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Authors
Solaiman, Sultana
Allard, Sarah M
Callahan, Mary Theresa
et al.

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Longitudinal Assessment of the Dynamics of *Escherichia coli*, Total Coliforms, *Enterococcus* spp., and *Aeromonas* spp. in Alternative Irrigation Water Sources: a CONSERVE Study

Sultana Solaiman,[a] Sarah M. Allard,[b*] Mary Theresa Callahan,[a] Chengsheng Jiang,[b] Eric Handy,[c] Cheryl East,[c] Joseph Haymaker,[d] Anthony Bui,[b] Hillary Craddock,[b] Rianna Murray,[b] Prachi Kulkarni,[b] Brienna Anderson-Coughlin,[e] Shani Craighead,[e] Samantha Gartley,[e] Adam Vanore,[e] Rico Duncan,[d] Derek Foust,[d] Maryam Taabodi,[d] Amir Sapkota,[b] Eric May,[d] Fawzy Hashem,[d] Salina Parveen,[d] Kalmia Kniel,[e] (ORCID) Manan Sharma,[c] Amy R. Sapkota,[b] (ORCID) Shirley A. Micallef[a,f]

[a]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland, USA
[b]Maryland Institute for Applied and Environmental Health, School of Public Health, University of Maryland, College Park, Maryland, USA
[c]Environmental Microbial and Food Safety Laboratory, USDA-ARS, Beltsville, Maryland, USA
[d]Department of Agriculture, Food and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, Maryland, USA
[e]Department of Animal and Food Sciences, University of Delaware, Newark, Delaware, USA
[f]Center for Food Safety and Security Systems, University of Maryland, College Park, Maryland, USA

ABSTRACT As climate change continues to stress freshwater resources, we have a pressing need to identify alternative (nontraditional) sources of microbially safe water for irrigation of fresh produce. This study is part of the center CONSERVE, which aims to facilitate the adoption of adequate agricultural water sources. A 26-month longitudinal study was conducted at 11 sites to assess the prevalence of bacteria indicating water quality, fecal contamination, and crop contamination risk (*Escherichia coli*, total coliforms [TC], *Enterococcus*, and *Aeromonas*). Sites included nontidal freshwater rivers/creeks (NF), a tidal brackish river (TB), irrigation ponds (PW), and reclaimed water sites (RW). Water samples were filtered for bacterial quantification. *E. coli*, TC, enterococci (~86%, 98%, and 90% positive, respectively; $n = 333$), and *Aeromonas* (~98% positive; $n = 133$) were widespread in water samples tested. Highest *E. coli* counts were in rivers, TC counts in TB, and enterococci in rivers and ponds ($P < 0.001$ in all cases) compared to other water types. *Aeromonas* counts were consistent across sites. Seasonal dynamics were detected in NF and PW samples only. *E. coli* counts were higher in the vegetable crop-growing (May-October) than nongrowing (November-April) season in all water types ($P < 0.05$). Only one RW and both PW sites met the U.S. Food Safety Modernization Act water standards. However, implementation of recommended mitigation measures of allowing time for microbial die-off between irrigation and harvest would bring all other sites into compliance within 2 days. This study provides comprehensive microbial data on alternative irrigation water and serves as an important resource for food safety planning and policy setting.

IMPORTANCE Increasing demands for fresh fruit and vegetables, a variable climate affecting agricultural water availability, and microbial food safety goals are pressing the need to identify new, safe, alternative sources of irrigation water. Our study generated microbial data collected over a 2-year period from potential sources of irrigation (rivers, ponds, and reclaimed water sites). Pond water was found to comply with Food Safety Modernization Act (FSMA) microbial standards for irrigation of fruit and vegetables. Bacterial counts in reclaimed water, a resource that is not universally allowed on fresh produce in the United States, generally met microbial standards or needed minimal mitigation. We detected the most seasonality and the highest microbial loads in river water, which emerged as the water type that would

require the most mitigation to be compliant with established FSMA standards. This data set represents one of the most comprehensive, longitudinal analyses of alternative irrigation water sources in the United States.

Agriculture consumes ~70% of global freshwater withdrawals annually (1), with much higher proportions used in agriculturally intensive countries. In 2015, irrigation accounted for 42% of freshwater (surface and groundwater) withdrawals for all uses in the United States (2). Increasing population growth and agricultural demands, competing interests for surface water, unsustainable groundwater abstraction, and changing precipitation and drought patterns are placing a strain on water availability for agriculture. As a result, concerns of long-term water scarcity in the United States are growing (3, 4), and there is a need to explore alternative water sources for agriculture to reduce dependence on high-quality, environmentally sensitive groundwater sources. In the mid-Atlantic region of the United States, such alternatives include tertiary treated wastewater (reclaimed water), pond and river surface water, and recycled vegetable processing wash water. The safe use of these alternative sources could reduce the heightened demand on existing groundwater resources.

Aside from availability, a major reason for using high-quality water, such as groundwater, in agriculture is to assuage food safety concerns in the irrigation of fruit and vegetable crops. Microbiologically contaminated irrigation water has the potential to spread infectious agents to crops (5, 6), and enteric pathogen-contaminated irrigation water has been implicated in several foodborne illness outbreaks (7, 8). Pathogens can survive for extended periods in surface and reclaimed water under favorable conditions, but bacterial dynamics are complex and water physicochemical parameters alone do not provide strong predictive potential (9, 10). Therefore, assessing the microbial quality of alternative water sources for the irrigation of fresh produce is a critical step in evaluating the suitability of those sources (11).

The earliest standards to evaluate the microbial quality of irrigation water made use of total coliforms (TC), a heterogenous group of bacteria (12). However, due to their inability to reliably indicate the presence of fecal contamination determined to be the most probable source of pathogens in water, microorganisms most frequently found in feces were selected later as more appropriate indicators. These included *Escherichia coli* for drinking water and *Enterococcus* spp. for recreational water use (13). The U.S. Environmental Protection Agency (EPA) recommends the use of *Enterococcus* as an indicator of fecal contamination for both freshwater and saltwater (14) because of their long survival in water. In recent years, the U.S. Food and Drug Administration (FDA) Food Safety Modernization Act (FSMA) established standards in a Produce Safety Rule (PSR; 21 CFR 112) specific to preharvest agricultural water that will come in direct contact with edible portions of fresh produce crops during cultivation. The rule requires yearly water testing and the generation of a rolling microbial water quality profile (MWQP), based on data from at least 20 samples collected over the most recent 2- to 4-year period. The PSR standard uses *E. coli* concentrations and sets a geometric mean (GM) not to exceed 126 CFU and a statistical threshold value (STV) not to exceed 410 CFU of *E. coli* in 100 ml of water.

