# UC San Diego UC San Diego Previously Published Works

### Title

Enteric Viruses and Pepper Mild Mottle Virus Show Significant Correlation in Select Mid-Atlantic Agricultural Waters

**Permalink** https://escholarship.org/uc/item/6730q2z9

**Journal** Applied and Environmental Microbiology, 87(13)

**ISSN** 0099-2240

### Authors

Anderson-Coughlin, Brienna L Craighead, Shani Kelly, Alyssa <u>et al.</u>

Publication Date 2021-06-11

## DOI

10.1128/aem.00211-21

Peer reviewed





# **Enteric Viruses and Pepper Mild Mottle Virus Show Significant Correlation in Select Mid-Atlantic Agricultural Waters**

Brienna L. Anderson-Coughlin,<sup>a</sup> Shani Craighead,<sup>a</sup> Alyssa Kelly,<sup>a</sup> Samantha Gartley,<sup>a</sup> Adam Vanore,<sup>a</sup> Gordon Johnson,<sup>b</sup> Chengsheng Jiang,<sup>c</sup> DJoseph Haymaker,<sup>d</sup> Chanelle White,<sup>d</sup> Derek Foust,<sup>d</sup> Rico Duncan,<sup>d</sup> Cheryl East,<sup>e</sup> Eric T. Handy,<sup>e</sup> Rhodel Bradshaw,<sup>e</sup> Rianna Murray,<sup>c</sup> Prachi Kulkarni,<sup>c</sup> Mary Theresa Callahan,<sup>f</sup> Sultana Solaiman,<sup>f</sup> Walter Betancourt,<sup>g</sup> Charles Gerba,<sup>9</sup> Sarah Allard,<sup>h</sup> <sup>(D)</sup> Salina Parveen,<sup>d</sup> Fawzy Hashem,<sup>d</sup> <sup>(D)</sup> Shirley A. Micallef,<sup>f</sup> Amir Sapkota,<sup>c</sup> Amy R. Sapkota,<sup>c</sup> Manan Sharma,<sup>e</sup> 
Kalmia E. Kniel<sup>a</sup>

<sup>a</sup>University of Delaware, Department of Animal and Food Sciences, Newark, Delaware, USA

<sup>b</sup>University of Delaware, Department of Plant and Soil Sciences, Newark, Delaware, USA

cMaryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland, USA

<sup>d</sup>University of Maryland Eastern Shore, Department of Agriculture and Resource Sciences, Princess Anne, Maryland, USA

eU.S. Department of Agriculture, Agricultural Research Service, Northeast Area, Beltsville Agricultural Research Center, Environmental Microbiology and Food Safety Laboratory, Beltsville, Maryland, USA

fUniversity of Maryland, Department of Plant Science and Landscape Architecture, College Park, Maryland, USA

9University of Arizona, Department of Animal and Comparative Biomedical Sciences, Tucson, Arizona, USA

<sup>h</sup>University of California, San Diego, Department of Pediatrics, La Jolla, California, USA

**ABSTRACT** Enteric viruses (EVs) are the largest contributors to foodborne illnesses and outbreaks globally. Their ability to persist in the environment, coupled with the challenges experienced in environmental monitoring, creates a critical aperture through which agricultural crops may become contaminated. This study involved a 17-month investigation of select human EVs and viral indicators in nontraditional irrigation water sources (surface and reclaimed waters) in the Mid-Atlantic region of the United States. Real-time quantitative PCR was used for detection of Aichi virus, hepatitis A virus, and norovirus genotypes I and II (GI and GII, respectively). Pepper mild mottle virus (PMMoV), a common viral indicator of human fecal contamination, was also evaluated, along with atmospheric (air and water temperature, cloud cover, and precipitation 24 h, 7 days, and 14 days prior to sample collection) and physicochemical (dissolved oxygen, pH, salinity, and turbidity) data, to determine whether there were any associations between EVs and measured parameters. EVs were detected more frequently in reclaimed waters (32% [n = 22]) than in surface waters (4% [n = 49]), similar to PMMoV detection frequency in surface (33% [n=42]) and reclaimed (67% [n=21]) waters. Our data show a significant correlation between EV and PMMoV ( $R^2 = 0.628$ , P < 0.05) detection levels in reclaimed water samples but not in surface water samples ( $R^2 = 0.476$ , P = 0.78). Water salinity significantly affected the detection of both EVs and PMMoV (P < 0.05), as demonstrated by logistic regression analyses. These results provide relevant insights into the extent and degree of association between human (pathogenic) EVs and water quality data in Mid-Atlantic surface and reclaimed waters, as potential sources for agricultural irrigation.

**IMPORTANCE** Microbiological analysis of agricultural waters is fundamental to ensure microbial food safety. The highly variable nature of nontraditional sources of irrigation water makes them particularly difficult to test for the presence of viruses. Multiple characteristics influence viral persistence in a water source, as well as affecting the recovery and detection methods that are employed. Testing for a suite of viruses in water samples is often too costly and labor-intensive, making identification of suitable indicators for viral pathogen contamination necessary. The results from this study address two critical data gaps, namely, EV prevalence in surface and reclaimed waters of the Mid-Atlantic region of the United States and subsequent evaluation of Citation Anderson-Coughlin BL, Craighead S, Kelly A, Gartley S, Vanore A, Johnson G, Jiang C, Haymaker J, White C, Foust D, Duncan R, East C, Handy ET, Bradshaw R, Murray R, Kulkarni P, Callahan MT, Solaiman S, Betancourt W, Gerba C, Allard S, Parveen S, Hashem F, Micallef SA, Sapkota A, Sapkota AR, Sharma M, Kniel KE. 2021. Enteric viruses and pepper mild mottle virus show significant correlation in select Mid-Atlantic agricultural waters. Appl Environ Microbiol 87:e00211-21. https://doi.org/10 .1128/AEM.00211-21.

Editor Edward G. Dudley, The Pennsylvania State University

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Kalmia E. Kniel, kniel@udel.edu.

Received 28 January 2021 Accepted 15 April 2021

Accepted manuscript posted online 23 April 2021 Published 11 June 2021

physicochemical and atmospheric parameters used to inform the potential for the use of indicators of viral contamination.

**KEYWORDS** norovirus, hepatitis A virus, Aichi virus, pepper mild mottle virus, surface water, reclaimed water

roundwater has traditionally been used to irrigate crops but, as populations lacksim increase and water resources become limited, alternative water sources are needed. Any water source other than groundwater, when used for crop irrigation, is referred to as a nontraditional water source, and sources can include runoff, surface, recycled, and reclaimed waste waters (1). Surface water is currently used for agricultural irrigation in some environments; however, surface water is more likely to be contaminated with zoonotic microorganisms with the potential to transmit disease (2). The Federal-Provincial-Territorial Committee on Drinking Water reviewed 22 studies in which enteric viruses (EVs) were detected in surface waters and groundwaters of North America; EVs were frequently detected in surface waters, and intact confined aquifers guarded groundwater against contamination; however, EVs could enter through infrastructure failures (3). Reclaimed or recycled water is also used for irrigation, to reduce the environmental impact of agriculture and to repurpose water derived from municipalities, rivers, lakes, and other available sources. According to the Department of Water Resources of the State of California, water reclamation for use in crop irrigation began over 100 years ago, in the late 1800s, and 46% of all reclaimed water produced in the state is used for crop irrigation (4).

