cycling parameters included: an initial denaturation of 10 min at 95°C, followed by 40 cycles of: 20 s at 95°C, 30 s at 64°C, and 30 s at 72°C, and a final extension of 7 min at 72°C. Details on primer sequences, reaction composition,

and thermal cycling conditions are provided with the data set. For *S. enterica*, primers targeted a *Salmonella*-specific gene (*yfiR*) (10).

S. enterica and *L. monocytogenes* detection and confirmation

For qPCR results from pre-enriched samples, positive detection was determined when the Cycle threshold (C_T) value was ≤ 35 . For culture results, the MMS were considered positive when a presumptive isolate from a water sample of either *S. enterica* or *L. monocytogenes* was confirmed by qPCR assay.

Statistical analysis

To compare qPCR method results with the culture method results, sensitivity (true positive percentage), specificity (true negative percentage), accuracy (percentage of true positives and true negatives), positive predictive value (PPV; the probability that a positive result was a true positive), and negative predictive value (NPV; the probability that a negative result was a true negative) were calculated using the epiR package version 2.0.57 (11) in R version 4.2.2 (12). Formulas for these terms are listed in Table 1. The culture method result was used as the basis for the determination of the aforementioned values. Samples that were positive or negative by both methods were considered true positives and true negatives, respectively. Samples with qPCR negative and culture positive results were categorized as false negatives; samples with qPCR positive and culture negative results were categorized as false positives.

To evaluate the influence of filtration volume, water type, and season on agreement between qPCR and culture method results, a mixed effects logistic regression model was constructed using the lme4 package version 1.1-31 (13) in R. The fixed effects of the model were filtration volume, water type, and season with site of water collection included as a random effect. Models were constructed separately for *S. enterica* and *L. monocytogenes* data. The dependent variable of these models was qPCR/culture method agreement, where a “1” indicated either a true positive or a true negative result. A “0” indicated mixed results, either a false positive or false negative. Logistic regression models for each water type (pond, reclaimed, and river) used the 0.1 L filtration volume, and the Fall season as the reference model. Odds ratios (ORs) were calculated from logistic regression coefficients to compare the likelihood of method agreement by sampling volume and season. Odds ratios >1 denote that a variable increases the likelihood that qPCR and culture results will agree, whereas odds ratios <1 denote that a variable decreases the likelihood of method agreement. Statistical significance in this study was defined by P values <0.05 .

Logistic regression models were visualized using the “effects” package version 4.2-2 (14) in R. The “effects” package calculates probabilities from logistic regression model coefficients by building separate models that consider the effects of each level of a variable (water type, season, or volume), while all other model coefficients are scaled by their means to represent their average effects on the model. Resulting model coefficients for the levels of each variable are converted from log-odds to probabilities using the inverse-logit function. The resulting plots demonstrate the individual effects that each

TABLE 1 Definitions of sensitivity, specificity, accuracy, positive predictive value, and negative predictive value^a

Term	Description	Formula ^a
Sensitivity	Percentage of true positives	$[TP/(TP + FN)]$
Specificity	Percentage of true negatives	$[TN/(TN + FP)]$
Accuracy	Percentage of true positives and true negatives (or culture, qPCR agreement)	$[(TP + TN)/(TP + FP + TN + FN)]$
Positive predictive value	Probability that a qPCR positive indicates a true positive	$[TP/(TP + FP)]$
Negative predictive value	Probability that qPCR negative indicates a true negative	$[TN/(TN + FN)]$

^aTP, true positive (both the culture method and qPCR method detected the pathogen from the same sample); FN, false negative—culture method detected pathogen but qPCR method did not; TN, true negative (both the culture method and qPCR method did not detect the pathogen from the same sample); FP, false positive (the culture method did not detect the pathogen in the sample but the qPCR method did).

^bValues listed in Tables 2 to 6 are percentages.

variable (filtration volume, water type, or season) has on the probability of qPCR and culture methods agreement.

RESULTS

Performance of qPCR in comparison to standard culture methods

In this study, the performance of a multiplex qPCR method versus standard culture methods was evaluated according to pathogen, water type, filtration volume, and season. Quantitative analysis of the culture data in this set has previously been reported from references (2, 3).

Table 2 shows the overall performance of this qPCR method for *S. enterica* and *L. monocytogenes* in all three water types, all three volumes, and all four seasons. Sensitivity, the percentage of true positives, was greater for *S. enterica* (70%) than for *L. monocytogenes* (39%). Specificity, the percentage of true negatives, was 86% for *S. enterica* and 95% for *L. monocytogenes*. The accuracy of the qPCR method was similar for *S. enterica* and *L. monocytogenes* at 81% and 83%, respectively. The PPV (68%) and NPV (87%) for *S. enterica* were similar to the PPV (67%) and NPV (85%) for *L. monocytogenes*.

Tables 3 and 4 show the performance of the qPCR method for detecting both pathogens in each water type at different filtration volumes. In this study, more samples were collected from river ($n = 1,049$) compared to pond ($n = 591$) and reclaimed water ($n = 350$) sources. For *S. enterica* in river water samples, sensitivity values increased as sample volume of MMS decreased. Accuracy values were lower in river water (76%) compared to pond (88%) or reclaimed water (86%). Across all water types, accuracy values increased as sample volume decreased, with the highest accuracy levels observed in the lowest volumes (0.1 L) and the lowest accuracy levels observed in the largest volumes (10 L). The largest increase in accuracy values across sample volumes occurred in river water, where the percentage increase from 10 L samples (68%) to 0.1 L samples (81%) was 13%, while the increases in accuracy levels were 7% and 4% for pond and reclaimed water, respectively, over the same volumes. Similar to accuracy levels, NPV values increased as sample volumes decreased across all water types. Conversely, PPV decreased as sample volume decreased. Overall, PPV was highest in river water samples (77%) compared to pond (32%) and reclaimed water (42%). The range of specificity values for all volumes of pond and reclaimed water samples were between 87% and 93%, whereas river water samples ranged from 70% to 83% for these performance metrics (Table 3).

