Comparison of methods to determine the microbial quality of alternative irrigation waters

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A B S T R A C T

The availability of water for crop irrigation is decreasing due to droughts, population growth, and pollution. Implementation of the Food Safety and Modernization Act (FSMA) for irrigation water standards will discourage growers to use poor microbial quality water for produce crop irrigation. We evaluated the applicability of a novel concentrator method for assessment of microbiological quality of alternative waters including secondary-treated wastewater (STWW), roof-harvest rainwater (RHW), and creek water (CW) in comparison to the standard membrane filtration method. Water samples of 100 ml were filtered through a 0.45 μm membrane filter using a vacuum manifold or concentrated to ~250 μl using the innovative concentrator. Then they were directly enumerated on specific agars, or enriched to monitor the populations of fecal bacterial indicators (*Escherichia coli*, enterococci, total and fecal coliforms) and bacterial pathogens (*Salmonella*, *Listeria monocytogenes*, and *E. coli* O157:H7). Presumptive pathogens were confirmed by real-time quantitative PCR. In total, 25 samples of alternative water were analyzed including 7 STWW, 9 RHW, and 9 CW. No significant differences between both detection methods were observed when enumerating indicator bacterial populations and detecting the presence of pathogens in RHW and CW samples. Recovery of fecal coliforms in STWW samples by concentrator analysis was significantly lower than the membrane filtration technique. Results suggest that performance of the concentrator method is equivalent to membrane filtration method in determining the microbiological quality of CW and RHW waters; the type of the water sources may influence the accuracy and sensitivity of the concentrator analysis.

1. Introduction

Water scarcity has become a major worldwide problem because of climate change and increased urbanization. Climate change altered the weather patterns and resulted in a higher frequency and intensity of droughts in the world (Meehl et al., 2007). Moreover, the world population has increased from 2.5 billion in 1950 to 6.5 billion in 2009 with doubled irrigated area and tripled water withdrawals (Pardey et al., 2014; Schierhorn et al., 2014) which could result in water scarcity in future. Therefore, to meet the growing demand for irrigation water, alternative water sources are imperative (Teklehaimanot et al., 2015).

The use of alternative water sources such as wastewater and roof-harvested rainwater in agriculture has gained more attention lately as a way to overcome water scarcity. Wastewater is commonly used in water scarce regions, especially in Asia and Africa countries. In China, 7% of the nation's farmland (4.1 million ha) are irrigated with polluted water (Xie, 2009); In Pakistan, 26% of the vegetables are dependent on irrigation with wastewater (Pedrero et al., 2010). In Europe, countries such as Spain, France, Italy, and Greece have allowed the use of treated wastewater for agriculture irrigation if it would not lead to the pollution of surface water by chemical and biological contaminants from the wastewater (EU, 2007; EC, 2016).

The approval of the Clean Water Act in 1972 made the secondary treatment a requirement for all wastewater treatment plants in the United States. The United States Environmental Protection Agency reported that 70% of the 16,000 facilities did not provide tertiary treatment of wastewater (USEPA, 2004), which was later recommended as an advance treatment (USEPA, 2012b). Thus, secondary-treated wastewater (STWW) was evaluated for its potential as irrigation water in the current study. Wastewater treatment plants process 130 gigalitre/day of wastewater in the United Sates (Seiple et al., 2017). The reuse of wastewater minimizes the discharge of treated effluent directly into freshwater (Scott et al., 2004). Moreover, wastewater contains nitrogen and phosphorus that are natural fertilizers for crops, which reduce the need for supplemental mineral fertilizers (Jimenez et al., 2010; Mojid

et al., 2010).

Roof-harvested rainwater (RHW) and creek water (CW) have also been considered as potential water sources for irrigation purposes (Ahmed et al., 2011; Chidamba and Korsten, 2015). Roof-harvested rainwater has been used as a potable- and nonpotable-water source in many countries such as Australia (Uba and Aghogho, 2000; Evans et al., 2006; Despins et al., 2009). Currently, applications of RHW for irrigation have not been well recognized and only 17 states have established guidelines to regulate the usage of RHW for agricultural irrigation in the United States (NCSL, 2017).

The consumption of fresh produce increased by 25% per capita during 1990s compared to 1970s due to changes in dietary trends and globalization (Pollack, 2001; Brandl, 2006). The proportion of all foodborne outbreak illnesses associated with raw fresh produce has also increased from < 1% to 12% during this period (Harris et al., 2003; Sivapalasingam et al., 2004). Approximately 48 million foodborne illnesses occur each year in the United States and ~ 46% of the illnesses are linked to fresh produce contamination (Painter et al., 2013). Studies have suggested that water is a significant source of contamination to fresh produce such as spinach, lettuce, and cabbage (Uyttendaele et al., 2015). The recently proposed Food Safety and Modernization Act (FSMA) for irrigation water standards discourage growers to use poor microbial quality water for produce crop irrigation (USFDA, 2013).