As part of a U.S. Department of Agriculture-funded center, called CONSERVE (Center of Excellence at the Nexus of Sustainable Water Reuse, Food and Health), with the long-term goal of facilitating the adoption of safe agricultural water reuse, a mid-Atlantic team conducted a longitudinal study of microbial water quality of 11 alternative irrigation water sources. With the impending implementation of the agricultural water provision of the PSR, the microbial quality of these potential irrigation water sources was evaluated. Sites were selected to represent various types of surface and reclaimed water, and four bacterial taxonomic groups (*Escherichia coli*, TC, *Enterococcus* spp., and *Aeromonas* spp.) were enumerated to monitor the bacterial quality of the
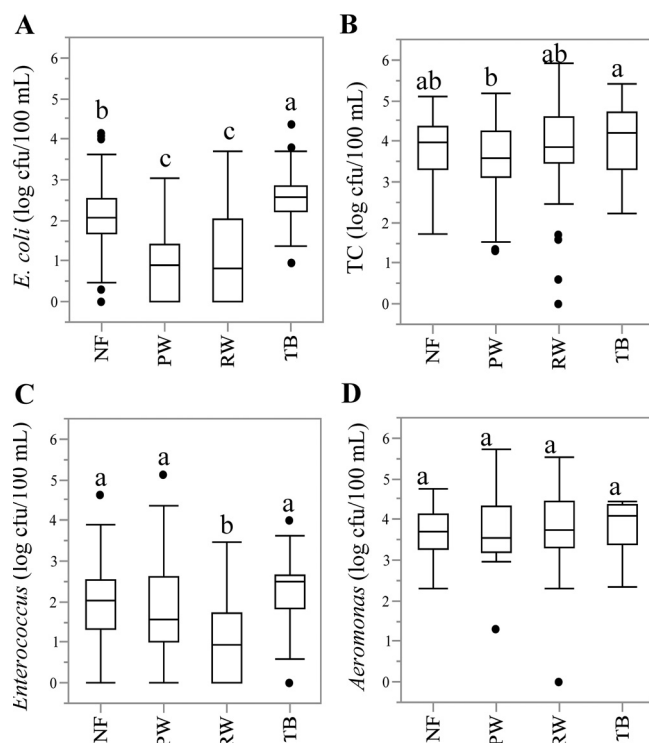
FIG 1 Bacterial prevalence in log CFU/100 ml in various water types for *E. coli* (A), total coliforms (TC) (B), *Enterococcus* spp. (C), and *Aeromonas* spp. (D). Data for each water type are pooled from various sites: nontidal fresh river (NF; $n = 166$ from 5 sites), pond water (PW; $n = 69$ from 2 sites), reclaimed water (RW; $n = 64$ from 3 sites), and tidal brackish rivers (TB; $n = 34$ from 1 site). The boxplots show the median and the 25th and 75th percentiles of the range. The whiskers show lower and higher observations than the 25th and 75th percentiles, respectively. Lowercase letters denote statistically significant differences at a *P* value of <0.05 among water types for each taxon.

water source over a 2-year period. *Escherichia coli*, TC, and *Enterococcus* spp. were selected for their role as indicator bacteria. *Aeromonas* spp. were included as an understudied group of potential human pathogens ubiquitously found in water environments. We hypothesized that despite seasonal and geographical variability, surface water from various sources would be microbiologically safe to use for irrigation based on the proposed PSR criteria. We also hypothesized that reclaimed water would meet the PSR criteria and, moreover, exhibit more consistent parameters over time. Seasonal dynamics, relationships between bacterial taxa and physicochemical parameters of water, and compliance with the proposed agricultural water provision of the PSR were explored to assess which of the various water types are appropriate sources for irrigation of fresh crops.

RESULTS

Bacterial prevalence and differences by water types. The 11 water sites were sampled longitudinally, resulting in 333 water samples. *E. coli* was detected in 288 (86.5%), TC in 327 (98.2%), and *Enterococcus* spp. in 299 (89.8%) of these. From the subset of 133 water samples tested for *Aeromonas* spp., 131 (98.5%) were positive. The highest microbial loads were generally detected in river water (NF and TB, respectively) (see Table S1 in the supplemental material). Higher *E. coli* counts were detected in TB and NF than PW and RW ($P < 0.001$). TC levels were higher in TB than PW ($P < 0.05$), and *Enterococcus* levels were higher in TB, NF, and PW than RW ($P < 0.001$) (Fig. 1). No water type variations were detected in *Aeromonas* levels. Overall, concentrations of *E. coli* ranged from 0 to 4.4 log CFU/100 ml, TC ranged from 0 to 5.9 log CFU/100 ml, *Enterococcus* ranged from 0 to 5.1 log CFU/100 ml, and *Aeromonas* spp. ranged from 0 to 5.7 log CFU/100 ml (Fig. 1). Comparing data between year 1 (September 2016 to
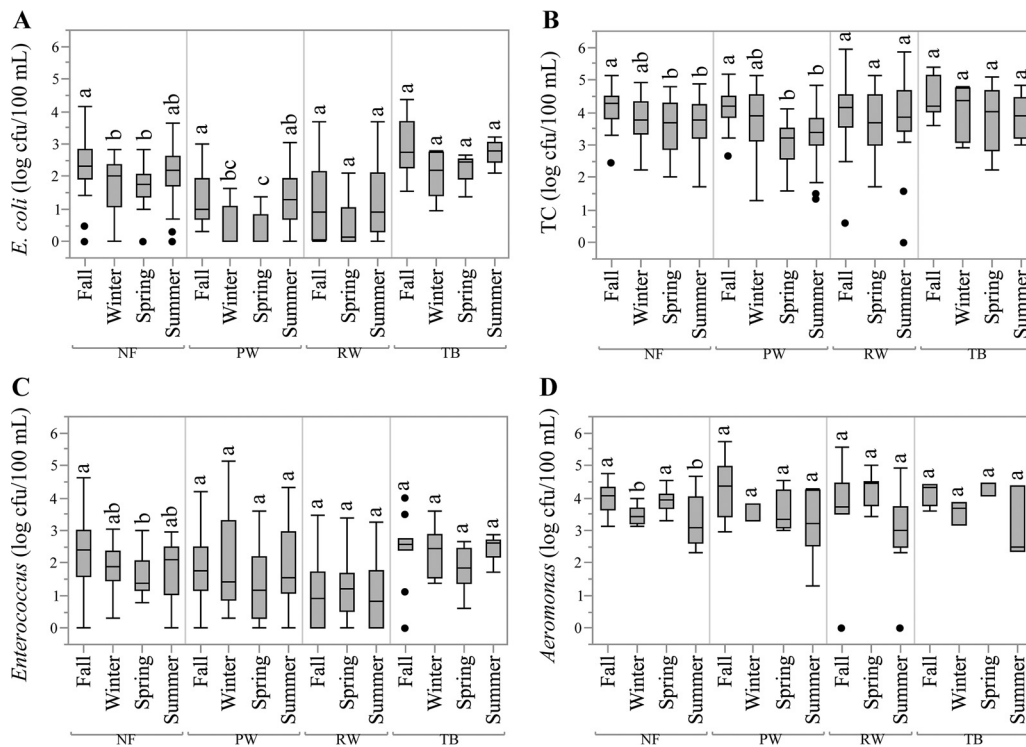
FIG 2 Seasonal variation in bacterial counts in log CFU/100 ml for *E. coli* (A), total coliforms (TC) (B), *Enterococcus* spp. (C), and *Aeromonas* spp. (D), enumerated in different water types. Data for each water type are pooled from various sites. The boxplots show the median and the 25th and 75th percentiles of the range. The whiskers show lower and higher observations than the 25th and 75th percentiles, respectively. Lowercase letters denote statistically significant differences at a *P* value of <0.05 among water types for each taxon.

August 2017) and year 2 (September 2017 to August 2018) of collection revealed little discrepancy, with only *Enterococcus* species counts differing significantly between years for PW (year 2 counts were 0.94 log CFU/100 ml higher than year 1, $P < 0.001$) and TB (year 1 counts were 0.52 log CFU/100 ml higher than year 2, $P < 0.05$).