For *L. monocytogenes*, accuracy levels followed similar trends as those for *S. enterica*—accuracy values and NPVs increased as sample volumes decreased over all water types (Table 4). However, accuracy levels were lower for river water (71%) compared to pond water (96%) or reclaimed water (95%). NPV was highest in pond (97%) and reclaimed water samples (98%) compared to river water (71%). Sensitivity values across all water types and volumes were generally low (0–44%), except when filtering 0.1 L of pond water (67%) (Table 4). Specificity levels were between 91% and 98% for *L. monocytogenes*. Overall PPV was highest in river water (72%), followed by pond water (52%) and reclaimed water (8%).

Tables 5 and 6 show the performance of qPCR for detecting both pathogens in each water type during different seasons. No reclaimed water samples were collected during the Winter. For *S. enterica*, sensitivity was higher in river water during Winter (92%)

TABLE 2 Performance of qPCR method for the detection of *Salmonella enterica* and *Listeria monocytogenes*

Pathogen	No. of samples					% (Lower value, upper value) ^a				
	Total	True positive	False positive	False negative	True negative	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
<i>Salmonella</i>	1,990	421	197	178	1,194	70 (66, 74)	86 (84, 88)	81 (79, 83)	68 (64–72)	87 (85–89)
<i>L. monocytogenes</i>	1,990	167	81	263	1,479	39 (34, 44)	95 (94, 96)	83 (81, 84)	67 (61–73)	85 (83–87)

^aNumbers in parenthesis represent the 95% confidence interval for the lower and upper values of sensitivity, specificity, accuracy, and positive and negative predictive values. True positive, positive by both methods; false positive, positive only by qPCR; false negative, positive only by culture; true negative, negative by both methods.

TABLE 3 Performance of qPCR method by water type within each water volume for *Salmonella enterica*

Water type	Vol (L)	No. of samples					% (lower value, upper value) ^a				
		Total	True positive	False positive	False negative	True negative	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
River	All	1,049	371	110	145	423	72 (68, 76)	79 (76, 83)	76 (73, 78)	77 (73–81)	74 (71–78)
	10	349	168	29	83	69	67 (61, 73)	70 (60, 79)	68 (63, 73)	85 (80–90)	45 (37–54)
	1	350	117	33	44	156	73 (65, 79)	83 (76, 88)	78 (73, 82)	78 (71–84)	78 (72–84)
	0.1	350	86	48	18	198	83 (74, 89)	80 (75, 85)	81 (77, 85)	64 (55–72)	92 (87–95)
Pond	All	591	23	49	23	496	50 (35, 65)	91 (88, 93)	88 (85, 90)	32 (21–44)	96 (93–97)
	10	197	19	15	14	149	58 (39, 75)	91 (85, 95)	85 (80, 90)	56 (38–73)	91 (86–95)
	1	198	3	20	7	168	30 (7, 65)	89 (84, 93)	86 (81, 91)	13 (3–34)	96 (92–98)
	0.1	196	1	14	2	179	33 (1, 91)	93 (88, 96)	92 (87, 95)	7 (0–32)	99 (96–100)
Reclaimed	All	350	27	38	10	275	73 (56, 86)	88 (84, 91)	86 (82, 90)	42 (29–54)	96 (94–98)
	10	116	12	10	8	86	60 (36, 81)	90 (82, 95)	84 (77, 91)	55 (32–76)	91 (84–96)
	1	117	8	14	2	93	80 (44, 97)	87 (79, 93)	86 (79, 92)	36 (17–59)	98 (93–100)
	0.1	117	7	14	0	96	100 (59, 100)	87 (80, 93)	88 (81, 93)	33 (15–57)	100 (96–100)

^aData grouped by water type and filtration volume (all, 10, 1, and 0.1 L). Numbers in parenthesis represent 95% confidence intervals for sensitivity, specificity, accuracy, positive predictive values, and negative predictive values. True positive, positive by both methods; false positive, positive only by qPCR; false negative, positive only by culture; true negative, negative by both methods.

compared to Spring (85%), Fall (67%), or Summer (61%) (Table 5). Similar to river water, sensitivity for pond water samples was 100% in the Winter and Spring, 61% in the Fall, and 26% in the Summer. Sensitivity for reclaimed water samples was highest in the Summer (90%), followed by Fall (67%) and Spring (45%). Specificity for all water types and seasons ranged from 85–98%, except for river water samples collected in the Fall and Winter which were 74% and 71%, respectively. Accuracy across all seasons ranged from 70% to 84% in river water, 83% to 98% in pond water, and 82% to 91% in reclaimed water samples. PPVs across seasons were higher in river water samples (67–83%) compared to pond (13–50%) and reclaimed water (33–47%). NPVs for all water types and seasons ranged from 88% to 100%, except for river water samples collected in the Fall (65%) and Summer (65%) (Table 5).