The 1965 Water Quality Act propelled water research in the United States. In 1976, the U.S. Environmental Protection Agency (EPA) released a report on the quality criteria for water (5), which identified *Escherichia coli* as a water quality and fecal pollution indicator and recommended that fecal coliform levels in recreational waters should not exceed 1 log CFU/100 ml. A Food Safety Modernization Act rule was released almost 40 years later by the U.S. Food and Drug Administration (FDA) and provided regulations regarding the use of water applied to growing produce (6). *E. coli* remains the primary indicator of water quality, and the maximum allowable level for agricultural water is set at 126 CFU/100 ml for most commodities. The rule, as stated currently, also requires increased testing for nontraditional water sources such as surface waters, which are more susceptible to contamination (7). While *E. coli* continues to be used as a microbiological indicator for water today, many studies have identified the deficiencies in this practice, and it does not accurately assess the potential for viruses to be in the water (8–11).

There are documented risks in the use of nontraditional water sources for irrigation of raw agricultural commodities, including increased microbial levels and complications experienced during water quality testing (12–15). Human feces have been shown to contain up to 12 log EV particles/g (16), and raw sewage has been shown to contain an average of 4.6 log genomic copies of norovirus (NoV)/liter (17). Given the potentially high levels of virus particles in sewage, even a small sewage contamination event in a water supply used for crop irrigation could result in subsequent contamination events and gastrointestinal illnesses long after the initial incident.

EVs, i.e., viruses affecting the gastrointestinal tract, are the most common cause of acute gastroenteritis globally. Foodborne illnesses globally exceed 600 million cases annually, and approximately 120 million cases are attributed to NoV alone (18). In the United States, more than one-half of foodborne illnesses are estimated to be caused by NoV (19). NoVs are nonenveloped, RNA viruses of the *Caliciviridae* family, which is divided into six genogroups and further divided into over 30 genotypes; genogroups I, II, and IV are infectious to humans, with the NoV GII.4 strain being the most prevalent cause of NoV outbreaks (20). Another notable EV is hepatitis A virus (HAV), a nonenveloped, RNA virus of the *Picornaviridae* family. HAV has seven genogroups, of which genogroups I, II, III, and VII affect humans and the rest affect simians (21). In 1995, a lifelong

immunity HAV vaccine was developed; however, HAV is still the second largest viral contributor to foodborne illness globally. Although the World Health Organization (WHO) initiated the Global Health Sector Strategy on Viral Hepatitis in 2016 and the HAV vaccine is considered a standard childhood vaccination in 16 countries, including the United States, there were still an estimated 6,700 new cases in the United States in 2017 (https://www.cdc.gov/hepatitis/hav/havfaq.htm#general). Aichi virus (AiV), like HAV, is in the *Picornaviridae* family, but it is a member of the *Kobuvirus* genus. AiV A targets human gastrointestinal tracts and can cause vomiting, nausea, and diarrhea (22). AiV seroprevalence and transmission were reviewed by Reuter et al. (23), who determined that up to 80 to 95% of adults were seropositive by 40 years of age.

The use of *E. coli* as an indicator may not sufficiently represent the viral communities of a water sample due to the vast differences in structure, persistence, and life cycles among the organisms. Viruses, unlike bacteria, cannot propagate in a water source, but they have been shown to remain detectable for more than 1 month in river water and after sewage treatment (24–26). Pepper mild mottle virus (PMMoV), an RNA plant virus, has been suggested as an indicator of pathogenic viruses due to its high prevalence in human waste, compared to environmental and animal sources (27). PMMoV has been detected in influent and effluent of a wastewater treatment plant at 6 and 5 log PFU/liter, respectively (28), corroborating the prevalence and persistence of the virus.

The fluctuation of atmospheric and physicochemical conditions contributes to the highly variable nature of environmental waters through a series of reactions. Atmospheric or meteorological conditions include temperature, humidity, cloud cover, barometric pressure, and other parameters. Physicochemical parameters are water properties influenced by both physical and chemical components and include dissolved oxygen levels, turbidity, salinity, pH, and more. Atmospheric and physicochemical parameters during and prior to sample collection create a unique set of water characteristics that can have an influence on the methods used for virus recovery and detection. Increased precipitation has been shown to negatively affect the ability to recover viruses from environmental waters due to the surge of suspended solids and decreases in concentration efficacy (29). Elevated turbidity has been positively correlated with detection of NoV genotype I (GI) (30), but the combination of increased virus levels and decreased recovery efficacy can lead to underestimates of viral prevalence. Temperature, relative humidity, and overall seasonality have been associated with viral detection, correlating both positively and negatively throughout previous studies. Theories about these contradictory results include the persistence of the viruses, the seasonality of gastrointestinal illness, and variations in the detection assays implemented (31-33).

Currently, there are significant data gaps and contradictory results in the literature regarding the potential correlations of viral presence and atmospheric or physicochemical parameters of water sources. The evaluation of these characteristics separately does not provide a complete image of the risks associated with the use of nontraditional water sources. An investigation of a multitude of attributes will provide the data necessary to develop a method to mitigate viral threats in these highly variable water sources when they are used for crop irrigation.

The objectives of this study were to perform surveillance of foodborne viruses in nontraditional water sources used for crop irrigation in the Mid-Atlantic region of the United States and to evaluate the effects of physicochemical and atmospheric parameters of water on the detection of pathogenic and indicator viruses. The combination of these data collected from a variety of locations and water types, repeatedly sampled over a prolonged time, and robust statistical analysis provides a unique insight into nontraditional water samples used for crop irrigation in the Mid-Atlantic region of the United States.

#### RESULTS

**EV and indicator virus detection.** EVs were detected in 4.1% of surface water samples (n = 49) and 31.8% of reclaimed water samples (n = 22), with an overall detection

<b>TABLE 1</b> Percentage of water samples in which enteric viruses ( $n = 71$ ), indicator virus
(n = 63), or both enteric and indicator viruses $(n = 63)$ were detected

	% of samples			
Viral target	Surface	Reclaimed	Overall	
Enteric virus	4.1 ( <i>n</i> = 49)	31.8 ( <i>n</i> = 22)	12.7	
Indicator virus	33.3 ( <i>n</i> = 42)	66.7 ( <i>n</i> = 21)	44.4	
Enteric and indicator viruses	0.0 ( <i>n</i> = 42)	28.6 ( <i>n</i> = 21)	9.5	

rate of 12.7% (Table 1). In surface water, HAV and NoV GII were detected in one and two occasions, respectively. In reclaimed water, AiV (3/22), HAV (2/22), and NoV GII (2/22) were detected more frequently. PMMoV was detected in 33.3% of surface water samples (n=42) and 66.7% of reclaimed water samples (n=21), with an overall detection rate of 44.4%. PMMoV detection was significantly more frequent in reclaimed water samples than in surface water samples (P < 0.05). There were fewer surface water samples in which EVs and indicator virus were both detected; however, 28.6% of reclaimed water samples were positive for both EVs and indicator virus. There was a significant correlation ( $R^2$  = 0.628, P < 0.05) in reclaimed water samples but not in surface water samples ( $R^2$  = 0.476, P = 0.78).