The microbiological quality of water is generally assessed by monitoring the fecal indicator bacteria, which are commonly found in the guts of the warm-blooded animals (Pinfold et al., 1993; Uba and Aghogho, 2000; Sazakli et al., 2007). The alternative water resources herein studied may contain high populations of coliforms, *Escherichia coli* or pathogenic bacteria including *Salmonella* spp., *Listeria monocytogenes*, and *E. coli* O157:H7 (Déportes et al., 1995; Steele and Odumeru, 2004; USEPA, 2012a). For instance, although microbial populations decrease during the wastewater reclamation process (Van der Steen et al., 2000), the secondary treated effluents may contain *Salmonella* (Maynard et al., 1999; Armon et al., 2002). Moreover, RHW might be contaminated by bird droppings on the roof (Ahmed et al., 2011). The creek water is also susceptible to contamination with pathogenic microorganisms by storm water runoff, animal fecal materials, and sewage discharges (Bagdasaryan, 1964; Alderisio and DeLuca, 1999; Steele and Odumeru, 2004).

Traditionally, the membrane filtration method has been used for the examination of bacterial populations from environmental waters (USEPA, 2002a,b; USEPA, 2012b). However, this method may be inappropriate for water with high turbidity due to clogging of the membrane filter by particulate matter (SIS, 1996; Eckner, 1998; Köster et al., 2003). In this study, a rapid, innovative biological concentrator developed for general microbiology purpose was introduced for irrigation water analysis. This is the first report on the evaluation of an innovative biological concentrator to determine the microbial quality of alternative irrigation waters in comparison with the traditional membrane filtration method.

2. Materials and methods

2.1. Sampling locations and sample collection

Sampling locations included water collected from rain-barrels of local households, creeks (Little Paint Branch creek, Beltsville, MD; Little Cove creek, Chambersburg, PA), and a wastewater treatment plant (Arlington, VA). All water samples were collected from November 2016 to January 2017. Water samples (4 liters/sample) were collected in sterile, labelled plastic bottles (Fisher Scientific, Fair Lawn, NJ), stored at 4 °C, and analyzed within 24 h.

Secondary-treated wastewater (STWW) was collected at the Arlington Water Pollution Control Plant (AWPCP) that purifies ~ 115 million liters of wastewater each day from residences and businesses through 5 wastewater treatments and solids handling systems

(Preliminary treatment, Primary treatment, Secondary treatment, Tertiary treatment/Chemical addition, and Treatment of solids). A total of 7 STWW samples were collected at the location where the waste water is passed through six-10 million liters, four-pass aeration tanks, configured for biological nutrient removal.

Seven creek water samples (CW) were collected from the Little Paint Branch creek (Beltsville, MD) and 2 CW samples were obtained from Little Cove Creek (Chambersburg, PA). In addition, 9 roof-harvest rainwater samples (RHW) were obtained from rain-barrels of local households located in the state of Maryland.

2.2. Indicator bacteria enumeration

Indicator bacteria including total coliforms, fecal coliforms, *E. coli*, and enterococci from each water sample were enumerated by the membrane filtration method and the concentrator method.

For the membrane filtration method, each water sample with appropriate dilution (total volume 100 ml) was filtered through a 0.45 μm (47 mm diameter) nitrocellulose membrane (Fisher Scientific) using vacuum manifold (Thermo Scientific, Waltham, MA). Immediately after filtration, membrane filters with trapped bacteria from the water samples were transferred onto specific agar plates and then transferred to incubator with appropriate incubation conditions. The specific agar media used were Violet Red Bile agar (VRB; Fisher Scientific), mFC agar (Fisher Scientific), mTEC agar (Neogen, Lansing, MI), and m-enterococcus agar (mE; Neogen) for the enumeration of total coliforms, fecal coliforms, *E. coli*, and enterococci, respectively. The VRB and mE agars were incubated at 35 °C for 24 h and 48 h, respectively, and mTEC and mFC agars were first incubated at 35 °C for 2 h, followed by at 44 °C for 24 h.

For the concentrator method, each water sample with appropriate dilution (total volume 100 ml) was concentrated using a bio-concentrator (InnovaPrep, Drexel, MO) and a 0.45 μm concentrating pipette (InnovaPrep) to a ~ 250 μl concentrate, and the entire ~ 250 μl concentrate was spread plated onto the aforementioned agars with the appropriate incubation conditions as previously prescribed.

For both methods, number of bacterial colonies between 20 and 60 per plate (USEPA, 2002a,b) from an original or diluted water sample was counted and expressed as log colony-forming units (CFU) per 100 ml. Plates with < 20 colonies were counted when counts were low in undiluted water sample. Detection thresholds of both detection methods for all water samples were 1 CFU/100 ml.

2.3. Anaerobic bacteria enumeration

Populations of anaerobic bacteria including *Clostridium perfringens* and *Bacteroides fragilis* from water samples were determined. Individual water samples (100 ml) were filtered or concentrated as previous described. Then the membrane and the ~ 250 μl concentrate were placed and spread plated onto Tryptose Sulfite Cycloserine agar (TSC; Sigma Aldrich) or Bacteroides Bile Esculin agar (BBE; Hardy Diagnostics, Santa Maria, CA) for the isolation of *C. perfringens* or *B. fragilis*, respectively. Agar plates were incubated anaerobically at 36 °C for 48 h (Bisson and Cabelli, 1979).