For *L. monocytogenes*, sensitivity was low across different seasons and water types ranging from 20% to 55% in river water, 0% to 54% in pond water, and 0% to 14% in reclaimed water samples (Table 6). Specificity was similar across water types and seasons, ranging from 93% to 100%, except for river water in the Winter (70%). Accuracy was higher in pond and reclaimed water samples (90–98%) compared to river water (62–74%)

TABLE 4 Performance of qPCR by water type within each water volume for *Listeria monocytogenes*

Water type	Vol (L)	No. of samples					% (Lower value, upper value) ^a				
		Total	True positive	False positive	False negative	True negative	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
River	All	1,049	155	60	240	594	39 (34, 44)	91 (88, 93)	71 (69, 74)	72 (66–78)	71 (68–74)
	10	349	72	19	92	166	44 (36, 52)	90 (84, 94)	68 (63, 73)	79 (69–87)	64 (58–70)
	1	350	44	19	80	207	35 (27, 45)	92 (87, 95)	72 (67, 76)	70 (57–81)	72 (67–77)
	0.1	350	39	22	68	221	36 (27, 46)	91 (87, 94)	74 (69, 79)	64 (51–76)	76 (71–81)
Pond	All	591	11	10	16	554	41 (22, 61)	98 (97, 99)	96 (94, 97)	52 (30–74)	97 (95–98)
	10	197	4	5	8	180	33 (10, 65)	97 (94, 99)	93 (89, 96)	44 (14–79)	96 (92–98)
	1	198	3	3	6	186	33 (7, 70)	98 (95, 100)	95 (92, 98)	50 (12–88)	97 (93–99)
	0.1	196	4	2	2	188	67 (22, 96)	99 (96, 100)	98 (95, 99)	67 (22–96)	99 (96–100)
Reclaimed	All	350	1	11	7	331	12 (0, 53)	97 (94, 98)	95 (92, 97)	8 (0–38)	98 (96–99)
	10	116	0	7	2	107	0 (0, 84)	94 (88, 97)	92 (86, 96)	0 (0–41)	98 (94–100)
	1	117	0	3	3	111	0 (0, 71)	97 (93, 99)	95 (89, 98)	0 (0–71)	97 (93–99)
	0.1	117	1	1	2	113	33 (1, 91)	99 (95, 100)	97 (93, 99)	50 (1–99)	98 (94–100)

^aData grouped by water type and filtration volume (all, 10, 1, and 0.1 L). Numbers in parenthesis represent 95% confidence intervals for sensitivity, specificity, accuracy, positive predictive values, and negative predictive values. True positive, positive by both methods; false positive, positive only by qPCR; false negative, positive only by culture; true negative, negative by both methods.

TABLE 5 Performance of qPCR by water type within each season for *Salmonella enterica*

Water type	Season	No. of samples					% (Lower value, upper value) ^a			Positive predictive value	Negative predictive value
		Total	True positive	False positive	False negative	True negative	Sensitivity	Specificity	Accuracy		
River	All	1,049	371	110	145	423	72 (68, 76)	79 (76, 83)	76 (73, 78)	77 (73–81)	74 (71–78)
	Fall	295	108	35	53	99	67 (59–74)	74 (66–81)	70 (65–75)	76 (68–82)	65 (57–73)
	Winter	165	59	29	5	72	92 (83–97)	71 (61–80)	79 (72–85)	67 (56–77)	94 (85–98)
	Spring	257	93	24	17	123	85 (76–91)	84 (77–89)	84 (79–88)	79 (71–86)	88 (81–93)
	Summer	332	111	22	70	129	61 (54–68)	85 (79–91)	72 (67–77)	83 (76–89)	65 (58–71)
Pond	All	591	23	49	23	496	50 (35, 65)	91 (88, 93)	88 (85, 90)	32 (21–44)	96 (93–97)
	Fall	216	14	28	9	165	61 (39–80)	85 (80–90)	83 (77–88)	33 (20–50)	95 (90–98)
	Winter	99	2	2	0	95	100 (16–100)	98 (93–100)	98 (93–100)	50 (7–93)	100 (96–100)
	Spring	116	2	13	0	101	100 (16–100)	89 (81–94)	89 (82–94)	13 (2–40)	100 (96–100)
	Summer	160	5	6	14	135	26 (9–51)	96 (91–98)	88 (81–92)	45 (17–77)	91 (85–95)
Reclaimed	All	350	27	38	10	275	73 (56, 86)	88 (84, 91)	86 (82, 90)	42 (29–54)	96 (94–98)
	Fall	107	4	8	2	93	67 (22–96)	92 (85–97)	91 (83–95)	33 (10–65)	98 (93–100)
	Winter	0	0	0	0	0	ND ^b	ND	ND	ND	ND
	Spring	90	5	10	6	69	45 (17–77)	87 (78–94)	82 (73–89)	33 (12–62)	92 (83–97)
	Summer	153	18	20	2	113	90 (68–99)	85 (78–91)	86 (79–91)	47 (31–64)	98 (94–100)

^aData grouped by water type and season (all, Fall, Winter, Spring, and Summer). Numbers in parenthesis represent 95% confidence intervals for sensitivity, specificity, accuracy, positive predictive values, and negative predictive values. True positive, positive by both methods; false positive, positive only by qPCR; false negative, positive only by culture; true negative, negative by both methods.