Atmospheric and physicochemical parameter analyses. Physicochemical data (dissolved oxygen levels [percentage], pH, salinity [practical salinity units [PSU]], and turbidity [formazin nephelometric units [FNU]]) according to water type (surface water and reclaimed water) are displayed in Table 2. The levels of dissolved oxygen were significantly higher (P < 0.05) in surface water (104.69  $\pm$  39.31%) than in reclaimed water  $(64.48 \pm 33.48\%)$ ; levels ranged from 60.10% to 254.55% in surface water and from 25.50% to 119.50% in reclaimed water. The pH values of surface water (7.43  $\pm$  0.90) and reclaimed water (7.46  $\pm$  0.86) were not significantly different (P = 0.93); the pH of surface and reclaimed water samples ranged from 6.27 to 9.81 and from 6.36 to 9.07, respectively. The turbidity of reclaimed water samples (49.81  $\pm$  67.91 FNU) was significantly higher (P < 0.05) than the turbidity of surface water samples (9.68 ± 16.61 FNU). Reclaimed water turbidity ranged from 2.53 FNU to 268.60 FNU, while surface water turbidity ranged from -4.10 FNU to 118.15 FNU. Surface water salinity levels (0.10  $\pm$  0.07 PSU) were significantly (P < 0.05) lower than those of reclaimed water samples (10.96  $\pm$  11.60 PSU). Reclaimed water salinity ranged from 0.05 PSU to 27.31 PSU, and surface water salinity ranged from 0.03 PSU to 0.44 PSU.

**TABLE 2** Calculated means and standard deviations of physicochemical parameters including dissolved oxygen, pH, salinity, and turbidity

	Mean $\pm$ SD <sup>a</sup>			
Water type and season	DO (%)	рН	Salinity (PSU)	Turbidity (FNU)
Reclaimed ( $n = 22$ )				
Spring	$91.33 \pm 15.67$	$\textbf{7.70} \pm \textbf{0.29}$	$18.54 \pm 7.02$	$\textbf{8.01} \pm \textbf{4.80}$
Summer	$54.34 \pm 32.27$	$\textbf{7.42} \pm \textbf{1.02}$	$8.11 \pm 12.38$	$\textbf{57.64} \pm \textbf{53.96}$
Autumn	$\textbf{73.03} \pm \textbf{36.36}$	$\textbf{7.41} \pm \textbf{0.74}$	$13.33 \pm 10.75$	$53.77 \pm 105.57$
Winter <sup>b</sup>	—	_	—	—
Overall	$64.48 \pm 33.48$	$\textbf{7.46} \pm \textbf{0.86}$	$10.96 \pm 11.60$	$49.81\pm67.91$
Surface ( <i>n</i> = 49)				
Spring	$107.12 \pm 37.84$	$\textbf{7.31} \pm \textbf{0.74}$	$0.07\pm0.01~\text{A}$	$8.37\pm5.31~\text{AB}$
Summer	$122.28 \pm 56.73$	$\textbf{7.41} \pm \textbf{0.94}$	$0.09\pm0.05~\text{AB}$	$6.24\pm4.05~\text{B}$
Autumn	$86.92 \pm 15.15$	$\textbf{7.53} \pm \textbf{0.82}$	$0.15\pm0.11~\text{B}$	$4.75\pm1.71~\text{B}$
Winter	$99.51 \pm 21.28$	$\textbf{7.52} \pm \textbf{1.24}$	$0.06\pm0.02~\text{A}$	$24.04\pm35.88~\text{A}$
Overall	$104.69\pm39.31$	$\textbf{7.43} \pm \textbf{0.90}$	$\textbf{0.10} \pm \textbf{0.07}$	$9.68 \pm 16.61$

<sup>a</sup>The connecting-letter report was generated by Tukey-Kramer honestly significant difference (HSD) analysis, and the assignment of the same letter represents no significant difference between groups ( $P \ge 0.05$ ); data in columns without connecting letters showed no significant seasonal difference. DO, dissolved oxygen. <sup>b</sup>Reclaimed samples were not collected during the winter season. —, no data available.



**FIG 1** Seasonal air and water temperatures recorded at the time of sample collection according to season, i.e., spring (n = 16) (light gray hatched bars), summer (n = 26) (light gray bars), autumn (n = 18) (dark gray hatched bars), and winter (n = 9) (dark gray bars). Each set of whiskers represents the range of data in that group, and the shaded region represents the interquartile range. Letters above each box are from the connecting-letter report generated by Tukey-Kramer honestly significant difference (HSD) analysis. Boxes with the same letter are not significantly different, and different letters indicate statistically significant differences (P < 0.05) within air or water temperatures.

There were no significant differences in any physicochemical parameters of samples collected during the growing seasons of May through September in year 1 versus year 2. However, there was significant (P < 0.05) variability observed seasonally in the salinity and turbidity of surface water. Salinity levels of surface water were significantly (P < 0.05) lower in the spring and winter ( $0.07 \pm 0.01$  PSU and  $0.06 \pm 0.02$  PSU, respectively) than in the autumn ( $0.15 \pm 0.11$  PSU); salinity levels of samples collected during the summer were not significantly different from those of samples collected in any season ( $0.09 \pm 0.05$  PSU). Turbidity was significantly greater (P < 0.05) in the winter ( $24.04 \pm 35.88$  FNU) than in the summer ( $6.24 \pm 4.05$  FNU) and autumn ( $4.75 \pm 1.71$  FNU), likely attributable to winter-related weather events; the turbidity of samples collected in other seasons ( $8.37 \pm 5.31$  FNU). There was no significant variability observed in the physicochemical parameters of reclaimed water samples when analyzed by season.

The atmospheric parameters evaluated included cloud cover (score of 0 to 4), air and water temperatures (degrees Celsius), and cumulative precipitation (centimeters) 24 h, 7 days, and 14 days prior to sample collection. Cloud cover was measured on an ordinal five-point scale of 0 to 4, representing the following percentages of cloud cover; 1, 0%; 2, 0 to <50%; 3, 50%; 4, 50 to <100%; 5, 100% (https://www.wpc.ncep .noaa.gov/html/stationplot\_printer.html). There was no significant difference in atmospheric parameters during the growing seasons of May through September in year 1 versus year 2. Air and water temperatures changed seasonally as expected in the U.S. Mid-Atlantic region, with temperatures rising from spring to summer and cooling through autumn to winter. There was no significant difference between air and water temperatures in spring (P = 0.45), summer (P = 0.42), autumn (P = 0.44), or winter (P = 0.68). Air and water temperatures of samples collected during each season are shown in Fig. 1 in box-and-whisker plots, along with a connecting-letter report representing statistically significant differences by season. Precipitation 24 h and 7 days prior to sample collection did not have significant fluctuations seasonally, although patterns were observed and significant changes might be observed with a larger data set. There was significantly greater (P < 0.05) precipitation 14 days prior to sample collection in the summer  $(7.50 \pm 5.45 \text{ cm})$ , compared to the autumn  $(2.45 \pm 2.14 \text{ cm})$ . Samples collected in the winter  $(5.60 \pm 5.69 \text{ cm})$  and spring  $(4.91 \pm 5.75 \text{ cm})$  were not significantly different from those collected in the autumn or summer. Precipitation data are displayed in Fig. 2 with a connecting-letter report representing statistically significant differences by season.

The relationships between virus detection and physicochemical and atmospheric parameters, presented as standardized coefficients, are presented in Fig. 3 and 4.



**FIG 2** Average precipitation 24 h, 7 days, and 14 days prior to sample collection according to season, i.e., spring (n = 16) (light gray hatched bars), summer (n = 26) (light gray bars), autumn (n = 17) (dark gray hatched bars), and winter (n = 9) (dark gray bars). Letters above each bar are from a connecting-letter report generated by Tukey-Kramer honestly significant difference (HSD) analysis. Bars with different letters indicate statistically significant (P < 0.05) differences in seasonal precipitation for each of the 24-h, 7-day, or 14-day periods prior to sample collection between seasons.