To detect low level contamination by these bacteria (< 1 CFU/ 100 ml), one additional membrane and a ~ 250 μl concentrate were separately transferred to a tube containing 10 ml of fluid thioglycollate broth (FTB, Fisher Scientific) and incubated anaerobically at 36 °C for 48 h for enrichment. After incubation, a loopful of FTB was streaked on TSC and BBE agars, and incubated at 36 °C for 24 h to detect these bacteria following enrichment.

2.4. Detection of pathogenic bacteria

Salmonella spp., *L. monocytogenes*, and *E. coli* O157:H7 were detected in water samples using primary enrichment in full-strength

peptone water (BPW; Neogen) followed by the secondary enrichment in selective media (APHA, 2005). Briefly, a membrane or a ~250 μl concentrate from each water sample was enriched in 50 ml of BPW at 37 °C for 24 h. After incubation, 5 ml of the enriched BPW from each sample was transferred to a tube containing 45 ml of tetrathionate broth (Neogen), Fraser broth (Neogen), or mEHEC broth (Biocontrol, Bellevue, WA), and incubated at 37 °C for 24 h. Following secondary enrichment, a loopful of these enrichment broths was streaked on xylose lysine tergitol-4 agar (XLT4; Neogen), modified oxford agar (MOX; Neogen), and sorbitol MacConkey agar (SMAC; Neogen), for the isolation of *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157:H7, respectively.

Presumptive isolates of the target pathogens on selective media were confirmed by real-time quantitative PCR (RT-qPCR). Two presumptive colonies of the target bacteria were selected from each selective agar plate (XLT4, MOX, or SMAC) and separately sub-cultured in 10 ml of Tryptic Soy Borth (TSB; Fisher Scientific). Two hundred microlitres (200 μl) of an overnight TSB culture was suspended in phosphate buffered saline (PBS, pH 7.0) and the genomic DNA was extracted using DNeasy Blood and Tissue Kits (Qiagen, Germantown, MD). Real-time quantitative PCR was performed using published gene specific primers as listed in Table 1 with a Stratagene Mx3005p instrument (Stratagene, La Jolla, CA).

Primers specific for the *invA* gene of *Salmonella* spp. (Rahn et al., 1992) and confirmed by Malorny et al. (2003) to be highly specific for *Salmonella* serotypes were used to confirm *Salmonella* isolates. Real-time quantitative PCR was performed in a 50 μl volume PCR reaction with SYBR Green reagents (Bio-Rad, Hercules, CA).

Two probe-based RT-qPCR assays were performed to detect the presence of virulence genes *stx1* and *stx2*, and *eae* gene for presumptive *E. coli* O157:H7 isolates (Sharma and Dean-Nystrom, 2003). Primers and probe for *eae* gene specifically targeted *E. coli* O157:H7 intimin, which allow the RT-qPCR assay to distinguish *E. coli* O157:H7 from non-O157 serotypes (Sharma and Dean-Nystrom, 2003). These two RT-qPCR assays were carried out in a 50 μl volume reaction with qPCR mastermix plus low ROX reagents (Eurogentec, San Diego, CA).

Presumptive *L. monocytogenes* colonies from the MOX were further confirmed by streaking on Listeria ottavani and agosti agar (ALOA, BioMerieux, Durham, NC) and RAPID'*L. mono* agar (LMONO, Bio-rad). Samples exhibited the *L. monocytogenes* phenotype from both ALOA and LMONO agars were subjected for DNA extraction and RT-qPCR confirmation using primers and probes targeting *iap* and *hly* genes as previously described (Rodríguez-Lázaro et al., 2004).

Table 2
List of the primers used in multiplex PCR for detecting *E. coli* virulence genes.

Gene	Name	Sequence	Size
stx1	stx1-F	5′-TGT AAC TGG AAA GGT GGA GTA TAC-3′	210 bp
	stx1-R	5′-GCT ATT CTG AGT CAA CGA AAA ATA AC-3′	
stx2	stx2-F	5′-GTT TTT CTT CGG TAT CCT ATT CCG-3′	484 bp
	stx2-R	5′-GAT GCA TCT CTG GTC ATT GTA TTA C-3′	
Rfb	RfbF	5′-GTG TCC ATT TAT ACG GAC ATC CAT G-3′	292 bp
	RfbR	5′-CCT ATA ACG TCA TGC CAA TAT TGC C-3′	
fliC	flic-F	5′-GCG CTG TCG AGT TCT ATC GAG C-3′	625 bp
	flifc-R	5′-CAA CGG TGA CTT ATC GCC ATT CC-3′	

F: forward primer; R: reverse primer.