Effect of season on bacterial dynamics. Seasonal dynamics in bacterial counts were dependent on water type. In NF rivers, seasonal differences were detected in *E. coli*, TC, *Enterococcus*, and *Aeromonas* (Fig. 2). In this water type, *E. coli* counts were higher in fall than in winter or spring ($P \leq 0.001$) (Fig. 2A). Total coliform counts were higher in fall than in spring ($P < 0.001$) and summer ($P < 0.01$) (Fig. 2B), and *Enterococcus* levels were elevated in fall compared to spring ($P < 0.01$) (Fig. 2C). *Aeromonas* spp. were detected at higher levels in fall and spring; statistically supported differences were detected between fall and both winter and summer (both $P < 0.01$) and between spring and summer ($P < 0.01$). A weaker difference was detected between spring and winter ($P < 0.05$) (Fig. 2D).

In PW, seasonal differences were only detected for *E. coli* and TC. As in NF, *E. coli* counts were higher in fall than winter ($P < 0.05$) and spring ($P < 0.01$) (Fig. 2A). *E. coli* concentrations were also higher in summer than spring ($P = 0.01$). The highest TC counts were found in fall and were different from counts obtained in the spring and summer seasons ($P < 0.01$) (Fig. 2B). No seasonal differences in bacterial prevalence were detected in TB or RW (Fig. 2).

Differences in bacterial counts between vegetable crop-growing and nongrowing seasons and PSR compliance. Significant differences were detected in bacterial counts between the vegetable crop-growing and nongrowing season for *E. coli* and TC (Fig. 3). The divergence was most consistently observed for *E. coli*, with higher population levels retrieved in the crop-growing season than the nongrowing season in NF, PW (both $P < 0.001$), RW ($P < 0.01$), and TB ($P < 0.05$) (Fig. 3A). Total coliform counts in
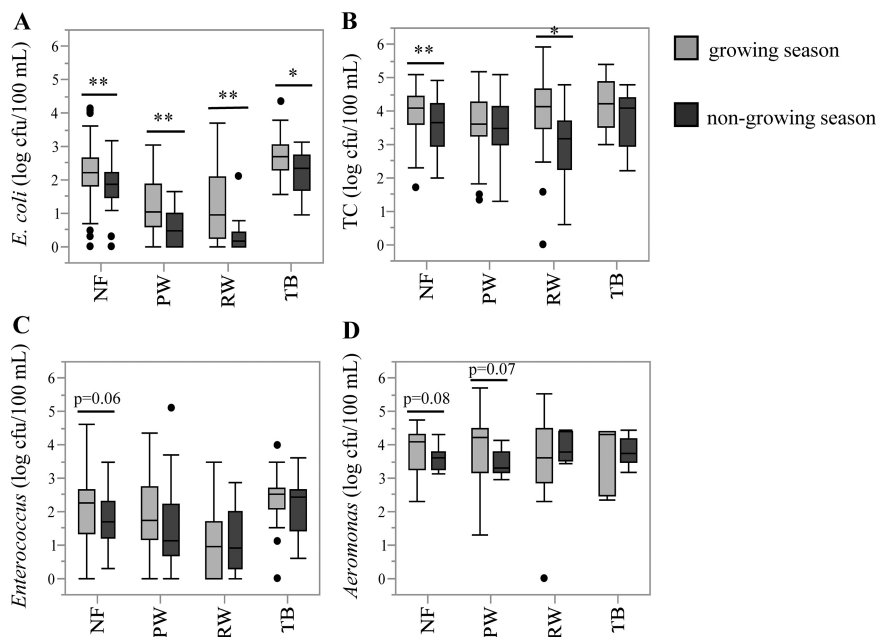
FIG 3 Bacterial counts in log CFU/100 ml for *E. coli* (A), total coliforms (TC) (B), *Enterococcus* spp. (C), and *Aeromonas* spp. (D), enumerated in different water types and categorized by vegetable crop-growing (light gray bars) and nongrowing (dark gray bars) seasons. Asterisks indicate a significant difference by Student's *t* test. **, $P \le 0.01$; *, $P \le 0.05$. The boxplots show the median and the 25th and 75th percentiles of the range. The whiskers show lower and higher observations than the 25th and 75th percentiles, respectively.

NF ($P = 0.01$) and RW ($P < 0.05$) water samples showed the same trend (Fig. 3B). No statistically significant differences in *Enterococcus* and *Aeromonas* species counts were detected by growing season; however, similar patterns were sometimes discernible. Counts were higher in the growing season for *Enterococcus* spp. in NF ($P = 0.06$) and *Aeromonas* spp. in PW ($P = 0.07$) and NF ($P = 0.08$) (Fig. 3C and D).

In the crop-growing season, 5 out of 11 sites had *E. coli* GM levels above the FSMA PSR threshold value of 2.10 log CFU/100 ml (exceeded by 0.01 to 0.61 log CFU/100 ml), whereas 8 out of 11 sites had noncompliant STV values above 2.61 log CFU/100 ml (exceeded by 0.19 to 0.89 log CFU/100 ml) (Fig. 4A). All sites exceeding the GM threshold also failed to meet the STV metric. None of the river sites (MA03, MA04, MA05, MA07, MA08, and MA09) were compliant (Fig. 4A). Only one RW (MA02) and both PW (MA10 and MA11) sites met the FSMA PSR GM and STV generic *E. coli* metric. Applying the FSMA PSR mitigation measure of letting up to 4 days elapse between irrigation and harvest to allow for bacterial die-off, stipulated at a decay rate of 0.5 log CFU/day per day, would bring all sites into compliance within 1 to 2 days (Fig. 4A). Breaking down *E. coli* counts by months within the crop-growing season reveals little variation (Fig. 4B to E). Fluctuations in *E. coli* densities throughout the growing season were detected only for NF (Fig. 4B), while counts in TB and RW remained relatively steady (Fig. 4C and E, respectively). In NF, *E. coli* counts were significantly lower at the beginning of the growing season in May (1.84 log CFU/100 ml) and in August (1.96 log CFU/100 ml) than in October (2.71 log CFU/100 ml, $P < 0.05$) (Fig. 5A). A substantial discrepancy of ~1 log CFU was also observed between May and October for PW (0.39 and 1.52 log CFU/100 ml, respectively) and RW (0.63 and 1.62 log CFU/100 ml, respectively), but these differences were not statistically supported (Fig. 4D and E).