^bND, not determined.

across all seasons. PPV was highly variable in river (59–85%), pond (0–80%) water, and reclaimed water samples (0–50%). NPV was higher in pond and reclaimed water samples (93–100%) compared to river water (60–74%) across all seasons. Several values for pond and reclaimed water samples could not be calculated because either no true positives, false positives, or false negatives were observed (Table 6).

TABLE 6 Performance of qPCR by water type within each season for *Listeria monocytogenes*

Water type	Season	No. of samples					% (Lower value, upper value) ^a			Positive predictive value	Negative predictive value
		Total	True positive	False positive	False negative	True negative	Sensitivity	Specificity	Accuracy		
River	All	1,049	155	60	240	594	39 (34, 44)	91 (88, 93)	71 (69, 74)	72 (66–78)	71 (68–74)
	Fall	295	17	12	70	196	20 (12–29)	94 (90–97)	72 (67–77)	59 (39–76)	74 (68–79)
	Winter	165	46	24	38	57	55 (44–66)	70 (59–80)	62 (55–70)	66 (53–77)	60 (49–70)
	Spring	257	52	9	61	135	46 (37–56)	94 (88–97)	73 (67–78)	85 (74–93)	69 (62–75)
	Summer	332	40	15	71	206	36 (27–46)	93 (89–96)	74 (69–79)	73 (59–84)	74 (69–79)
Pond	All	591	11	10	16	554	41 (22, 61)	98 (97, 99)	96 (94, 97)	52 (30–74)	97 (95–98)
	Fall	216	0	5	0	211	ND	98 (95–99)	98 (95–99)	0 (0–52)	100 (98–100)
	Winter	99	7	4	6	82	54 (25–81)	95 (89–99)	90 (82–95)	64 (31–89)	93 (86–97)
	Spring	116	0	0	1	115	0 (0–98)	100 (97–100)	99 (95–100)	ND	99 (95–100)
	Summer	160	4	1	9	146	31 (9–61)	99 (96–100)	94 (89–97)	80 (28–99)	94 (89–97)
Reclaimed	All	350	1	11	7	331	12 (0, 53)	97 (94, 98)	95 (92, 97)	8 (0–38)	98 (96–99)
	Fall	107	0	3	0	104	ND	97 (92–99)	97 (92–99)	0 (0–71)	100 (97–100)
	Winter	0	0	0	0	0	ND	ND	ND	ND	ND
	Spring	90	1	1	6	82	14 (0–58)	99 (93–100)	92 (85–97)	50 (1–99)	93 (86–97)
	Summer	153	0	7	1	145	0 (0–98)	95 (91–98)	95 (90–98)	0 (0–41)	99 (96–100)

^aData grouped by water type and season (all, Fall, Winter, Spring, and Summer). Numbers in parenthesis represent 95% confidence intervals for sensitivity, specificity, accuracy, positive predictive values, and negative predictive values. True positive, positive by both methods; false positive, positive only by qPCR; false negative, positive only by culture; true negative, negative by both methods. ND, not determined.