Logistic regression (LR) chi-square values for physicochemical and atmospheric data are displayed in Table 3. Of the physicochemical parameters analyzed, salinity had the greatest positive standardized coefficient for both EV and indicator virus detection (0.51  $\pm$  0.22 and 0.53  $\pm$  0.24, respectively). pH coefficients were negligible for both the EV (0.09  $\pm$  0.33) and indicator virus (0.00  $\pm$  0.20) models. Turbidity and dissolved oxygen coefficients were negative for both models, although values were greater for EV detection  $(-0.93 \pm 0.84$  and  $-0.80 \pm 0.49)$  than for indicator virus detection  $(-0.11 \pm 0.17 \text{ and } -0.23 \pm 0.22)$ . Of the atmospheric parameters analyzed, the standardized coefficients varied for EV and indicator virus detection models. While coefficients for water temperature (1.28  $\pm$  0.66) and air temperature ( $-1.06 \pm 0.66$ ) for EV detection were both large, water temperature ( $0.19 \pm 0.38$ ) and air temperature  $(-0.11 \pm 0.39)$  for PMMoV detection were much smaller. Cloud cover (0.26  $\pm$  0.28) and precipitation 24 h (0.23  $\pm$  0.32), 7 days ( $-0.28 \pm$  0.65), and 14 days ( $-0.13 \pm$  0.54) prior to sampling were all moderate for EV detection. However, coefficients for the PMMoV detection model ranged from cloud cover (0.00  $\pm$  0.17) to precipitation 24 h  $(-0.29 \pm 0.25)$ , 7 days  $(0.80 \pm 0.44)$ , and 14 days  $(-0.82 \pm 0.40)$  prior to sampling. Through the LR chi-square analysis, salinity was found to significantly impact (P < 0.05) the detection of both EVs and indicator virus, while dissolved oxygen, pH, and turbidity effects were not significant for either. The LR chi-square analysis for atmospheric parameters returned significant results (P < 0.05) for water tempera-



**FIG 3** Standardized coefficients and standard errors of the physicochemical parameters for enteric virus (dark gray bars) and indicator virus (light gray bars) detection binary logistic regression models. Standardized coefficients were calculated to compare parameters with different units for their impact on the detection of enteric and indicator viruses.



**FIG 4** Standardized coefficients and standard errors of the atmospheric parameters for enteric virus (dark gray bars) and indicator virus (light gray bars) detection binary logistic regression models. Standardized coefficients were calculated to compare parameters with different units for their impact on the detection of enteric and indicator viruses.

ture with EV detection and for precipitation 14 days prior to sample collection with indicator virus detection.

The principal-component analysis loading plots generated (Fig. 5) provide a visual representation of relationships between variables according to surface and reclaimed water types. Air and water temperatures were approximal in both model plots, as were pH and dissolved oxygen. Precipitation (24 h, 7 days, and 14 days prior to collection), cloud cover, and turbidity were closely and similarly oriented in both plots. The most notable difference between the surface and reclaimed water models is that turbidity and salinity are oriented similarly in the surface water plot and are oriented in opposing directions in the reclaimed water plot. In both surface and reclaimed water Pearson's product moment correlated (P < 0.05) and precipitation 24 h, 7 days, and 14 days prior to sample collection were significantly correlated (P < 0.05). Dissolved oxygen and pH were also significantly correlated for both water types (P < 0.05). Additionally, dissolved oxygen, pH, salinity, and turbidity were all significantly correlated in reclaimed water (P < 0.05). Turbidity was also correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitation 24 h and 7 days prior to sample collection and cloud cover was correlated with precipitating 24

TABLE 3 Binary logistic regression analysis for EV and indicator virus detection models<sup>a</sup>

	LR chi-square <sup>a</sup>		
Parameter	EVs ( <i>n</i> = 71)	PMMoV ( <i>n</i> = 63)	
Physicochemical			
Dissolved oxygen	3.26 (P = 0.07)	1.24 (P = 0.27)	
рН	0.07 ( <i>P</i> = 0.79)	0.00 (P = 0.99)	
Salinity	6.91 ( <i>P</i> < 0.05)	7.81 ( <i>P</i> < 0.05)	
Turbidity	2.54 ( <i>P</i> = 0.11)	0.48 (P = 0.49)	
Atmospheric			
Cloud cover	0.91 (P=0.34)	0.00 ( <i>P</i> = 0.98)	
Precipitation			
24 h	0.53 (P = 0.47)	1.52 (P = 0.22)	
7 days	0.20 ( <i>P</i> = 0.66)	3.76 ( <i>P</i> = 0.05)	
14 days	0.05 (P = 0.81)	5.17 ( <i>P</i> < 0.05)	
Temperature			
Air	3.23 (P=0.07)	0.08 (P = 0.78)	
Water	5.48 ( <i>P</i> < 0.05)	0.25 ( <i>P</i> = 0.62)	

<sup>a</sup>Physicochemical and atmospheric parameters were evaluated in separate models, and the logistic regression chi-square coefficients and associated P values are presented. Significant coefficients and P values (P < 0.05) are in bold. LR, logistic regression; EVs, enteric viruses; PMMoV, indicator virus.



**FIG 5** Principal-component analysis loading plots, including all atmospheric and physicochemical parameters analyzed, for reclaimed (A) and surface (B) water types. The direction and length of the arrows are indicative of the strength of the relationships between parameters. The -1.0 to 1.0 axis labels are arbitrary values commonly used to provide reference for comparison of parameters. D.O., dissolved oxygen.

tion 24 h prior to sample collection in reclaimed water (P < 0.05). Cloud cover and air and water temperatures were significantly correlated in surface waters, as were water temperature and precipitation 14 days prior to sample collection (P < 0.05). All other combinations of atmospheric and physicochemical parameters evaluated, excluding those stated above, were not significantly correlated in either water type, surface or reclaimed.

#### DISCUSSION

To the best of our knowledge, this is the first study performed in the Mid-Atlantic region of the United States investigating nontraditional irrigation waters for the prevalence of foodborne EVs and potential viral indicators. Comprehensive analyses were performed on the atmospheric and physicochemical water parameters to determine whether any parameters could provide a labor- and cost-effective indicator of EV contamination. Examinations of surface and reclaimed waters are essential for the Mid-Atlantic region due to the general decrease in groundwater levels experienced throughout Delaware, Maryland, and Virginia (34). This area of the United States may serve as a model system in many ways, and a few are described here. The watershed here is critical to a large ecoregion consisting of coastal plains, flatwoods, and mountain ranges, which compose one the greatest biologically and geographically diverse regions of the country (35, 36). There are 14 watersheds throughout the Mid-Atlantic region and one subwatershed alone, Brandywine Creek, supports \$10.3 billion of farmland and over \$425 million of urban ecosystems (37, 38). The \$15.6 billion agricultural industry supports approximately 41 million residents of the Mid-Atlantic region, spread from rural environments to densely populated metropolitan areas (39, 40).

Groundwater depletion combined with rising sea levels exacerbates saltwater intrusion and results in the decline of the quality of the remaining water supplies (41). This too is an issue facing many regions around the world. Changes in climate, population, and agricultural production in the Mid-Atlantic region create difficulties in predicting the future impacts of these threats on the availability of groundwater as the primary source of agricultural irrigation water (42–44). However, there is a consensus that more research is needed to proactively determine suitable alternatives, including the use of and risk associated with nontraditional water sources, to supplement groundwater for irrigation.