Relationships between bacterial taxa by season and water type. *E. coli*, TC, and *Enterococcus* spp. were positively correlated with each other in all seasons and water type, with some exceptions (Fig. 5). *E. coli* counts were positively correlated with TC in winter and fall ($r = 0.51$, $P < 0.001$) and summer ($r = 0.38$, $P < 0.001$) but weakly associated in spring ($r = 0.22$, $P = 0.05$) (Fig. 5A). Likewise, all correlations between *E.*
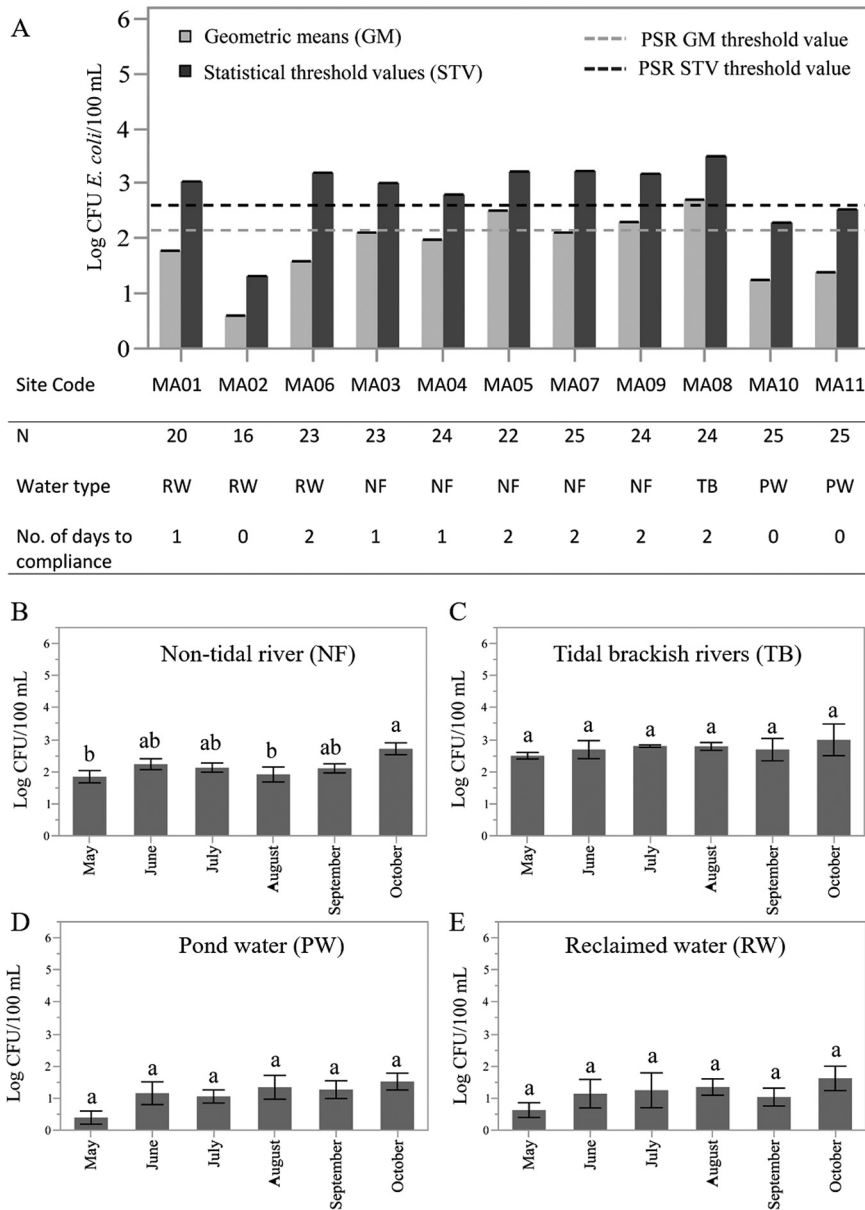
FIG 4 (A) Geometric means (GM) and statistical threshold values (STV) of *E. coli* counts in log CFU/100 ml for vegetable crop-growing season months only for 11 sites, including nontidal rivers (NF), tidal rivers (TB), pond water (PW), and reclaimed water (RW) sites, in relation to the Produce Safety Rule (PSR) standards. Light gray bars indicate GM, dark gray bars indicate STV, the light gray dashed line indicates PSR GM threshold value of 2.1 log CFU/100 ml, and the dark gray dashed line indicates PSR STV threshold value of 2.61 log CFU/100 ml. The table in the figure displays delays needed between application of irrigation water and harvest to allow for bacterial die-off, stipulated in the PSR to occur at a rate of 0.5 log CFU/day. (B to E) Month-to-month variation in average *E. coli* counts over two growing seasons in nontidal rivers (NF) (B), tidal brackish water (TB) (C), pond water (PW) (D), and reclaimed water (RW) (E). Different lowercase letters denote statistical differences at a *P* value of <0.05 in bacterial counts by month of collection, and error bars denote standard errors.

coli and *Enterococcus* levels were positive in all seasons (*P* < 0.01) but strongest in fall and summer (*r* = 0.59 and 0.51, respectively, *P* < 0.001) (Fig. 5B). Significant positive relationships were also apparent between TC and *Enterococcus* counts in all seasons (*P* < 0.01) (Fig. 5C).

The strongest correlation between *E. coli* and TC levels was detected in TB (*r* = 0.62; *P* < 0.001), followed by RW (*r* = 0.51; *P* < 0.001), NF, and PW (both *P* < 0.01) (Fig. 5D). *E. coli* counts were positively correlated with *Enterococcus* counts in all water types, with
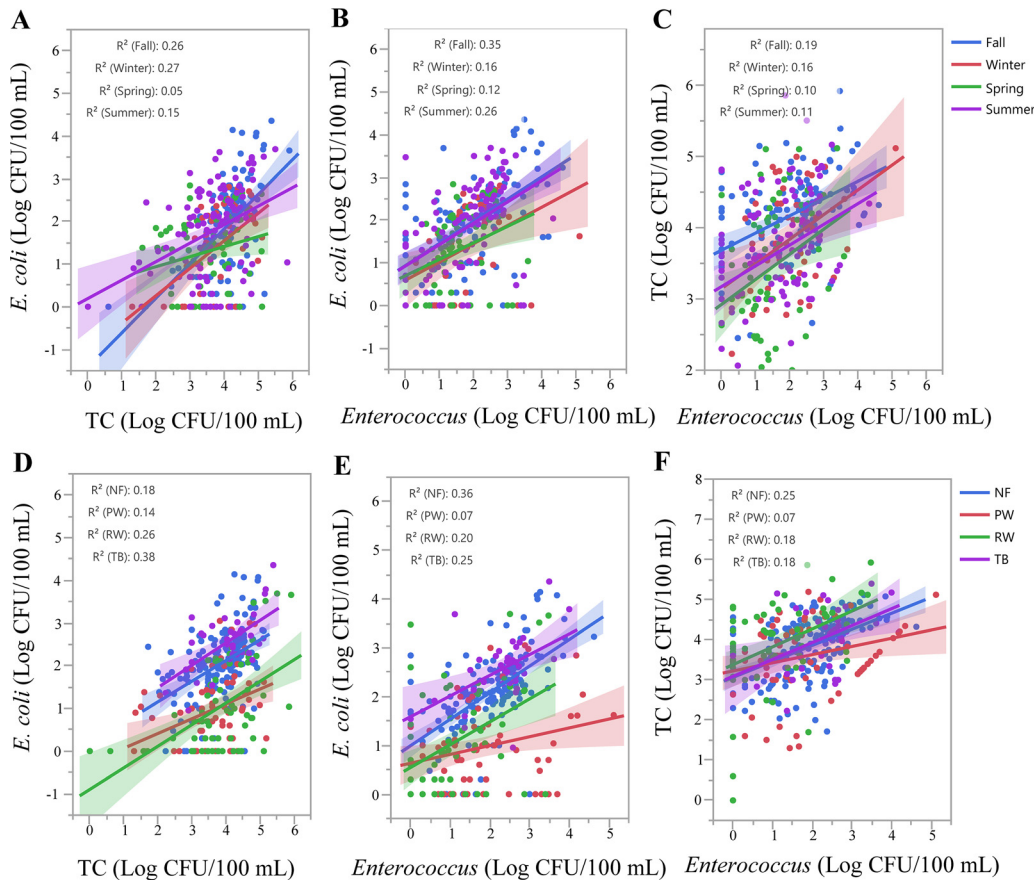
FIG 5 Relationships between bacterial indicator counts for *E. coli*, total coliforms (TC), and *Enterococcus* spp. in log CFU/100 ml by season (A to C) and water type (D to F). R^2 values indicate goodness of fit of the line using Pearson correlation analysis. NF denotes nontidal river, PW denotes pond water, RW denotes reclaimed water, and TB denotes tidal river.