2.5. Prevalence of E. coli virulence genes

The prevalence of *E. coli* virulence genes including *stx1*, *stx2*, *rfb*, and *fliC* from water samples was determined by conventional PCR and gel electrophoresis. Specific primers for the detection of these genes listed in Table 2 were previously described by Hu et al. (1999). One hundred milliliters of each water sample was filtered or concentrated and the filtered membrane or the ~250 μl concentrate was transferred to 50 ml BPW and incubated at 37 °C for 24 h. After incubation, BPW cultures were centrifuged at $5000 \times g$ for 15 min and DNA was extracted using DNeasy Blood and Tissue Kits (Qiagen, Germantown, MD). A multiplex PCR was performed in a 25 μl reaction volume with Platinum SuperFi PCR Master Mix reagents (Thermo Scientific), 0.8 μM *stx1* forward and reverse primer, 1.6 μM *stx2* forward and reverse primer, 0.5 μM *rfb* forward and reverse primer, 0.5 μM *fliC* forward and reverse primer, and ~50 ng of DNA template in Mastercycler pro (Eppendorf, Westbury, NY). The amplified DNA was visualized in 2% agarose gels stained with SYBR-safe (Sigma Aldrich, St. Louis, MO, USA) and a 100-bp ladder as the standard.

2.6. Chemistry of the water sample: chlorine, pH, electrical conductivity and temperature

At each sampling, 100 ml of water samples collected from different sources were measured for chlorine, pH, electrical conductivity (EC), and temperature (Castro et al., 2009). Water pH and temperature were measured with a pH meter (Orion, St. Louis, MO) and EC was determined with a conductivity meter (HM Digital, Culver City, CA). Total chlorine and free chlorine of each water sample were measured using chlorine Pocket Photometer (HF Scientific, Fort Myers, FL).

Table 1
List of primers and probes used for RT-qPCR in detecting genes specific for pathogenic bacteria.

Target pathogen	Gene	Name	Sequence
Salmonella	*invA*	invA-F	5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′
		invA-R	5′-TCA TCG CAC CGT CAA AGG AAC C-3′
L. monocytogenes	*hly*	hly-F	5′-CAT GGC ACC ACC AGC ATC T-3′
		hly-R	5′-ATC CGC GTG TTT CTT TTC GA-3′
		hly-P	5′-FAM-CGC CTG CAA GTC CTA AGA CGC CA-TAMRA-3′
	iap	iap-F	5′-AAT CTG TTA GCG CAA CTT GGT TAA-3′
		iap-R	5′-CAC CTT TGA TGG ACG TAA TAA TAC TGT T-3′
		iap-P	5′-FAM-CAA CAC CAG CGC CAC TAC GGA CG-TAMRA-3′
E. coli O57:H7	*stx1*	stx1-F	5′-GAC TGC AAA GAC GTA TGT AGA TTC G-3′
		stx1-R	5′-ATC TAT CCC TCT GAC ATC AAC TGC-3′
		stx1-P	5′-FAM-TGA ATG TCA TTC GCT CTG CAA TAG GTA CTC-TAMRA-3′
	stx2	stx2-F	5′-ATT AAC CAC ACC CCA CCG-3′
		stx2-R	5′-GTC ATG GAA ACC GTT GTC AC-3′
		stx2-P	5′-HEX-CAG TTA TTT TGC TGT GGA TAT ACG AGG GCT TG-TAMRA-3′
	eae	eae-F	5′-GTA AGT TAC ACT ATA AAA GCA CCG TCG-3′
		eae-R	5′-TCT GTG TGG ATG GTA ATA AAT TTT TG-3′
		eae-P	5′-FAM-AAA TGG ACA TAG CAT CAG CAT AAT AGG CTT GCT-TAMRA-3′

F: forward primer; R: reverse primer.

Table 3
Bacterial indicator populations in water samples analyzed by the membrane filtration method and the concentrator method.[1]

	Creek water log CFU/100 ml		Roof-harvest rainwater log CFU/100 ml		Secondary-treated wastewater log CFU/100 ml	
	Membrane	Concentrator	Membrane	Concentrator	Membrane	Concentrator
Total coliforms	2.31 ± 0.18^{bcx}	2.59 ± 0.14^{bx}	1.41 ± 0.32^{dx}	1.91 ± 0.22^{cdx}	3.61 ± 0.11^{ax}	3.51 ± 0.08^{ax}
Fecal coliforms	1.81 ± 0.32^{bcx}	1.17 ± 0.25^{cdy}	0.95 ± 0.31^{cdx}	0.52 ± 0.29^{dy}	3.51 ± 0.12^{ax}	2.82 ± 0.16^{bx}
E. coli	1.57 ± 0.27^{bx}	1.02 ± 0.18^{by}	0.23 ± 0.10^{cy}	0.43 ± 0.25^{cy}	3.04 ± 0.19^{ax}	3.04 ± 0.14^{ax}
enterococci	1.43 ± 0.32^{ax}	1.29 ± 0.30^{ay}	1.35 ± 0.39^{ax}	1.21 ± 0.39^{axy}	1.96 ± 0.23^{ay}	1.73 ± 0.30^{ay}

[a–d] Means with different superscripts in a row differ significantly ($P < 0.05$).