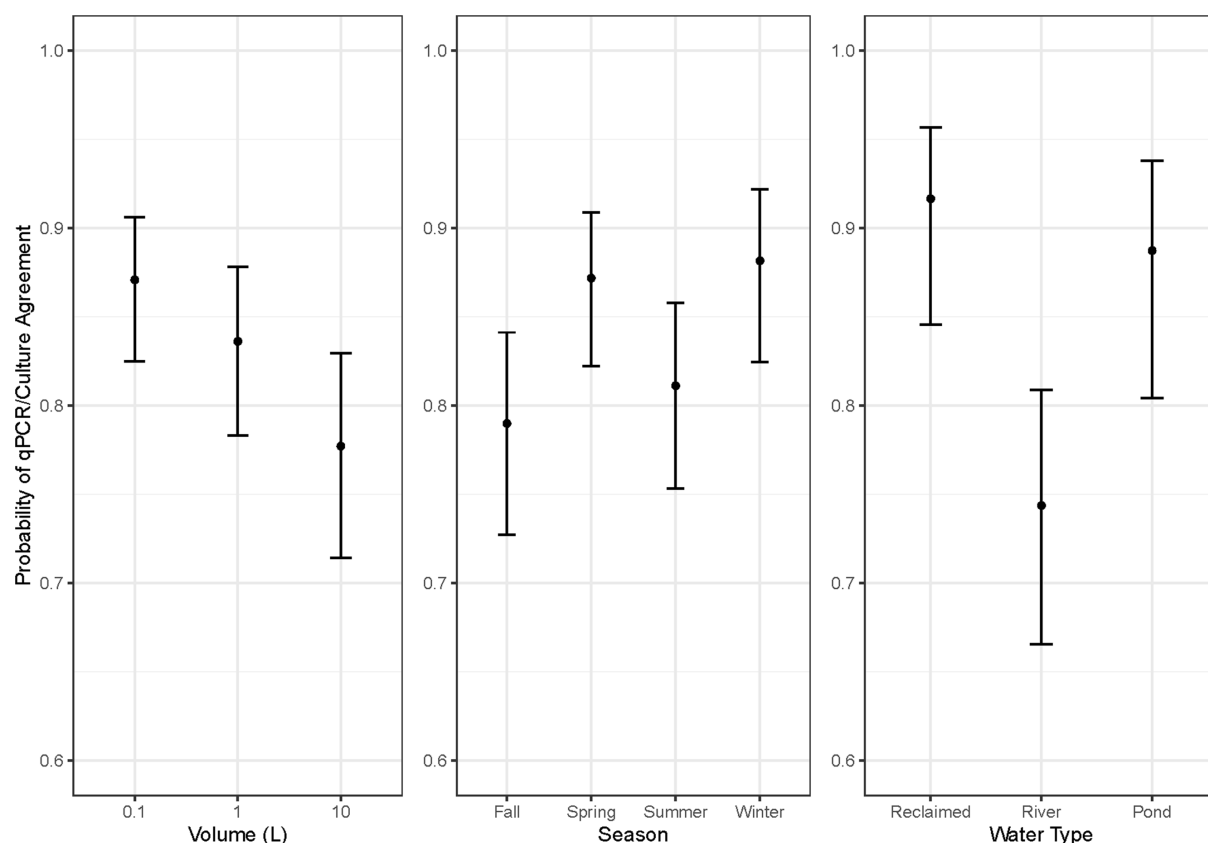


FIG 1 Probability of qPCR and culture results agreement with 95% confidence intervals by filtration volume, season, and water type for the detection of *S. enterica*.

Odds ratios and probabilities comparing qPCR and standard culture methods agreement by water type, filtration volume, and season

Figures 1 and 2 demonstrate the estimated probabilities that the qPCR and culture method results will agree (i.e., a true positive or true negative) for both pathogens at each water type, season, and filtration volume. For *S. enterica*, the probability of method agreement decreased as filtration volumes increased, with 0.87 at 0.1 L, 0.84 at 1 L, and 0.78 at 10 L. Among different seasons, the probability of agreement was higher in the Winter (0.88) and Spring (0.87) compared to the Summer (0.81) and Fall (0.79). For water types, qPCR/culture method agreement was higher for reclaimed (0.92) and pond water (0.89) than river water (0.74) (Fig. 1). For *L. monocytogenes*, the probability of method agreement was highest at the 0.1 L filtration volume (0.90), followed by 1 L (0.88) and 10 L (0.85). Seasonal agreement was similar in the Fall, Spring, and Summer (0.89) and decreased in the Winter (0.81). Among different water types, pond, and reclaimed water both had high agreement probabilities of 0.96 compared to 0.72 for river water samples (Fig. 2).

The calculated ORs demonstrate similar effects for filtration volume, season, and water type on the likelihood of agreement between qPCR and culture method results. OR for each water type was based on a reference model for *S. enterica* and *L. monocytogenes*. Volume of water samples and season of collection significantly influenced the agreement of culture and PCR results more frequently for *S. enterica* than for *L. monocytogenes*. Agreement of culture and qPCR results for *S. enterica* was influenced by a variety of seasonal and collection volume factors. For reclaimed water, 0.1 L samples collected in Spring (OR = 0.38, $P < 0.030$), and samples collected in Summer (OR = 0.44, $P < 0.050$), was significantly (<0.05) less likely to have qPCR and culture results in agreement