NF exhibiting the strongest correlation ($r = 0.60$; $P < 0.001$), followed by TB and RW ($P < 0.001$) (Fig. 5E). Correlations between TC and *Enterococcus* counts were detected in NF ($r = 0.50$; $P < 0.001$), RW, and TB (both $r = 0.42$; $P < 0.001$) (Fig. 5F). Associations between enterococci and *E. coli*/TC in PW exhibited a weaker relationship (both $r = 0.27$; $P < 0.05$) (Fig. 5E and F).

Significant positive relationships were observed between bacterial indicators and *Aeromonas* counts but not in the winter months. Although *Aeromonas* was positively correlated with *E. coli* only in the fall ($r = 0.42$, $P < 0.01$) (Fig. 6A), a strong positive relationship was found with TC in summer ($r = 0.70$, $P < 0.001$), fall, and spring ($r = 0.5$, $P < 0.01$) (Fig. 6B). *Aeromonas* and *Enterococcus* spp. were only associated in the fall ($r = 0.50$, $P < 0.001$) and summer ($r = 0.46$, $P < 0.01$) (Fig. 6C).

A correlation was detected between *Aeromonas* and *E. coli* in RW and NF ($r = 0.44$ and 0.26, respectively, both $P < 0.05$), while no association was seen in TB and PW (Fig. 6D). On the other hand, the relationship between *Aeromonas* and TC was discernible in all water types ($r = 0.6$; $P < 0.05$) (Fig. 6E). *Enterococcus* and *Aeromonas* were most positively associated in PW and RW ($r = 0.56$ and 0.53, respectively, $P < 0.01$) and less strongly in NF ($r = 0.41$, $P < 0.001$) (Fig. 6F).

Physicochemical parameters of water and relationship with bacterial counts. Counts of the four bacterial taxa displayed significant correlations with various parameters in a water type-dependent manner (Fig. 7 and Table S2). Variations in physicochemical levels measured during the study period, grouped by water type, are compiled in Fig. S1. Temporal variation was observed in water temperature at all sites ($P \leq 0.001$), with highest mean temperatures recorded in the RW samples MA02
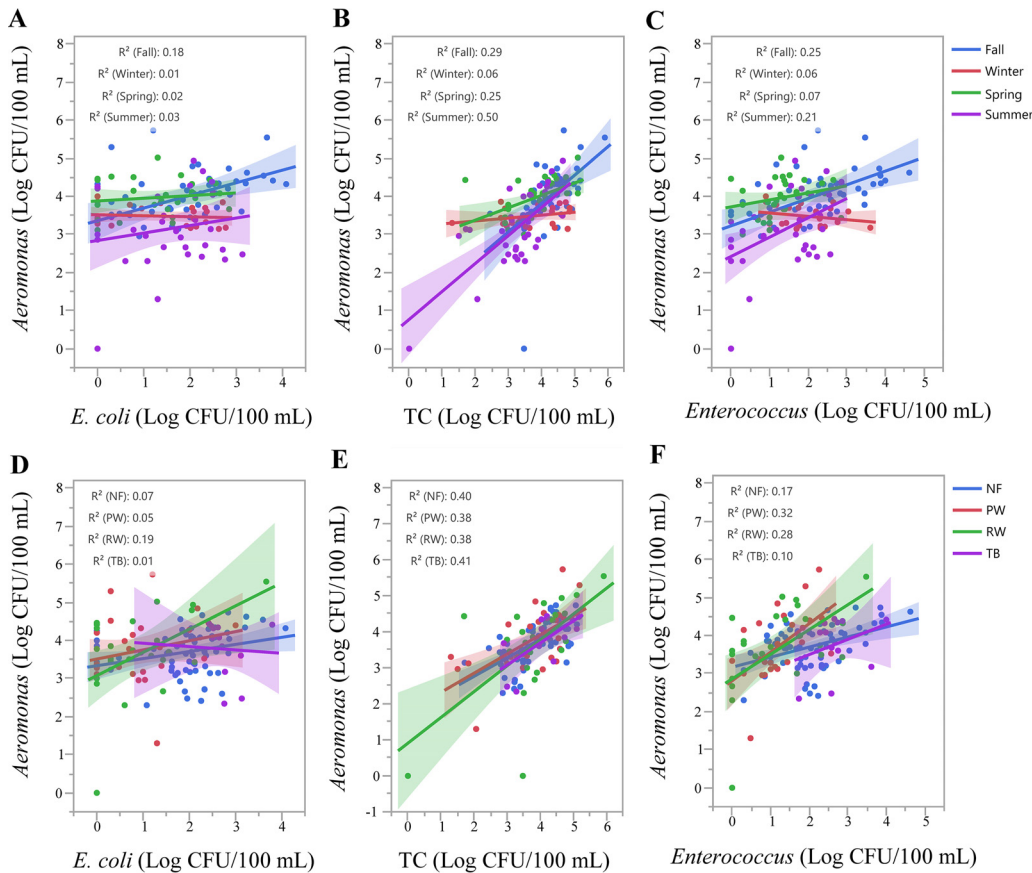
FIG 6 Relationships between *Aeromonas* species counts and bacterial indicator counts (*E. coli*, total coliforms [TC], and *Enterococcus* spp.) in log CFU/100 ml by season (A to C) and water type (D to F). R^2 values indicate goodness of fit of the line using Pearson correlation analysis and are given in each panel. NF denotes nontidal river, PW denotes pond water, RW denotes reclaimed water, and TB denotes tidal river.

(21.9°C) and MA01 (21.5°C) and PW sample MA11 (20.1°C). These means were all statistically different from the mean water temperature recorded at the NF site MA05 (14.3°C) (Fig. S1A). However, temperature was only positively correlated with *E. coli* and TC in PW and RW, respectively ($P \le 0.05$) (Fig. 7 and Table S2). The tidal river MA08, the only brackish site, showed seasonal variation in turbidity ($P < 0.01$), with highest measurements recorded from January to March. A positive relationship ($r = 0.34$, $P = 0.06$) was detected between *Enterococcus* spp. and turbidity in this water type and in NF (0.21, $P < 0.05$). MA08 (TB) had the highest nitrate (mean, 18.1 mg/liter) and
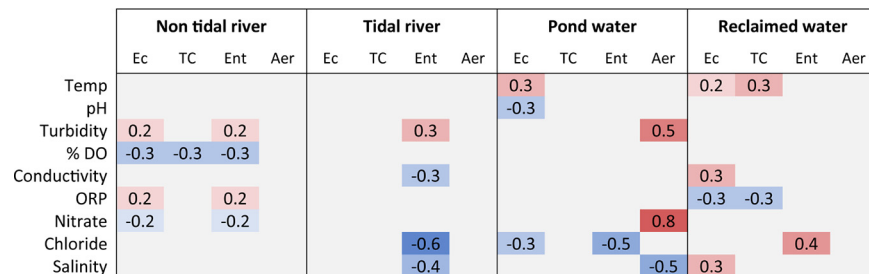