**EV and indicator virus detection.** Unlike respiratory viruses, foodborne EVs can persist for extended periods within a contaminated water source. EVs (e.g., NoV and

adenovirus [AdV]) have been shown to remain detectable in groundwater for more than 3 years (45). In surface water under laboratory conditions, EVs were shown to remain infectious for 25 days and persisted for more than 70 days (46). The sources and biological characteristics (e.g., size and persistence) of PMMoV allow the virus to potentially be used, in addition to bacterial water quality indicators, as a marker for human fecal contamination, and this concept has been explored previously (8, 47–51). Compared to other viral or bacterial indicators, PMMoV prevalence is limited in animal feces (18, 47); however, PMMoV has been found to be ubiquitous in both wastewater and surface water samples (47–49). PMMoV has been shown to persist in surface water for more than 21 days without a significant reduction (50). The prevalence and persistence of PMMoV, compared to EVs and other traditional indicators, further support its suitability as an indicator (51).

In our study, EVs and indicator virus were analyzed in surface and reclaimed waters over a 17-month period between June 2017 and October 2018. EVs (AiV, HAV, NoV GI, and NoV GII) were detected in 4.1% of surface water samples and 31.8% of reclaimed water samples. PMMoV was detected in 44.4% of total samples, 33.3% of surface water samples, and 66.7% of reclaimed water samples. Overall, 9.8% of samples were positive for detection of both EVs and indicator virus. There was no correlation between the detection of the EVs and that of the indicator virus in surface waters; however, there was a significant correlation in the detection of these viruses in reclaimed waters.

Detection of EVs in this study was comparable to that in other studies. A study performed in California, United States, detected NoV GI in 20.1% and NoV GII in 11.9% of surface water samples (n = 860) (52). A study in China surveyed 108 surface water samples for NoV and detected the virus in 4.6% of samples (53). Jurzik et al. evaluated surface water samples in Germany for NoV GII over 20 months and detected the virus in 25.7% of the 187 samples processed (8). Brazilian surface waters (n = 52) were examined for EV presence, and NoV was detected in only 5.8% of samples; however, 59.6% of samples contained at least one EV (54).

The removal of EV and PMMoV through wastewater treatment was investigated by Kitajima et al. (28). Both PMMoV and AiV were detected in all influent samples from two treatment facilities, averaging ~6 log copies/liter and ~4 to 6 log copies/liter, respectively. There was also a <3-log reduction observed for NoV GI and GII, AiV, and PMMoV, with PMMoV showing the smallest reduction (28). In 2011, wastewater treatment influent (n = 81) and effluent (n = 79) samples were collected in France. NoV GI and GII were detected in 43% and 88% of influent samples and 24% and 14% of effluent samples, respectively (55). In Norway, influent (n = 64) and effluent (n = 59) wastewater samples were evaluated for NoV GI and GII. NoV GI and GII were detected in 29.7% and 43.8% of influent samples and 33.9% and 57.6% of effluent samples, respectively (56).

The rates of EV and indicator virus detection in our study are within the ranges assessed previously and reported above. The variation in results across studies is expected, due to the wide array of recovery and detection methods employed, water quality parameters, and atmospheric conditions. Methods for recovery may include centrifugal ultrafiltration as performed in this study, polyethylene glycol (PEG) precipitation, or direct extraction without a concentration step for small sample volumes. Detection methods may include infectivity assays such as plaque or 50% tissue culture infective dose assays and molecular assays such as PCR and gel electrophoresis, quantitative PCR (qPCR), and digital droplet PCR. Results from these methods can be reported according to the total volume processed or viral copies detected as presence or absence or quantifiable levels, as well as infectious virus or viral genomic copies. All of these variables contribute to the lack of uniformity throughout the literature.

The correlation between EV and PMMoV in reclaimed water samples but not surface water samples is likely due in part to the higher concentrations of viruses in reclaimed water than in surface water. The limited number of positive virus detections in surface water also could have contributed to the lack of correlation between EV and PMMoV in

surface water. Selection of sites characterized in this study, where both EV and indicator viruses were detected, for a future study could allow repeated sampling of a source for the viruses without the need for the suite of parameters analyzed here.

Atmospheric and physicochemical parameter analyses. Dissolved oxygen levels were significantly lower in reclaimed water samples than in surface water samples. In contrast, the turbidity of reclaimed water samples was significantly higher than that of surface water samples. pH was not significantly different between surface and reclaimed water samples. An 11-month study was performed in Chile in which surface water samples were collected from 18 sites along the Chillán river. Decreases in dissolved oxygen levels were observed at sites downstream of a wastewater discharge, and no significant difference in pH was observed (57). The inconsistencies observed in dissolved oxygen, pH, and turbidity measurements provide valuable information regarding the quality of the water; however, these parameters had no significant impact on detection of EVs or indicator virus, and thus these parameters are not likely to be suitably indicative of EV presence.

Salinity was the most likely parameter to affect the detection of both EVs and indicator virus, with coefficients of 0.51  $\pm$  0.22 and 0.53  $\pm$  0.24, respectively, as well as statistically significant LR chi-square results. Salinity levels were significantly lower in surface water samples than in reclaimed water samples ( $0.10 \pm 0.07$  PSU and  $10.96 \pm 11.60$  PSU, respectively). Seasonal changes in salinity were also observed in surface water samples, with higher levels in the autumn than in the spring and winter. Interestingly, salinity was not correlated with any other parameter in surface water samples but was correlated with dissolved oxygen, pH, and turbidity in the reclaimed water samples. A Nigerian river was evaluated monthly by Abowei (58) at four separate locations over a 1-year period. Nigeria has a wet season from April to October and a dry season from November to March. Water temperatures ranged from 27°C to 31°C, increasing significantly in the dry season. Higher salinity values were observed in the dry season than in the wet season. Salinity levels ranged from 17 ppt in February and March to 5 ppt in September, averaging 13 ppt annually (58). Salinity initially showed promise as a suitable indicator, due to its impact on virus detection; however, the seasonally and source variability observed in this study and previous studies suggests that salinity is not a universally appropriate indicator of EV contamination. Studies have shown that some viruses are protected against heat inactivation by the presence of certain cations (59).

Atmospheric effects on virus detection varied between EV and indicator virus models. Air ( $-1.06 \pm 0.66$ ) and water ( $1.28 \pm 0.66$ ) temperatures had the largest standardized coefficients for the EV detection model, while coefficients for precipitation 7 days (0.80  $\pm$  0.44) and 14 days ( $-0.82 \pm 0.40$ ) prior to sample collection were largest for the PMMoV model. The chi-square analyses aligned with the standardized coefficients and returned significant results for water temperature in EV detection and for precipitation 14 days prior to sample collection in PMMoV detection. Temperature is known to affect the persistence of viruses, including HAV, enteroviruses, and AdVs (59). EVs, such as AdV41, can be detected in surface water after temperatures of up to 37°C for more than 70 days and are minimally affected by UV exposure of up to 400 mJ/cm<sup>2</sup> (46). Increased precipitation can subsequently increase the turbidity of a water source, which negatively affects the recovery and detection of those viruses. However, the concentrations of EVs have still been shown to increase in surface water sources following elevated precipitation (29). The relationship between extreme precipitation events and waterborne outbreaks in the United States between 1948 and 1994 was reviewed by Curriero et al. (60). The majority (68%) of waterborne outbreaks attributed to contaminated surface water occurred after precipitation above the 80th percentile in the month prior to the outbreak (60), suggesting that not only recent but also prolonged rainfall can be consequential. In this study, the rainfall over an extended period was of particular importance because reclaimed waters were held in a lagoon after treatment, prior to irrigation. Temperature and precipitation combined are promising

for use as possible indicators of EV contamination and should be investigated further to determine potential thresholds at which the risks increase.