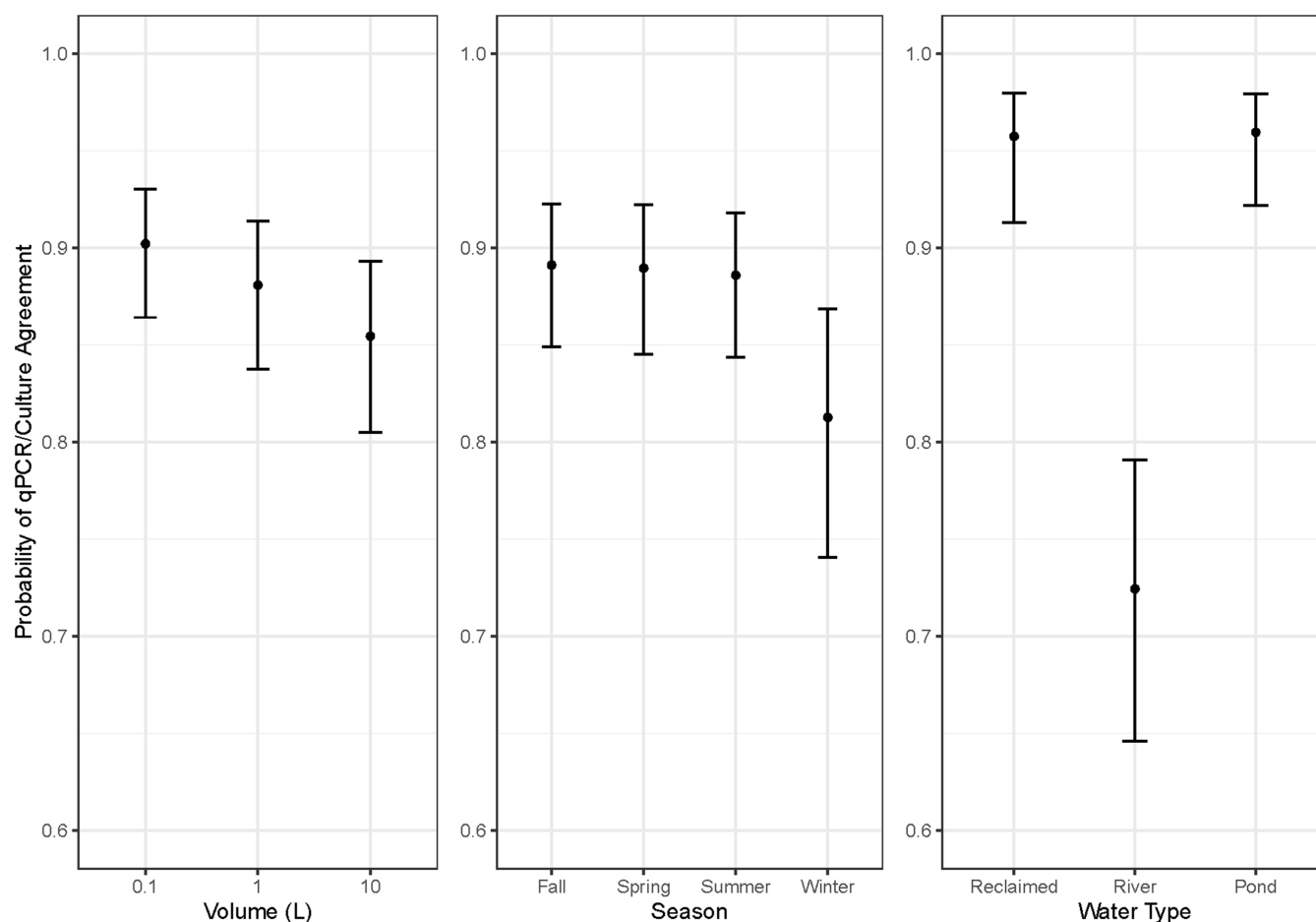


FIG 2 Probability of qPCR and culture results agreement with 95% confidence intervals by filtration volume, season, and water type for the detection of *L. monocytogenes*.

compared to the reference model (reclaimed water, 0.1 L, Fall). In comparison to the reference model used for river water (river water, 0.1 L, Fall), 10 L samples was significantly ($P < 0.001$) less likely to support the agreement of qPCR and culture results (OR = 0.48). For river water samples, 0.1 L samples collected in the Spring was significantly ($P < 0.001$) more likely to have agreement between qPCR and culture results compared to the reference model (OR = 2.57). Similarly, 0.1 L samples collected in Winter was also significantly ($P < 0.030$) more likely to support agreement of qPCR and culture results compared to river water reference conditions (OR = 1.67). Pond water results were similar to those of river water for 10 L samples taken in the fall, and for 0.1 L water samples taken in the winter. Compared to the reference model for pond water (pond water, 0.1 L, Fall), 10 L water samples was significantly ($P < 0.041$) less likely to support the agreement of qPCR and culture results (OR = 0.51). For 0.1 L water samples taken in Winter compared reference model, samples were significantly ($P < 0.002$) more likely to support the agreement of qPCR and culture results (OR = 10.06).

For *L. monocytogenes*, qPCR and culture results from 0.1 L river water samples collected in Winter were significantly ($P < 0.040$) less likely to agree (OR = 0.65) when compared with the reference conditions for river water (river water, 0.1 L, Fall). For pond water, 0.1 L samples collected in Winter were also significantly ($P < 0.005$) less likely to show qPCR and culture agreement (OR = 0.21) compared to the reference conditions (pond water, 0.1 L, Fall).

DISCUSSION

Numerous surveys have reported the presence of *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* in irrigation water sources (15–22). Irrigation water is a known risk for the pre-harvest contamination of fresh produce, and effective water quality monitoring tools are needed to help growers ensure the safety of their irrigation water sources (23). For bacterial pathogens like *S. enterica* and *L. monocytogenes*, the process of reliably detecting pathogens requires several days of pre- and selective culture enrichment. An accurate, qPCR assay specific for *S. enterica* and *L. monocytogenes* could expedite this process for those making decisions on the use of water. For both *S. enterica* and *L. monocytogenes*, specificity values (86–95%) were greater than sensitivity values (39–70%), and NPV (85–87%) were greater than PPV (67–68%) (Table 2). Since both specificity and NPVs are based on the percentages of true negatives, these results broadly indicate that qPCR results would be useful and reliable to indicate a negative test result (absence of the pathogen) in these water samples. As shown above, water type and volume influenced the magnitude of several of the values that were quantified in this study. The low sensitivity value (39%) for *L. monocytogenes* is a reflection of the agreement between culture and qPCR method detection. The high number of false negatives (where *L. monocytogenes* is detected by culture but not qPCR) indicates the culture method was more sensitive than the qPCR method for the pathogen. The qPCR assay may not be as sensitive for the target genes in the non-selective enrichment broth, where multiple organisms and perhaps low levels of *L. monocytogenes* are present. Previous work has shown that as little as 2 pg of *L. monocytogenes* DNA was detected from unenriched broth in the presence of *E. coli* O157:H7 and *S. enterica* DNA (9), so enriched water samples in our study may have contained less than 2 pg *L. monocytogenes* DNA. Alternatively, the presence of DNA from other bacterial species may have interfered with the qPCR assay. The use of UPB as a non-selective enrichment for 24 h without the addition of selective agents (acriflavin, cycloheximide nalidixic acid used in *Listeria* spp. enrichment) may have allowed other bacteria to grow to high levels, increasing the amount of non-*L. monocytogenes* DNA present, and decreasing the sensitivity of the *L. monocytogenes* specific qPCR assay. Specific PCR inhibitors (mentioned later in this section) may have also affected the sensitivity of the qPCR assay. Another factor that may impact the qPCR detection is the serogroup of *L. monocytogenes* isolates recovered. In previous work, several *L. monocytogenes* isolates belonging to serogroup 4b isolates (7/17) were shown not to possess the *hlyA* gene (the target of the qPCR assay used in this study), which potentially indicates that several isolates may have been recovered by culture methods but not detected by qPCR methods (24).

qPCR performance metrics for both pathogens were affected by the different water types tested. Accuracy values for *S. enterica* were lower in river water compared to pond or reclaimed water (Table 3), and a similar trend was observed for *L. monocytogenes* (Table 4), with smaller differences in accuracy values. While river water had higher PPVs for *S. enterica* (77%) and *L. monocytogenes* (72%) compared to other water types, these values essentially indicate approximately 25% of positive samples would not be detected by qPCR assay in river samples. Similarly, the NPV for *S. enterica* (71%) and *L. monocytogenes* (74%) also indicate an approximate 25% chance of mischaracterizing a negative result as positive.