	Non tidal river				**Tidal river**				**Pond water**				**Reclaimed water**			
	Ec	TC	Ent	Aer	Ec	TC	Ent	Aer	Ec	TC	Ent	Aer	Ec	TC	Ent	Aer
Temp									0.3				0.2	0.3		
pH									-0.3							
Turbidity	0.2		0.2				0.3					0.5				
% DO	-0.3	-0.3	-0.3													
Conductivity							-0.3						0.3			
ORP	0.2		0.2										-0.3	-0.3		
Nitrate	-0.2		-0.2				-0.6		-0.3		-0.5	0.8			0.4	
Chloride									-0.3		-0.5		0.3			
Salinity							-0.4					-0.5	0.3			

FIG 7 Relationship between bacterial counts and physicochemical parameters in different water types. EC indicates *E. coli*, TC indicates total coliforms, Ent indicates *Enterococcus* spp., and Aer indicates *Aeromonas* spp. Values in each box indicate Pearson correlation coefficient (*r*) at a *P* value of <0.05. Pink indicates positive and blue indicates negative association between two variables (bacterial counts and physicochemical parameter). The intensity of the box color increases with increasing value of *r*.

chloride levels, conductivity, and salinity (mean, 7.3 PSU) (all $P < 0.001$) of all the water types. Only *Enterococcus* spp. exhibited any significant associations with these parameters in TB, where negative relationships were observed with conductivity, chloride levels, and salinity ($P < 0.05$) (Fig. 7 and Table S2). *Aeromonas* spp. were highly associated with turbidity ($r = 0.47$, $P < 0.05$) and nitrate ($r = 0.76$, $P < 0.05$) in PW and negatively associated with salinity ($r = -0.52$, $P < 0.05$). Interestingly, salinity and conductivity were positively correlated with *E. coli* in RW ($P \leq 0.01$). pH was higher in PW (7.9) and RW (7.7) than in NF (7.0) and TB (6.8) ($P < 0.001$).

Pooling data from all sites revealed a positive relationship between *E. coli* and conductivity ($r = 0.25$, $P < 0.001$), nitrate ($r = 0.14$, $P < 0.05$), chloride ($r = 0.17$, $P = 0.01$), and salinity ($r = 0.25$, $P < 0.001$) and negative relationships between *E. coli* and percent dissolved oxygen (DO) ($r = -0.23$, $P < 0.001$) and pH ($r = -0.41$, $P < 0.001$) (Table S3). A positive association between enterococci and turbidity ($r = 0.15$, $P = 0.01$) and a negative association between enterococci and pH ($r = -0.25$, $P < 0.01$) were also detected.

We also investigated the relationship between bacterial counts and precipitation 1 day and 7 days prior to sampling for each site. We noted that *E. coli* counts from several of the bacterial counts from surface water sites correlated positively with rainfall 1 day prior to sampling but not with 7 days prior to sampling (Table S4). Only the NF site MA03 (TC) and the PW sites MA10 (enterococci) and MA11 (*Aeromonas*) exhibited relationships between rainfall and other bacterial groups. For MA03 and MA10, these relationships were noted for the previous 1-day and 7-day rainfall measurements.

DISCUSSION

Increasing demands for fresh crops and concerns for food safety, coupled with variability in climate that is disrupting surface and groundwater availability, are pressing the need to identify alternative sources of agricultural water. Characterizing the microbiological quality of alternative sources of water for irrigation of fresh crops is critical for the integrity of the crop for human consumption. Understanding the spatiotemporal population dynamics of bacteria used for proposed irrigation water standards (*E. coli*) and other common bacterial indicators (*Enterococcus*) will assist growers in implementing new U.S. standards. Although the mid-Atlantic region of the United States is not considered a water insecure geographical area, subregions within the mid-Atlantic are prone to water availability concerns. Fluctuations in rainfall and periods of drought, depletion of aquifers, and coastal saltwater intrusion have raised the need to identify microbiologically safe alternative sources of irrigation water to expand this precious resource. Farmers in the mid-Atlantic are concerned about water availability and are interested in tapping into alternative sources of irrigation water (15). This study characterized the microbial quality of typical rivers, ponds, and reclaimed water sources found in the mid-Atlantic region. We found that fresh and brackish river surface water had higher bacterial indicator counts than reclaimed and pond water. We also detected seasonal dynamics specific to water type and a difference in *E. coli* counts in all water types between growing and nongrowing seasons. The strength of this study lies in the longitudinal approach taken to sample and analyze a variety of water types at a high sampling frequency. As a result, this study allows for the detection of patterns and dynamics that are otherwise indiscernible from more limited sampling scopes.

Approximately 86% of our water samples ($n = 333$) tested positive for *E. coli*. This incidence is higher than the 78% ($n = 255$) reported for irrigation water samples collected from farms along the central California coast (16) and the 59% ($n = 120$) of samples from greenhouses and open-field farms in Belgium (17). The latter study also reported that 37% of samples were positive for *Enterococcus*, which differed from the 90% prevalence we found in our study. An extensive study conducted in southern Ontario, Canada, which included 501 irrigation water samples from 17 farms, found that 81% of samples had fewer than 20 CFU *Enterococcus* per 100 ml of water (18). In this report, 83% of samples met the Canadian Council of Ministers of the Environment (CCME) *E. coli* standards for irrigation water (100 CFU/100 ml). Using these same data,

11 of 20 ponds met the British Columbia criteria that required testing the same water source at least five times over at least 30 days (5). In the United States, the FSMA PSR water standards are also based on a microbial water quality profile, i.e., the geometric mean and statistical threshold value of 20 samples collected over 2 to 4 years (PSR; 21 CFR 112). Although this sampling frequency may be deemed low in terms of determining microbial quality, collecting five to 10 samples per crop-growing season for all water sources used for fresh crops on a single farm is considered a financial and time burden by growers (19). Regardless, available data remain too scant to support any given sampling schedule applicable nationally. Considering the PSR criteria using the data from this study, when growing season data were analyzed, only 3 out of 11 sites were acceptable for irrigation without the need for any mitigation. Irrigation water supplied from these sites would require implementation of die-off times or be treated by chemical disinfection or filtration. Also noteworthy was the correlation between rainfall and *E. coli* counts, and although growers are unlikely to irrigate after heavy rainfall, they should be advised to avoid collecting water samples for microbiological testing immediately following rain to avoid skewing their MWQP.

The low compliance rates noted above also signal the importance of making better links between standards based on generic bacterial counts and actual food safety risk. Havelaar et al. found a correlation between *E. coli* levels and *Salmonella* presence in Florida ponds but noted a higher variability in *E. coli* than is accommodated for by the PSR criterion for STV (20). Our data support that finding; considering only the GM criterion, 6 of 11 sites in this study would have met the PSR standard, as opposed to 3 of 11 not meeting the combined GM and STV criterion. Moreover, two of the NF river sites (MA03 and MA07) exceeded the GM metric only slightly, at 2.11 log CFU/100 ml, but were well over the STV metric at 3.01 and 3.23 log CFU/100 ml, respectively. This means that MA03 would require 1 day and MA07 2 days of bacterial die-off delay between irrigation and harvest, despite coming close to meeting the GM standard. GM and STV calculations were based on crop-growing season dates only, as recommended by the PSR. This requirement was supported by our data, with *E. coli* counts being significantly higher in the crop-growing season than the nongrowing season in all water types tested. Finally, when bacterial counts were influenced by season, counts were higher in the fall (September to November). This was the case for *E. coli*, TC, *Enterococcus*, and *Aeromonas* in nontidal freshwater rivers. Despite these observations, we detected little variation in *E. coli* counts between June and September, months with the highest irrigation activity. Understanding the seasonal dynamics of *E. coli* levels in various water types and locations would inform the development of MWQPs in the mid-Atlantic region.