[x–y] Means with different superscripts in a column differ significantly ($P < 0.05$).

[1] Results are mean values $\pm$ standard deviation (Detection threshold: 1 CFU/100 ml).

2.7. Statistical analysis

Bacterial populations (log CFU/100 ml) were subjected to ANOVA and analyzed using the Statistical Analysis Software (version 9.4 SAS Institute Inc, Cary, NC). Differences among the means were analyzed at $P < 0.05$ using Fisher's least significance difference test. To ensure that the data were normally distributed, standardized skewness was verified. In addition, Pearson product moment correlation coefficients (r) among the chemical and microbial properties of the water samples were analyzed ($P < 0.05$).

3. Results

3.1. Bacterial indicator populations

Bacterial indicator populations of the CW, RHW, and STWW samples are shown in Table 3. Among the three types of water sources, RHW had the lowest bacterial indicator populations; followed by CW and STWW. In RHW samples, total coliforms were detected in all 9 samples (100%), whereas fecal coliforms were found in 6 samples (67%; 6/9) and generic *E. coli* in 2 samples (22%; 2/9).

Populations of total coliforms and *E. coli* in STWW samples were significantly higher than in the other two types of waters as recovered by both detection methods. However, no significant differences were observed in populations of enterococci among the three types of waters. Additionally, there were no significant differences between the two methods in enumerating total coliforms, *E. coli*, and enterococci populations of CW, RHW, and STWW (Table 9). However, membrane filtration method detected significantly higher fecal coliforms in STWW than the concentrator method.

3.2. Detection of anaerobic bacteria

Table 4 shows the prevalence of *B. fragilis* and *C. perfringens* from the tested water samples. High prevalence of *B. fragilis* was detected in STWW (86%; 6/7 positive), but none in CW (0/9) and RHW (0/9). In

STWW samples, *B. fragilis* populations enumerated by the membrane filtration method and the concentrator method were different ($P < 0.05$). Although no *C. perfringens* populations were enumerated by direct plating in all three types of water sources by both detection methods, high prevalence of *C. perfringens* was detected in STWW (100%; 7/7), CW (78%; 7/9), and RHW (66%, 6/9) following enrichment. Recovery of *C. perfringens* in RHW was higher by the membrane filtration method which detected 67% positive compared to 44% positive by the concentrator method.

3.3. Detection of pathogenic bacteria

Results of the RT-qPCR confirmed the presence of bacterial pathogens in the tested water samples as shown in Table 5. In CW samples, *L. monocytogenes* was the only pathogen detected by both methods with further confirmation by chromogenic agars and RT-qPCR assays. Although the presence of *stx1* gene was detected in one of the CW samples by the membrane filtration method, none of the presumptive colonies isolated from CW samples was confirmed positive for *E. coli* O157:H7. Results of the CW and RHW samples showed that both detection methods exerted similar performance in detecting *L. monocytogenes* and *E. coli* O157:H7 in these two water sources.

Salmonella spp. were only detected in STWW samples; detection of *Salmonella* positive samples varied with the detection method. *Salmonella* were recovered from 43% (3/7) and 29% (2/7) STWW samples by the membrane filtration method and the concentrator method, respectively. Similarly, *E. coli* O157:H7 was recovered from 2 STWW samples using membrane filtration method; however, only one sample was detected using the concentrator method.

3.4. Prevalence of E. coli virulence genes

Table 6 shows the prevalence of *E. coli* virulence genes including *fliC* (H7 antigen), *rfb* (O157 antigen), and shiga-toxin genes (*stx1* and *stx2*). High prevalence of *fliC*, *rfbE*, and *stx 1* genes were found in all three water sources. Shiga-toxigenic gene (*stx1*) was detected in 6 RHW

Table 4
Anaerobic bacterial populations in water samples recovered by direct plating and enrichment.

	Creek water log CFU/100 ml		Roof-harvest rainwater log CFU/100 ml		Secondary-treated wastewater log CFU/100 ml	
	Membrane	Concentrator	Membrane	Concentrator	Membrane	Concentrator
B. fragilis	ND[1]	ND	ND	ND	$1.36 \pm 0.35^{[2]a}$	0.53 ± 0.16^{b}
Enrichment	0/9[3]	0/9	0/9	0/9	6/7	6/7
C. perfringens	ND	ND	ND	ND	ND	ND
Enrichment	7/9	7/9	6/9	4/9	7/7	7/7

[a–b] Means with different superscripts in a row of the same type of water sample differ significantly ($P < 0.05$).

[1] ND: below detection limit (< 1 CFU/100 ml).

[2] Results are mean values $\pm$ standard deviation (Detection threshold: 1CFU/100 ml).

[3] Number of the positive samples/total number of samples.

Table 5
Detection of bacterial pathogens in water samples following confirmation of presumptive positive isolates.