For both *S. enterica* and *L. monocytogenes*, the probability of culture method and qPCR agreement were lower in river water compared to pond or reclaimed water (Fig. 1 and 2). The inherent variability in river water quality and microbial flux may affect the agreement of culture and qPCR results. Previously published findings for these same water samples reported higher prevalence and levels of *S. enterica* and *L. monocytogenes* in river water compared to pond and reclaimed waters. Specific water quality attributes associated with water types may have explained the differences in culture method and qPCR method agreement. Previous work has shown that C_T values for qPCR detection of *invA* in *S. Typhimurium* inoculated into undiluted river water were significantly ($P < 0.05$) greater than C_T values in 1:10 dilutions of the same water, indicating that PCR

inhibitors from undiluted river water can affect qPCR detection of *S. Typhimurium* (25). Although previous work using the same water samples from the current study has not shown dramatic differences between turbidity values measured from four river sites (average 6.6 FNU) and two pond sites (6.4 FNU) (26), the specific chemical elements which compose the turbidity may be important to consider when considering qPCR results. The chemical composition of turbidity may vary among water sources and water types with varying consequences for the detection of pathogens or fecal indicators (27). Humic substances (humic acid, fulvic acid, and humin) can often be a component of turbidity but are not generally measured separately during routine water testing. Humic acid and fulvic acid can interfere with DNA polymerase activity, binding DNA, and disrupting ion concentrations in PCR reactions (28). Humic acid was shown to also decrease the intensity of fluorescence signals associated with qPCR detection, potentially decreasing the sensitivity of qPCR assays in environmental samples (28). In addition, the variability in qPCR performance across water types may have been affected by differing microbial communities and physiochemical properties in the water samples collected. In our current study, diluting enriched cultures or using a specific PCR inhibitor removal kit in addition to the silica-based/column DNA kit before DNA extraction may have potentially improved the agreement of culture and qPCR methods. Similarly, the use of propidium monoazide (PMA) may have decreased the number of false positives (culture negative and qPCR positive) obtained in our study. However, the number of samples (1,990) processed and analyzed in this study may the use of PMA unwieldy in the laboratory workflow when frozen samples of non-selective enrichment broth were analyzed after culture analysis.

Previous work with this same culture result data noted that filtering 10 L of water through an MMS significantly improved the odds of *S. enterica* recovery compared to 0.1 L [2,3]. For *L. monocytogenes*, Sharma et al. (2) observed a significant increase in the likelihood of recovery from 10 L filtration compared to 0.1 L, but Acheamfour et al. (3) found no significant differences in *L. monocytogenes* recovery by volume of water filtered from different sites of water collection. For *S. enterica* and *L. monocytogenes*, our results found that the likelihood of agreement between qPCR and culture methods was significantly lower when filtering 10 L of water compared to 0.1 L for both pathogens in river water (Table 7). We hypothesize that the greater volume filtered may have increased the amount of PCR inhibitors present in the enriched sample, leading to more false negatives (culture positive and qPCR negative) among samples. Similar trends were observed for *S. enterica* in pond water. The effects of seasonality on method agreement differed for both pathogens. For *S. enterica*, the likelihood (odds ratio) and probability of qPCR and culture method agreement were higher in the Spring and Winter, which were also the seasons with the lowest prevalence of *Salmonella* (2, 3). For both river and pond water, winter was more likely ($OR > 1$) to yield a likelihood of culture and qPCR method agreement for *Salmonella*. Conversely, *L. monocytogenes* was more prevalent in the Winter (2, 3), but had the lowest probability of qPCR and culture agreement ($OR < 1$) during this season for both and river and pond water.

Several studies have compared qPCR-based and culture methods for the detection of pathogenic and indicator bacteria in irrigation water sources (18, 25–28). However, these studies do not use performance metrics such as sensitivity, specificity, accuracy, NPV, and PPV. Using similar culture and qPCR methods for the detection of *S. enterica* and *L. monocytogenes*, Zhu et al. (29) evaluated reclaimed and return flow waters in Arizona for the presence of pathogenic bacteria, and reported that results from both qPCR and culture methods for the detection of *S. enterica* and *L. monocytogenes* were comparable (27). In our current study, the qPCR method performed better for the detection of *S. enterica* than *L. monocytogenes*, which had poor sensitivity and PPV in all water types. Li et al. (18) developed a new scheme for *S. enterica* recovery in surface water samples using qPCR as a screening step after pre-enrichment based on the FDA's Bacteriological Analytical Manual (BAM) methodology for *Salmonella* recovery. Their qPCR-based *Salmonella* recovery scheme reduced the turnaround time for results to 4

TABLE 7 Odds ratios of qPCR and culture method results agreement for filtration volumes and seasons of each water type for *Salmonella enterica* and *Listeria monocytogenes*