In the mid-Atlantic, the use of surface water, including pond water, declined between 2010 and 2013 from 48.5% ($n = 130$) to 23% ($n = 183$), while water testing increased from 11.5% in 2010 to 32% in 2013 (21). Likelihood of water testing was associated with farm scale, with 22% ($n = 18$) of small farms versus 54% ($n = 13$) of large farms reported testing their water in a survey of mid-Atlantic leafy greens and tomato growers (22). In a more recent study (2016 to 2018) of 263 mid-Atlantic growers, only 30% reported using surface water compared to 59% using groundwater (15). The decline in surface water use and increase in water testing indicate the growing unease around microbial safety concerns of using surface water for irrigation. However, this water type was the only one that consistently met the proposed PSR standards. Similarly, the study conducted in Florida that analyzed 6 ponds also reported that all ponds met the GM and STV criteria of the PSR, based on 90 samples per pond (20). The majority of farms in the mid-Atlantic region possess natural or man-made ponds, frequently recharged by underground springs or rainfall. Although farmer surveys along the east coast of the United States have reported use of surface water for irrigation (21, 23), none of these differentiated among pond, rivers, and other types of surface water. The discrepancy in microbial quality between pond and river water revealed in this study emphasizes the need for this distinction. Future investigations in farmer practices should explore the various types of surface water of which farmers may

be availing themselves. Lower *E. coli* levels in pond water than river water may influence the use of specific surface water sources for irrigation of crops.

The reuse of reclaimed water for irrigation purposes varies by state in the United States and ranges from agricultural use for animal feed and human food crops to use on landscapes (https://www.epa.gov/waterreuse). The level of treatment also varies by treatment plant, and state guidelines determine reuse of water based on treatment class. More extensive water reuse would benefit communities and economies, and the U.S. Environment Protection Agency (EPA) has released an Action Plan (https://www.epa.gov/waterreuse/water-reuse-action-plan) to accelerate the adoption of this resource. Knowledge of the microbial safety of reclaimed water under various treatment processes and from different regions is crucial to ensure its adequacy for use on fresh crops. In this study, reclaimed water met the GM standards but exceeded the STV criteria in the PSR. Interestingly, we still detected a difference in *E. coli* levels between crop-growing and non-crop-growing seasons in this water source. Investigating relationships between temporal fluctuations in indicator bacteria and presence of foodborne pathogens (24) will reveal important food safety parameters for the various water types.

In the present study, we also quantify and report levels of *Aeromonas* spp. for irrigation water in the United States. Although gastroenteritis caused by *Aeromonas* is not a reportable foodborne illness, this genus is an emerging foodborne pathogen and of increasing importance for food safety (25, 26). This organism has caused several outbreaks around the world (27–31). It is a well-known inhabitant of water environments and can attach to plant surfaces (32). It has also been detected on market fruit and vegetables (33, 34). Hence, the potential for *Aeromonas* transfer from irrigation water to crops exists. Our study provides novel and relevant data for this emerging foodborne pathogen on the prevalence and lack of seasonal influence across surface water types, as well as persistence in treated reclaimed water. These types of environmental data, in combination with assessment of food safety risk associated with this genus, aid in determining the potential need for inclusion of *Aeromonas* assessment in agricultural water quality management.

Much research has been conducted to investigate the relationships among indicator bacteria, enteric pathogens, and physicochemical parameters in water environments (10, 16, 18, 35–37). The membrane filtration method that we used for bacterial enumeration was not expected to recover all aerobic heterotrophic bacteria in our samples (38) or to correlate strongly with pathogens (10, 35). However, the method was appropriate to detect indicator bacteria for the purpose of assessing temporal shifts in microbiological quality and to compare water types. Moreover, both TC and *E. coli* showed strong correlations with *Enterococcus* and *Aeromonas* spp., genera that may include human pathogens. We found strong correlations between indicator bacteria in all water types tested but less predictive use of physicochemical parameters. Other related but useful measurements, such as biological oxygen demand and total dissolved solids, could have aided interpretation of our data and could be included in future studies. In some cases, we observed bias from having a skewed distribution of data, such as was the case with *E. coli* and salinity. Most samples had salinity of <0.1 PSU, but for the RW sites the average salinity was 0.35 PSU, supporting a positive correlation between *E. coli* and salinity (see Table S2 in the supplemental material). This association held up when all data were pooled to include MA08, a brackish site with average salinity of 7.3 PSU (Table S3). *Aeromonas* exhibited the strongest association with TC and the weakest with *E. coli*. This is attributed to the psychrotrophic nature of *Aeromonas*, which may align well with some members of the diverse TC group but weakly with *E. coli*, which was positively associated with temperature in PW. Considering the strong associations we detected between indicators but less so with *Aeromonas*, in combination with the lack of seasonal effect on *Aeromonas*, it appears that *Aeromonas* quantification would not be of predictive use for water quality assessment but may be important based on the risk it poses as a potential foodborne pathogen.

Our study provides a baseline for temporal microbial data for water sources that

TABLE 1 Sampling site description and sampling frequency

Site code	Water type	Description
MA01	RW	Influent is treated through activated sludge processing (sequential batch reactor), filtration, UV light, and chlorination and then stored in an open-air lagoon before land application; the spray fields are wooded with grass lanes; samples were collected from a spigot in the irrigation line of sprinkler heads
MA02	RW	Influent is treated through activated sludge processing (sequential batch reactor), filtration, UV light, and chlorination and then stored in an open-air lagoon before land application; water irrigates agronomic cropland (corn and soybeans) through center pivots; samples were collected from a spigot at the base of the center pivot
MA03	NF	Nontidal freshwater creek, tributary of the Nanticoke River that runs through Delaware and Maryland into the Chesapeake Bay; at sampling site, width was ~3 m and depth was ~1 m; wooded, agronomic cropland adjacent to the creek (~30–50 m); within 1.6 km downstream from wastewater treatment discharge facility
MA04	NF	Nontidal freshwater creek, tributary of the Choptank River that runs through Delaware and Maryland into the Chesapeake Bay; at sampling site, width was ~76 m and depth was ~0.3–0.6 m; catchment area was marshland/forested; parts of this creek could be tidal
MA05	NF	Nontidal freshwater creek, tributary of the Patuxent River along the western shore of the Chesapeake Bay; at sampling site, width was ~3–4 m and depth was ~0.2–0.5 m; catchment area was forested, with grasses on shoreline
MA06	RW	Influent is treated through grinding, activated sludge processing, and secondary clarification and then stored in an open-air lagoon; it is chlorinated prior to land application on grass; samples collected from spigot along sprinkler line, between chlorine contact chamber and field application
MA07	NF	Nontidal freshwater creek, tributary of the Nanticoke River; at sampling site width was ~10 m and depth was ~1 m; catchment area was flood plain grasses and woodland (hardwoods); within 4 km downstream from several poultry houses
MA08	TB	Tidal brackish river flowing into the Chesapeake Bay; at sampling site, width was ~15 m and depth was ~2–3 m; marsh grasses on both sides (~25–50 m wide), then pine woods. Located within 1.5–2.5 km downstream from broiler concentrated animal feeding operations (CAFOs)
MA09	NF	Nontidal freshwater creek, tributary of the Pocomoke River; at sampling site, width was ~8 m and depth ~1 m; catchment area was forested and agronomic cropland; located less than 1.5 km downstream from several poultry houses
MA10	PW	Collected from the surface of a freshwater pond with a maximum depth of ~3.4 m and a surface area of ~0.26 ha; at sampling site, width was ~20 m and depth was ~1 m; catchment area was agricultural
MA11	PW	Collected from the surface of a freshwater pond with a maximum depth of ~3 m and a surface area of ~0.40 ha; at sampling site, width was ~52 m and depth was ~0.6 m; catchment area was agricultural