		Creek water		Roof-harvest water		Secondary-treated wastewater	
		Membrane	Concentrator	Membrane	Concentrator	Membrane	Concentrator
L. monocytogenes	*hly*	3/9[1]	3/9	1/9	1/9	0/7	0/7
	iap	3/9	3/9	1/9	1/9	0/7	0/7
Salmonella spp.	*invA*	0/9	0/9	0/9	0/9	3/7	2/7
E. coli O157:H7	*eae*	0/9	0/9	1/9	1/9	2/7	1/7
	stx1	1/9	0/9	1/9	1/9	2/7	1/7
	stx2	0/9	0/9	0/9	0/9	0/7	0/7

[1] Number of the positive sample/total samples; presumptive positive isolates on selective media were confirmed by molecular procedure.

Table 6
Prevalence of *E. coli* virulence genes in water samples recovered by the membrane filtration method and the concentrator method.

		fliC	*rfb*	*stx1*	*stx2*
Creek water	Membrane	5/9[1]	5/9	6/9	0/9
	Concentrator	7/9	8/9	8/9	0/9
Roof-harvest rainwater	Membrane	7/9	6/9	6/9	1/9
	Concentrator	6/9	6/9	6/9	1/9
Secondary-treated wastewater	Membrane	4/7	4/7	5/7	0/7
	Concentrator	4/7	5/7	5/7	0/7

[1] Number of the positive sample/total samples.

(67%), 5 STWW (71%), and 8 CW (89%) samples analyzed by the concentrator method whereas only 6 CW (67%) samples were identified as *stx1* positive when analyzed by the membrane filtration method. The concentrator method also detected higher prevalence of the *rfb* gene than the membrane filtration method. The *rfb* gene was detected in 89% (8/9) and 71% (5/7) CW and STWW samples by the concentrator method as compared to 56% (5/9) and 57% (4/7) *rfb* positive samples, respectively, by the membrane filtration method. Prevalence of *stx2* gene was very low, only one of the RHW (1/9) contained *stx2* and none in CW or STWW.

3.5. Chemistry of water samples

The average values of pH, chlorine (total and free chlorine), and EC of all tested water samples met irrigation water quality standards according to FAO's guideline (Ayers and Westcot, 1985), with the exception of the slightly higher EC recorded in STWW, as shown in Table 7. The average EC of STWW was 0.98 ± 0.14 dS/m, which was in the range of the "slight to moderate degree of restriction" on use for irrigation water according to FAO guidelines (0.7–3.0 dS/m).

3.6. Correlation between chemistry and microbial indicators

Results of all water samples from CW, RHW, and STWW were pooled and analyzed for Pearson product moment correlation coefficients (r) and P value as shown in Table 8. Significant positive relationships (P < 0.05) were observed among all the microbial indicators (total coliforms, fecal coliforms, *E. coli,* and enterococci) and

the highest correlation was between the *E. coli* and fecal coliform populations (r = 0.8559; P < 0.05), followed by between *E. coli* and total coliforms (r = 0.7978, P < 0.05). Further, the EC of the water samples was significantly correlated with total coliforms (0.4943), fecal coliforms (0.4418), and *E. coli* (0.5877) populations.

4. Discussion

It is estimated that 44% of the population in the world will be living in water-stressed condition by 2050 (Scheierling et al., 2011). When water becomes limited for human consumption, it will be difficult to acquire water for irrigation purpose. As the world faces increasing water scarcity, the use of waste-water, roof-harvest rainwater, and creek water for irrigation would represent advantageous alternative sources. To determine the microbiological safety of these sources as irrigation waters for fresh produce, quantification and detection of indicator and pathogenic bacteria are important to ensure the fresh produce safety for human consumption. A RT-qPCR method was compared with the membrane filtration method for enumeration of *E. coli* in surface water (Vital et al., 2017). In their study, RT-qPCR method recovered significantly lower *E. coli* populations than the membrane filtration method. Detection of enterococci in recreational water by the membrane filtration and the defined substrate-based Enterolert® showed lower recovery (P < 0.05) by the latter method and reported that Enterolert® method was not equivalent to the membrane filtration method (Valente et al., 2010). During a long-term study, Buckalew et al. (2006) reported that *E. coli* populations detected by Colilert® in environmental water samples were similar to those detected by the membrane filtration method. In the current study, we also compared a new concentrator method against membrane filtration.

The comparative evaluation of the membrane filtration method and the concentrator method revealed no significant differences in recovery of indicator bacteria, except for lower recovery of fecal coliforms in STWW with the concentrator method. The results suggest that the concentrator method may be used to accurately determine the populations of total coliforms, *E. coli,* and Enterococci in alternative irrigation waters. The method may recover lower populations of fecal coliforms in STWW samples. Moreover, *C. perfringens* and *Bacteroides* spp. have also been used as indicators of fecal contamination in water (Bisson and Cabelli, 1979; Sartory et al., 1998; Fogarty and Voytek,

Table 7
Chemical quality of the creek water, roof-harvest water, and secondary-treated wastewater.[1]

	Temperature (˚C)	pH	EC (dS/m)[2]	Chlorine (µg/l)	
				Total	Free
Creek water	22.8 ± 0.88	7.3 ± 0.06	0.51 ± 0.87	0.4 ± 0.10	0.3 ± 0.10
Roof-harvest water	22.9 ± 1.37	7.7 ± 0.13	0.12 ± 0.82	0.4 ± 0.10	0.3 ± 0.10
Secondary-treated wastewater	22.6 ± 1.42	7.1 ± 0.09	0.98 ± 0.14	0.1 ± 0.03	0.1 ± 0.02

[1] Data are the mean values ± standard deviation.
[2] EC: electric conductivity.