**Conclusions.** The disparity in correlations between EV detection and PMMoV detection, as well as variations in relationships between parameters, is likely due to a combination of factors. Salinity, pH, and turbidity levels have been shown to affect the sediment adsorption and elution of viruses and to impact viruses differently (61). Precipitation events can cause the resuspension of sediment in the water and increase turbidity prior to sample collection (62), which has been shown to adversely affect the adsorption of some EVs to positively charged filters, likely due to suspended particles inhibiting binding of the negatively charged virus capsids to the positively charged membrane (63). The rod-shaped structure of PMMoV, as opposed to the icosahedral shape of EVs, could exacerbate the effects of physicochemical parameters on the variation in efficacy of recovery and detection methods, resulting in missed opportunities for detection and subsequent correlation if PMMoV is used as an indicator. Thresholds for each parameter, at which PMMoV and EV recovery and detection are analogous and PMMoV or other indicators, depending on further research, can be reliably used as an indicator, need to be established.

The wide variability of physicochemical parameters in both reclaimed and surface water samples, differences between the water types, and seasonal variations increase the complexity of determining indicators that can be used universally. Combined with the fluctuations in atmospheric conditions, particularly in the Mid-Atlantic region of the United States, there may not be a single indicator suitable for all water types throughout all seasons. These data suggest that detection of PMMoV and elevated precipitation and salinity levels may signify EV presence; however, wide variations in measured parameters and climate anomalies will continue to hinder the reliability of indicators for water contamination by EVs.

#### **MATERIALS AND METHODS**

Water sample and physicochemical and atmospheric data collection. Water samples were collected from six sites in the Mid-Atlantic region of the United States between June 2017 and October 2018. Sites were selected and characterized by CONSERVE collaborators prior to the initial sampling event, and universal codes were created and used by all researchers at collaborating universities and research facilities. Sites included two ponds (MA10 and MA11), one tidal-freshwater river (MA04), two wastewater reclamation sites (MA01 and MA02), and one produce wash water site (MA12). Samples were collected from sites twice monthly during the growing season from May through September and once monthly from October through April, as described by Haymaker et al. (64).

During sample collection, physicochemical water parameters were recorded in triplicate using a YSI ProDSS meter (EquipCo, Yellow Springs, OH) and included dissolved oxygen (percentage), pH, salinity (PSU), and turbidity (FNU). Atmospheric data collected included air and water temperatures (degrees Celsius), cloud cover (score of 0 to 4), and precipitation (centimeters) 24 h, 7 days, and 14 days prior to sample collection. Cloud cover was recorded similarly to the National Oceanic and Atmospheric Administration (NOAA) five-point scale, as follows: 0, 0%; 1, 0 to <50%; 2, 50%; 3, 50 to <100%; 4, 100% (https://www.wpc.ncep.noaa.gov/html/stationplot\_printer.html). Our study examined dissolved oxygen, pH, salinity, and turbidity of surface and reclaimed waters seasonally and overall, for the effects on EV and indicator virus detection. These parameters were selected because they are easily monitored and evaluated. They also provide an overall water quality profile describing the charge, concentration of suspended particles, dissolved gases, and inorganic compounds in the water.

Physicochemical and atmospheric parameters were compared seasonally and by water type using *t* tests and binary logistic regression analyses performed to evaluate the effects of parameters of different units on the detection of EV and PMMoV individually. The logistic regression method of analysis considers all independent continuous variables within the model in relation to each other and the dependent binary variables. The standardized coefficient describes the predicted change in a dependent variable resulting from a 1-standard deviation change in an independent variable.

**Virus recovery and detection.** A modified virus adsorption-elution (VIRADEL) method was employed for the recovery of viruses, as described previously (65). Water (25 liters, on average) was pumped through a 5-inch, positively charged NanoCeram filter, and viruses were adsorbed to the filter. An additional filter was used if the total sample volume was not able to pass through a single filter. Filters were eluted with 300 ml of sodium polyphosphate buffer at pH 9.3 during a 15-min incubation period at room temperature. The eluates were adjusted to neutral pH and concentrated via centrifugal ultrafiltration (100-kDa Centricon Plus-70 centrifugal filters; EMD Millipore, Billerica, MA). Eluate was added to the filter in 60-ml aliquots and centrifugal of 8 min at  $1,900 \times g$ . Centrifugation was repeated with sterile water, and then the filter was inverted and centrifuged for 2 min at  $800 \times g$ . The concentrate was collected, and extraction of nucleic acids was performed using the Qiagen AllPrep PowerViral RNA/

DNA kit. Nucleic acids were aliquoted and stored at -80°C until detection was performed with a realtime (RT) qPCR molecular assay. Probe-based RT-qPCR was performed using the Qiagen Rotor-Gene Q apparatus and primers and probes selected for their specificity and successful detection of the target from water samples in the literature. AiV primers and probe (66) designed to target the viral protein region of AiV genotypes A and B were used because of the association of those genotypes with human and bovine infections (22). The HAV primers and probe selected were able to detect all human genotypes (IA, IB, IIA, IIB, and III) by targeting the 5' untranslated region, which codes for viral polypeptides and is conserved among genotypes (67). Kageyama et al. developed the primers and probes used for NoV GI and GII detection, which targeted the conserved open reading frame 1 (ORF1)-ORF2 junction (68). PMMoV was detected using primers and probes designed to target the coat protein gene, which is the most conserved region among the genetically diverse sequences of PMMoV (69, 70).

**Molecular control.** Tulane virus (TV) (a gift from Xi Jiang, University of Cincinnati College of Medicine, Cincinnati, OH) was selected for use as a process control to evaluate extraction efficiency and detection inhibition. The virus was propagated in LLC-MK2 cells in medium 199 according to the protocol described by Wang et al. (71). TV RNA was extracted in the same manner as described above for environmental samples and serially diluted to create a standard curve. The TV stock was also added to each virus concentrate, at a concentration of 5 log genomic copies/reaction, prior to extraction. TV was detected using a primer and probe set designed to target the P289 to P290 region of the RNA polymerase gene (72). The control was successfully detected in all environmental samples (>1 log genomic copies/reaction), and no further processing was required.

**Data analysis.** Data were analyzed and figures were created using JMP, Microsoft Excel, and XLSTAT statistical software. Binary logistic regression was employed for analysis involving viral detection data, in which 1 represents detection and 0 represents no detection. Standardized coefficients were used for comparison of variables with different units of measurement and are presented with standard error values. Models were created and evaluated for goodness of fit using the receiver operating characteristic area under the curve (AUC) values. Only models with AUC values of >0.700 were included for analysis. Atmospheric and physicochemical data were analyzed according to season using the seasonal dates designated by the Farmer's Almanac (https://www.farmersalmanac.com/the-seasons). The significance level was set to  $\alpha$  of 0.10 for all analyses; only *P* values less than 0.05 were considered significant.

#### ACKNOWLEDGMENTS

This work was supported by the U.S. Department of Agriculture, National Institute of Food and Agriculture (grant 2016-68007-25064), which established CONSERVE: a center of excellence at the nexus of sustainable water reuse, food, and health.

We thank the entire CONSERVE Mid-Atlantic sampling team for project support.