could be used for irrigation. The data support the use of pond water, a widely available source of surface water on farms in the mid-Atlantic, for irrigation of crops. We found that bacterial counts in reclaimed water, a resource whose use on fresh food crops varies by state in the United States, were among the lowest we detected. This water type always met the *E. coli* GM standard in the PSR, although STV thresholds were exceeded. We detected the most seasonality and the highest microbial loads in river water, which emerged as the water type that would require the most frequent mitigation. However, based on the PSR standards alone, even this water source could be brought to compliance within 2 days of implementing the die-off delay recommended in the PSR. This report complements other objectives of CONSERVE that are investigating foodborne pathogens (24, 39) and chemical contaminants (40) in water sources. This data set represents one of the most comprehensive, longitudinal analyses of alternative irrigation water sources in the United States, and it can help identify safe irrigation water sources for the most sensitive of food crops.

MATERIALS AND METHODS

Sampling sites and sample collection. Samples were collected from 11 locations (current and prospective irrigation water sources) in the mid-Atlantic region over a period of 2 years, from September 2016 to October 2018, twice a month from May to October and once a month from November to April. Sites included 3 highly treated reclaimed wastewater effluents (RW) (MA01, MA02, and MA06), five nontidal, freshwater rivers (NF) (MA03, MA04, MA05, MA07, and MA09), one tidal brackish river (TB) (MA08), and two on-farm ponds (PW) (MA10 and MA11). Overall descriptions of the sites are given in Table 1. Since the sites included farms, the study was reviewed by the University of Maryland College Park Institutional Review Board (IRB) (project number 964795-1) and was approved as exempt due to minimal risk to farm owners.

From each site, 1 liter of water was collected into cleaned and sterile 1-liter polypropylene bottles (Thermo Fisher Scientific, Waltham, MA, USA). For surface water sites, bottles held with a sampling stick (Zenport Industries, Portland, OR, USA) were inverted, submerged 15 to 30 cm below the water surface, and turned sideways until full. For reclaimed water sites, water was collected from spigots close to field

release sites (e.g., sprinklers used for groundwater recharge or irrigation of animal feed crops). Water was allowed to run for 1 min prior to collection. Immediately after sample collection, 1 ml of 10% sodium thiosulfate (Alfa Aesar, Heysham, England) solution was added to reclaimed water samples to quench residual hypochlorite added as part of the water reclamation process. For all water types, bottles were immediately transferred to coolers containing ice packs for transport to the laboratory. Samples were processed within 12 h of collection. Physicochemical parameters, i.e., water temperature, dissolve oxygen (DO), conductivity, turbidity, nitrate, chloride, salinity, pH, and oxidation reduction potential (ORP), were measured at each sampling site right after the collection of water samples with an EXO2 or ProDSS mulitparameter water quality sonde/meter (YSI, Yellow Springs, OH, USA). Precipitation measurements 24 h and 7 days before sampling were obtained from a weather forecast website (https://www .wunderground.com/history/), using appropriate locations for each sampling site.

Sample processing and bacterial enumeration. Quantification of bacterial taxa was conducted using standard membrane filtration methods according to EPA method 1604 for *E. coli* and TC (41) and EPA method 1600 for *Enterococcus* (42) on all samples collected. EPA method 1605 was used for *Aeromonas* spp. (43) for a subset of samples collected between September 2016 and September 2017. Serial volumes of each sample (0.1, 1, 10, and 100 ml) were filtered through 0.45-μm, 47-mm cellulose ester membrane filters (Pall Corporation, Ann Arbor, MI, USA). Smaller volumes were made up to 10 ml with sterile water before filtration. Filters were placed aseptically onto ml agar plates (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) for quantification of *E. coli*/TC and on mEI agar plates (BD) for *Enterococcus*. For *Aeromonas* spp., filters were transferred to ampicillin dextrin agar (ADA) (Hardy Diagnostics, Santa Maria, CA, USA) supplemented with ampicillin, sodium salt (10 mg/10 ml; Fisher Scientific, Hampton, NH, USA), and vancomycin (V) (1 mg/1 ml) (AMRESCO, Solon, OH, USA). All ml and ADA-V plates were incubated for 24 h at 37°C, and mEI plates were incubated at 41°C for 48 h for improved visualization and differentiation between enterococci and nonenterococci. Blue colonies on ml plates were counted and recorded as *E. coli*. The fluorescent colony count under UV light (365 nm) was added to the nonfluorescent blue colony count to obtain a TC count. All colonies ≥0.5 mm in diameter (regardless of color) with a blue halo on mEI were recorded as enterococcus colonies. Yellow colonies on ADA-V plates were counted and recorded as *Aeromonas* spp. The limit of detection for *E. coli*, TC, *Enterococcus* spp., and *Aeromonas* was 1 CFU/100 ml.

Calculation of GM and STV. Calculations of geometric mean (GM) and statistical threshold value (STV) were performed on all data points collected during May to October, as described by the Produce Safety Alliance (PSA) (44). For GM, *E. coli* counts per 100 ml of water from the same site were log transformed and then averaged. The STV was calculated by using the formula log(STV) = average (log values) + 1.282 × SD (log values), where average (log values) are the GM values for each site, SD is the standard deviation, and 1.282 is a constant to calculate the STV (90th percentile of the data set). STVs were calculated for each sampling site separately.

Data management and statistical analysis. For statistical analyses of the effect of water type and seasonality on bacterial levels, data were pooled by water type (5 NF, 1 TB, 2 PW, and 3 RW sites) and the year was divided into four groups, defined as spring from 01 March to 30 May, summer from 01 June to 31 August, fall from 01 September to 30 November, and winter from 01 December to 28 February. The whole year was also categorized into vegetable crop-growing (May to October) and nongrowing (November to April) seasons, based on the region's recommended frost dates (https://extension.umd .edu/hgic/topics/when-plant-vegetables-maryland). Vegetable refers specifically to raw agricultural commodities. Although certain vegetable crops can be grown outside these periods, they are unlikely to be irrigated regularly during due to lower temperatures and high moisture levels. A mixed-effect model was used to assess the effect of season or water type on each bacterial taxon. The repeated measurements effects were controlled by using the random effect of site in the model, while season and water type were fixed effects. Tukey's honestly significant difference test with $\alpha = 0.05$ was employed to assess differences within groups. Physicochemical parameters were analyzed for temporal variation by month for each site using analysis of variance. Pearson correlation analysis was applied to explore correlations among bacterial indicators and between indicators and water physicochemical parameters ($\alpha = 0.05$). Linear regression analysis was used to assess the relationship between bacterial counts and rainfall 1 day and 7 days before sampling by site. Statistical analyses were conducted in JMP Pro 14.1 (Cary, NC, USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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