Table 8

Pearson correlation coefficients (r) with P-value determined between each of the chemical and microbiological quality of creek water, roof-harvest water, and secondary-treated wastewater.

	Total coliforms	Fecal coliforms	*E. coli*	enterococci	Temperature	pH	EC[1]	Total Chlorine
Fecal coliforms	0.7796[*]							
	(0.0000)[2]							
E. coli	0.7978[*]	0.8559[*]						
	(0.0000)	(0.0000)						
enterococci	0.4275[*]	0.5233[*]	0.3676[*]					
	(0.0020)	(0.0001)	(0.0086)					
Temperature	−0.0936	−0.0594	−0.0712	−0.1664				
	(0.5181)	(0.6819)	(0.6234)	(0.2481)				
pH	−0.2644	−0.2634	−0.4100[*]	0.0659	−0.2753			
	(0.0635)	(0.0646)	(0.0031)	(0.6495)	(0.0530)			
EC	0.4943[*]	0.4418[*]	0.5877[*]	0.0487	−0.2180	−0.5801[*]		
	(0.0003)	(0.0013)	(0.0000)	(0.7371)	(0.1284)	(0.0000)		
Total Chlorine	−0.3409[*]	−0.2261	−0.2305	−0.0114	0.5166[*]	−0.1252	−0.3065[*]	
	(0.0154)	(0.1144)	(0.1073)	(0.9372)	(0.0001)	(0.3865)	(0.0304)	
Free Chlorine	−0.3824[*]	−0.2998[*]	−0.2194	−0.0587	0.4880[*]	−0.1127	−0.2667	0.9453[*]
	(0.0061)	(0.0344)	(0.1257)	(0.6854)	(0.0003)	(0.4356)	(0.0612)	(0.0000)

[1] EC: electric conductivity.

[*] Correlation coefficients are statistically significant (P < 0.05).

[2] (P-value).

2005; Layton et al., 2006). Similar to the populations of traditional indicator bacteria, high prevalence of *C. perfringens* was detected in all three alternative water sources by both detection methods.

According to the proposed FSMA Produce Safety Rule, the irrigation water that is directly applied to growing produce should contain no more than 2.61 log CFU/100 ml *E. coli* per sample (statistical threshold value, STV) and 2.10 log CFU/100 ml of a geometric mean (n = 5 samples) (USFDA, 2013). Therefore, it is important that a detection method should provide accurate enumerations for *E. coli* populations in irrigation water. In the current study, *E. coli* was detected in 8 CW, 2 RHW, and all 7 STWW samples ranging from 0.6 to 4.0 log CFU/100 ml. *Escherichia coli* populations in CW and RHW samples were within FSMA regulation. However, 5 STWW samples contained *E. coli* populations higher than the STV for direct irrigation application. Results of the current study not only support the usage of concentrator method to enumerate *E. coli* in alternative irrigation waters, but also indicate that STWW may only be used for drip or furrow irrigation where water is not in direct contact with the harvestable portion of crop. Subsurface drip irrigation has been shown to reduce contamination magnitude of crops as compared to other irrigation systems such as overhead irrigation (Stine et al., 2005).

In this study, both detection methods were comparable in detecting pathogenic bacteria in CW and RHW samples and detected equal number of pathogen positive samples. However, the concentrator method missed one of the three *Salmonella* positive and one of the two

E. coli O157:H7 positive STWW samples. Overall, we observed that the accuracy and sensitivity of the concentrator method for microbiological quality assessments in STWW were compromised for bacterial pathogen detection and fecal coliform enumeration. Presence of solid particulates and/or chemicals in the STWW samples might have interfered with the target organisms in the concentrating pipette thereby negatively affecting the recovery of these bacteria.

As per USEPA's guidelines, secondary treatment standards are minimum requirements for wastewater treatment plants (USEPA, 2017). The STWW was selected for this study because it is more accessible wastewater source and may resemble to poor quality water. Moreover, STWW samples that contained higher bacterial populations than tertiary-treated wastewater were preferred in order to differentiate the accuracy and sensitivity between the two methods.