### REFERENCES

- Dery JL, Suri MR, Brassill N, Pee D, Goeringer P, Sapkota AR, Rock C, Goldstein RER. 2017. Recycled water and related terms relevant for agriculture. U.S. Department of Agriculture, Washington, DC. https://static1.squarespace .com/static/578101761b631b1a87aa0a3c/t/5ad4cbb1562fa7487dd68ca6/1 523895222928/Recycled+Water+and+Related+Terms.pdf.
- Pandey PK, Kass PH, Soupir ML, Biswas S, Singh VP. 2014. Contamination of water resources by pathogenic bacteria. AMB Express 4:51. https://doi .org/10.1186/s13568-014-0051-x.
- Federal-Provincial-Territorial Committee on Drinking Water. 2019. Guidelines for Canadian drinking water quality: technical document: enteric viruses in drinking water. Health Canada, Ottawa, Ontario, Canada. https://www.canada .ca/en/health-canada/services/publications/healthy-living/guidelines-canadian -drinking-water-quality-guideline-technical-document-enteric-viruses.html.
- 4. California Department of Water Resources. 2018. Making water conservation a California way of life: primer of 2018 legislation on water conservation, AB 1668: Friedman and SB 606: Hertzberg. California Department of Water Resources, Sacramento, CA. https://water.ca.gov/-/media/DWR-Website/ Web-Pages/Programs/Water-Use-And-Efficiency/Make-Water-Conservation -A-California-Way-of-Life/Files/PDFs/Final-WCL-Primer.pdf.
- U.S. Environmental Protection Agency. 1976. Quality criteria for water. EPA 440-9-76-023. U.S. Environmental Protection Agency, Washington, DC. https://www.epa.gov/sites/production/files/2018-10/documents/ guality-criteria-water-1976.pdf.
- U.S. Food and Drug Administration. 2011. FSMA final rule on produce safety standards for the growing, harvesting, packing, and holding of produce for human consumption. FDA-2011-N-0921. U.S. Food and Drug Administration, Silver Spring, MD. https://www.regulations.gov/document/ FDA-2011-N-0921-18558.
- U.S. Food and Drug Administration. 2011. Food Safety Modernization Act: Produce Safety Rule: Title 21 CFR part 112, subpart E. https://www.accessdata .fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=112.

- Jurzik L, Hamza IA, Puchert W, Uberla K, Wilhelm M. 2010. Chemical and microbiological parameters as possible indicators for human enteric viruses in surface water. Int J Hyg Environ Health 213:210–216. https://doi .org/10.1016/j.ijheh.2010.05.005.
- Espinosa AC, Arias CF, Sanchez-Colon S, Mazari-Hiriart M. 2009. Comparative study of enteric viruses, coliphages and indicator bacteria for evaluating water quality in a tropical high-altitude system. Environ Health 8:49. https://doi.org/10.1186/1476-069X-8-49.
- Hörman A, Rimhanen-Finne R, Maunula L, von Bonsdorff C-H, Torvela N, Heikinheimo A, Hänninen M-L. 2004. *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., noroviruses and indicator organisms in surface water in southwestern Finland, 2000–2001. Appl Environ Microbiol 70:87–95. https://doi.org/10.1128/AEM.70.1.87-95.2004.
- 11. Lin J, Ganesh A. 2013. Water quality indicators: bacteria, coliphages, enteric viruses. Int J Environ Health Res 23:484–506. https://doi.org/10 .1080/09603123.2013.769201.
- Steyer A, Torkar KG, Gutierrez-Aguirre I, Poljsak-Prijatelj M. 2011. High prevalence of enteric viruses in untreated individual drinking water sources and surface water in Slovenia. Int J Hyg Environ Health 214:392–398. https://doi.org/10.1016/j.ijheh.2011.05.006.
- Ijabadeniyi OA, Debusho LK, Vanderlinde M, Buys EM. 2011. Irrigation water as a potential preharvest source of bacterial contamination of vegetables. J Food Saf 31:452–461. https://doi.org/10.1111/j.1745-4565.2011 .00321.x.
- Li X, Watanabe N, Xiao C, Harter T, McCowan B, Liu Y, Atwill ER. 2014. Antibiotic-resistant *E. coli* in surface water and groundwater in dairy operations in northern California. Environ Monit Assess 186:1253–1260. https:// doi.org/10.1007/s10661-013-3454-2.
- Megchun-Garcia JV, Landeros-Sanchez C, Soto-Estrada A, Castaneda-Chavez MR, Martinez-Davila JP, Nikolskii-Gavrilov I, Galaviz-Villa I, Lango-Reynoso F. 2015. Total coliforms and *Escherichia coli* in surface and

subsurface water from a sugarcane agroecosystem in Veracruz, Mexico. J Agric Sci 7:110–119. https://doi.org/10.5539/jas.v7n6p110.

- Cronin AA, Rueedi J, Morris BL. 2006. The effectiveness of selected microbial and chemical indicators to detect sewer leakage impacts on urban groundwater quality. Water Sci Technol 54:145–152. https://doi.org/10 .2166/wst.2006.579.
- Eftim SE, Hong T, Soller J, Boehm A, Warren I, Ichida A, Nappier SP. 2017. Occurrence of norovirus in raw sewage: a systematic literature review and meta-analysis. Water Res 111:366–374. https://doi.org/10.1016/j .watres.2017.01.017.
- Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, de Silva NR, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, Devleesschauwer B. 2015. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLoS Med 12:e1001923. https://doi.org/10 .1371/journal.pmed.1001923.
- Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States: major pathogens. Emerg Infect Dis 17:7–15. https://doi.org/10 .3201/eid1701.P11101.
- Verhoef L, Hewitt J, Barclay L, Ahmed SM, Lake R, Hall AJ, Lopman B, Kroneman A, Vennema H, Vinje J, Koopmans M. 2015. Norovirus genotype profiles associated with foodborne transmission, 1999–2012. Emerg Infect Dis 21:592–599. https://doi.org/10.3201/eid2104.141073.
- Lemon SM, Robertson BH. 1994. Taxonomic classification of hepatitis A virus, p 50–53. *In* Nishioka K, Suzuki H, Mishiro S, Oda T (ed), Viral hepatitis and liver disease. Springer, Tokyo, Japan.
- Yates MV. 2014. Emerging viruses, p 529–533. In Percival SL, Yates MV, Williams DW, Chalmers RM, Gray NF (ed), Microbiology of waterborne diseases, 2nd ed. Academic Press, London, England.
- 23. Reuter G, Boros A, Pankovics P. 2011. Kobuviruses: a comprehensive review. Rev Med Virol 21:32–41. https://doi.org/10.1002/rmv.677.
- Ngazoa ES, Fliss I, Jean J. 2008. Quantitative study of persistence of human norovirus genome in water using Taqman real-time RT-PCR. J Appl Microbiol 104:707–715. https://doi.org/10.1111/j.1365-2672.2007 .03597.x.
- Payment P, Plante R, Cejka P. 2001. Removal of indicator bacteria, human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts at a large wastewater primary treatment facility. Can J Microbiol 47:188–193. https://doi.org/10.1139/w00-143.
- Hewitt J, Leonard M, Greening GE, Lewis GD. 2011. Influence of wastewater treatment process and the population size on human virus profiles in wastewater. Water Res 45:6267–6276. https://doi.org/10.1016/j.watres .2011.09.029.
- Hamza IA, Jurzik L, Uberla K, Wilhelm M. 2011. Evaluation of pepper mild mottle virus, human picobirnavirus, and torque teno virus as indicators of fecal contamination in river water. Water Res 45:1358–1368. https://doi .org/10.1016/j.watres.2010.10.021.
- Kitajima M, Iker BC, Pepper IL, Gerba CP. 2014. Relative abundance and treatment reduction of viruses during wastewater treatment processes: identification of potential viral indicators. Sci Total Environ 488-489:290–296. https:// doi.org/10.1016/j.scitotenv.2014.04.087.
- Hata A, Katayama H, Kojima K, Sano S, Kasuga I, Kitajima M, Furumai H. 2014. Effects of rainfall events on the occurrence and detection efficiency of viruses in river water impacted by combined sewer overflows. Sci Total Environ 468-469:757–763. https://doi.org/10.1016/j.scitotenv.2013.08.093.
- Lopez-Galvez F, Truchado P, Sanchez G, Aznar R, Gil MI, Allende A. 2016. Occurrence of enteric viruses in reclaimed and surface irrigation water: relationship with microbiological and physicochemical indicators. J Appl Microbiol 121:1180–1188. https://doi.org/10.1111/jam.13224.
- Lopman B, Armstrong B, Atchison C, Gray JJ. 2009. Host, weather and virological factors drive norovirus epidemiology: time-series analysis of laboratory surveillance data in England and Wales. PLoS One 4:e6671-10. https://doi.org/10.1371/journal.pone.0006671.
- Levy K, Hubbard AE, Eisenberg JNS. 2009. Seasonality of rotavirus disease in the tropics: a systematic review and meta-analysis. Int J Epidemiol 38:1487–1496. https://doi.org/10.1093/ije/dyn260.
- Farkas K, Cooper DM, McDonald JE, Malham SK, de Rougemont A, Jones DL. 2018. Seasonal and spatial dynamics of enteric viruses in wastewater and in riverine and estuarine waters. Sci Total Environ 634:1174–1183. https://doi.org/10.1016/j.scitotenv.2018.04.038.
- Dong Y, Jiang C, Suri MR, Pee D, Meng L, Rosenberg Goldstein RE. 2019. Groundwater level changes with a focus on agricultural areas in the Mid-