Variable	<i>S. enterica</i>		<i>L. monocytogenes</i>	
	Odds ratio (95% CI) ^a	P value ^b	Odds ratio (95% CI)	P value
Reclaimed water reference model (reclaimed water, 0.1 L volume, Fall) ^b	32.99 (6.79–160.13)	<0.001	141.06 (19.36–1027.98)	<0.001
1 L volume	0.85 (0.39–1.87)	0.684	0.48 (0.12–1.95)	0.305
10 L volume	0.72 (0.33–1.57)	0.414	0.31 (0.08–1.15)	0.080
Spring	0.38 (0.16–0.91)	0.030	0.29 (0.07–1.17)	0.081
Summer	0.44 (0.20–1.00)	0.050	0.43 (0.11–1.66)	0.219
River water reference model (river water, 0.1 L volume, Fall)	2.82 (1.81–4.40)	<0.001	3.03 (1.86–4.94)	<0.001
1 L volume	0.82 (0.56–1.19)	0.289	0.87 (0.62–1.23)	0.430
10 L volume	0.48 (0.34–0.69)	<0.001	0.73 (0.52–1.02)	0.065
Spring	2.57 (1.67–3.96)	<0.001	1.08 (0.73–1.59)	0.703
Summer	1.23 (0.86–1.76)	0.256	1.13 (0.78–1.62)	0.519
Winter	1.67 (1.05–2.65)	0.030	0.65 (0.42–0.98)	0.040
Pond water reference model (pond water, 0.1 L volume, Fall)	7.73 (4.06–14.72)	<0.001	94.76 (26.33–340.99)	<0.001
1 L volume	0.55 (0.29–1.07)	0.079	0.43 (0.13–1.44)	0.173
10 L volume	0.51 (0.26–0.97)	0.041	0.29 (0.09–0.91)	0.034
Spring	1.63 (0.82–3.22)	0.160	2.72 (0.31–23.62)	0.364
Summer	1.46 (0.81–2.64)	0.206	0.36 (0.12–1.06)	0.065
Winter	10.06 (2.37–42.65)	0.002	0.21 (0.07–0.63)	0.005

^aOdds ratios and 95% confidence intervals reported for each model variable. Odds ratios >1 denote that a variable increased likelihood of method agreement; odds ratios <1 denote decreased likelihood of method agreement. P values ≤0.05 denote significance.

^bWithin each variable and water type, all conditions of the reference model remain the same except the variable listed in that specific row. For example, in reclaimed water, where 1 L, the odds ratio listed is compared conditions for the reference model to reclaimed water, 1 L, Fall).

days compared to 5–9 days with the BAM method and significantly increased recovery efficiency (18). In our current study, the turnaround time was reduced from 6 days with the culture method to 2 days with the qPCR method. The performance of the multiplex qPCR method described in our study could be improved by modifying the qPCR method itself or the steps before qPCR. For example, Volpe et al. (30) developed an effective qPCR method for the detection of low levels of *Salmonella* (1–10 CFU/L) in irrigation waters which incorporated a locked nucleic acid (LNA) fluorescent probe and an internal amplification control (IAC). The addition of an LNA probe could improve the sensitivity of the qPCR method and an IAC would minimize false negative results caused by PCR inhibitors (31). The use of propidium monoazide in combination with qPCR methods also provided similar estimates of levels of *E. coli* in irrigation water (32). McEgan et al. (33) combined immunomagnetic separation (IMS) beads with qPCR to demonstrate a 100% recovery for low levels of *Salmonella* (1 CFU/L) in inoculated surface water compared to 83% recovery using a culture method. The use of IMS beads after pre-enrichment may significantly improve the sensitivity of the current qPCR method for both pathogens. These studies were done in the same types of surface water.

As fruit and vegetable farmers seek alternative irrigation water sources, evaluating the microbial quality of these sources will be essential to prevent pre-harvest contamination of fresh produce. Detecting pathogens in water more quickly can provide farmers with options to reduce the risk of contamination to crops, like treating water from the irrigation source with an antimicrobial sanitizer like sodium hypochlorite or peroxyacetic acid (PAA) to reduce the potential presence of these pathogens in irrigation water. There will be a constant need for the development and validation of rapid, sensitive, and accurate qPCR-based tests to monitor water quality. The use of qPCR-based methods allows for faster results (≤2 days) compared to traditional culture-based methods (≥6 days) (17). However, qPCR methods require more expensive equipment and reagents, as well as training for personnel to properly conduct the tests. Culture-based methods require only basic microbiology equipment necessary for the recovery, which can be further analyzed with traditional and molecular subtyping methods to track sources of contamination (18).

Results in our study show that the type of surface water, volume collected, and seasonality all affect the agreement of qPCR and culture results for *S. enterica* and *L. monocytogenes*. Specifically, qPCR determination of the absence of *S. enterica* and *L. monocytogenes* in pond water and reclaimed water was accurate, based on NPVs and accuracy values for these water types. In both pond and reclaimed water, prevalence of these pathogens was lower than for river water. If lower prevalence is linked to lower microbial diversity or microbial load compared to river water, as previously determined for these sites (2, 3), this could indicate that qPCR may provide more reliable results under these conditions. qPCR results were less determinative of the presence of these pathogens in river water, where their prevalence was increased, and overall could not provide a conclusive result that would not require culture confirmation of these pathogens. Future work could explore the effects of bacterial levels and diversity of water samples on the accuracy of this method.

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DATA AVAILABILITY

The data set generated for this study is available at Ag Data Commons: <https://figshare.com/s/072a6b7d00feb3ab22b6>.

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