In this study, STWW samples had a relatively higher average EC than the other two sources. The higher EC indicates higher salinity of water, which reduces plant biomass and plant growth when used for crop irrigation thereby reducing the crop yield and its quality (Lazof and Bernstein, 1999; Bernstein and Kafkafi, 2002). Electric conductivity of CW depends on the surrounding geology; for example, minerals in clay and limestone soils ionize as they dissolve and contribute to water conductivity (Wetzel, 2001). The higher EC of STWW than the other two water sources might be due to higher concentrations of calcium ions and organic carbon solutes (USEPA, 2012b). Djaouda et al. (2013) reported that water with higher EC supported the survival of *E. coli* and

Table 9

Analysis of Variance (ANOVA) table for comparing differences in detection of bacterial indicators by membrane filtration and concentrator methods.

Indicator bacteria	Source	Degrees of freedom		Sum of Squares	Mean Square	F Value	Pr > F
Total coliforms	Water[1]	2	28.4896		14.2448	40.13	< 0.0001
	Method[2]	1	0.7938		0.7938	2.24	0.1419
	Water*Method	2	0.6933		0.3467	0.98	0.3846
Fecal coliforms	Water	2	47.6520		23.8260	39.14	< 0.0001
	Method	1	4.1876		4.1876	6.88	0.0119
	Water*Method	2	0.1662		0.0831	0.14	0.8728
Escherichia coli	Water	2	55.5961		27.7980	80.96	< 0.0001
	Method	1	0.0360		0.0360	0.10	0.7477
	Water*Method	2	1.7456		0.8728	2.54	0.0905
Enterococci	Water	2	2.8506		1.4253	1.51	0.2328
	Method	1	0.3411		0.3411	0.36	0.5512
	Water*Method	2	0.0232		0.0116	0.01	0.9878

[1] The main effect "Water" included creek water, roof-harvest rainwater, and secondary-treated wastewater.

[2] The main effect "Method" included the membrane filtration and the concentrator method.

Salmonella spp., which supports the findings of the current study that STWW samples contained significantly higher *E. coli* populations than CW and RHW. Additionally, *Salmonella* was only detected in STWW samples among all three types of waters. Previously, the presence of *Salmonella* had been reported in wastewater (Blumenthal et al., 1989; Mara and Cairncross, 1989; Shuval, 1991; Melloul et al., 2001).

Investigating the prevalence of *E. coli* virulence genes in irrigation water may be helpful in determining the microbiological safety of these alternative water sources. A published multiplex PCR assay was conducted in this study for rapidly detecting the prevalence of all four *E. coli* virulence genes in one single reaction (Hu et al., 1999). We observed high prevalence of *fliC*, *rfb*, and *stx1* genes but low prevalence of *stx2* gene in all water sources studied. The results concur with our previous study where 36%–80% of CW samples collected in Pennsylvania contained shiga-toxin genes (Shelton et al., 2011). Recent study by Topalcengiz et al. (2017) confirmed high prevalence of *fliC* (52%) and *stx1* (33%) gene but low prevalence of *stx2* (9%) in Central Florida irrigation water ponds. Interestingly, although CW and RHW met the FSMA standard as irrigation water sources, at least one virulence gene of *E. coli* was detected in these water samples. Detection of *E. coli* virulence genes by the concentrator was superior to the membrane filtration method in CW, and similar in other waters, which suggest that the novel concentrator method can also be used for detecting virulence genes in these waters.

Traditionally, the membrane filtration method has been widely used for water quality analysis. However, this technique is not applicable for waters with a level of turbidity that would cause the filter to become blocked before an adequate volume of water has passed through (SIS, 1996; Eckner, 1998; Köster et al., 2003). Further, background heterotrophic bacteria may decrease the recovery of coliforms in water by membrane filtration (Burlingame et al., 1984). The concentrator method possessed one significant practical advantage when used for water samples with high particulate content. In the current study, membrane filters were clogged while processing some of the CW and STWW samples due to water turbidity; however, there was no clogging issue observed using the concentrator method. Turbid water sample can be diluted with a sterile water or multiple filters can be used to avoid clogging in the membrane filtration method.

The concentrator examined in this study uses tangentially-loaded filtration concentrating pipette to capture microorganisms and the trapped microorganisms are released through a patented Wet Foam elution process into ~ 250 µl PBS-based elution fluid. It does not require vacuum and filter manifold setup. Previously, Hunter et al. (2011) used ultrafiltration and the concentrator to detect *E. coli* O157:H7 in spiked-recreational water and reported that this method provided accurate detection of this pathogen. The current study was the first report using the concentrator method to determine populations of indicator bacteria, the presence of pathogenic bacteria, and the prevalence of *E. coli* virulence genes in alternative irrigation waters, where results were comparable to the membrane filtration method in CW and RHW samples.

5. Conclusions

The concentrator method is comparable to membrane filtration method for analyzing microbiological quality of creek water and roof-harvested water. It is superior in detecting virulence genes associated with *E. coli* O157:H7 in creek water. The concentrator method is simple, highly effective, and rapid in detecting bacterial populations in irrigation water and can be used as an alternative to traditional tedious membrane filtration method. The type of water sources may influence the accuracy and efficiency of the concentrator.

Disclosure

The mention of trade names or commercial products does not imply

recommendation or endorsement to the exclusion of other products by the U.S. Department of Agriculture.

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