Atlantic region of the United States, 2002–2016. Environ Res 171:193–203. https://doi.org/10.1016/j.envres.2019.01.004.

- Nave LE, DeLyser K, Butler-Leopold PR, Sprague E, Daley J, Swanston CW. 2019. Effects of land use and forest management on soil carbon in the ecoregions of Maryland and adjacent eastern United States. For Ecol Manage 448:34–47. https://doi.org/10.1016/j.foreco.2019.05.072.
- 36. Butler-Leopold PR, Iverson LR, Thompson FR, III, Brandt LA, Handler SD, Janowiak MK, Shannon PD, Swanston CW, Bearer S, Bryan AM, Clark KL, Czarnecki G, DeSenze P, Dijak WD, Fraser JS, Gugger PF, Hille A, Hynicka J, Jantz CA, Kelly MC, Krause KM, La Puma IP, Landau D, Lathrop RG, Leites LP, Madlinger E, Matthews SN, Ozbay G, Peters MP, Prasad A, Schmit DA, Shephard C, Shirer R, Skowronski NS, Steele A, Stout S, Thomas-Van Gundy M, Thompson J, Turcotte RM, Weinstein DA, Yáñez A. 2018. Mid-Atlantic ecosystem vulnerability assessment and synthesis: a report from the Mid-Atlantic Climate Change Response Framework Project. General Technical Report NRS-181. U.S. Department of Agriculture, Forest Service, Northern Research Station, Newtown Square, PA. https://doi.org/10.2737/NRS-GTR-181.
- U.S. Department of Agriculture, National Agricultural Statistics Service.
   2012. 2012 Census of agriculture watersheds report, p 10–17. National Agricultural Statistics Service, Washington, DC.
- Cruz-Ortiz C, Miller K. 2013. Economic value of the Brandywine Creek watershed. Water Resources Agency, School of Public Policy and Administration, University of Delaware, Newark, DE.
- U.S. Census Bureau. 2020. Resident population in the Middle Atlantic census division, 13 January 2020 revision date. https://fred.stlouisfed.org/ series/CMATPOP.
- 40. Servidio C. 27 March 2020. Mid-Atlantic farmers bring food to the table. The EPA Blog https://blog.epa.gov/2020/03/27/mid-atlantic-farmers-bring -food-to-the-table.
- Sawyer AH, David CH, Famiglietti JS. 2016. Continental patterns of submarine groundwater discharge reveal coastal vulnerabilities. Science 353:705–707. https://doi.org/10.1126/science.aag1058.
- Abler DG, Shortle JS. 2000. Climate change and agriculture in the Mid-Atlantic Region. Clim Res 14:185–194. https://doi.org/10.3354/cr014185.
- Neff R, Chang H, Knight CG, Najjar RG, Yarnal B, Walker HA. 2000. Impact of climate variation and change on Mid-Atlantic Region hydrology and water resources. Clim Res 14:207–218. https://doi.org/10.3354/cr014207.
- Russo TA, Lall U. 2017. Depletion and response of deep groundwater to climate-induced pumping variability. Nat Geosci 10:105–110. https://doi .org/10.1038/ngeo2883.
- Kauppinen A, Pitkanen T, Miettinen IT. 2018. Persistent norovirus contamination of groundwater supplies in two waterborne outbreaks. Food Environ Virol 10:39–50. https://doi.org/10.1007/s12560-017-9320-6.
- Prevost B, Goulet M, Lucas FS, Joyeux M, Moulin L, Wurtzer S. 2016. Viral persistence in surface and drinking water: suitability of PCR pre-treatment with intercalating dyes. Water Res 91:68–76. https://doi.org/10.1016/j .watres.2015.12.049.
- Rosario K, Symonds EM, Sinigalliano C, Stewart J, Breitbart M. 2009. Pepper mild mottle virus as an indicator of fecal pollution. Appl Environ Microbiol 75:7261–7267. https://doi.org/10.1128/AEM.00410-09.
- Kuroda K, Nakada N, Hanamoto S, Inaba M, Katayama H, Do AT, Nga TTV, Oguma K, Hayashi T, Takizawa S. 2015. Pepper mild mottle virus as an indicator and a tracer of fecal pollution in water environments: comparative evaluation with wastewater-tracer pharmaceuticals in Hanoi, Vietnam. Sci Total Environ 506-507:287–298. https://doi.org/10.1016/j.scitotenv.2014 .11.021.
- 49. Shrestha S, Shrestha S, Shindo J, Sherchand JB, Haramoto E. 2018. Virological quality of irrigation water sources and pepper mild mottle virus and tobacco mosaic virus as index of pathogenic virus contamination level. Food Environ Virol 10:107–120. https://doi.org/10.1007/s12560-017-9324-2.
- Rachmadi AT, Kitajima M, Pepper IL, Gerba CP. 2016. Enteric and indicator virus removal by surface flow wetlands. Sci Total Environ 542:976–982. https://doi.org/10.1016/j.scitotenv.2015.11.001.
- 51. Kitajima M, Sassi HP, Torrey JR. 2018. Pepper mild mottle virus as a water quality indicator. npj Clean Water 1:19. https://doi.org/10.1038/s41545 -018-0019-5.
- 52. Tian P, Yang D, Shan L, Wang D, Li Q, Gorski L, Lee BG, Quinones B, Cooley MB. 2017. Concurrent detection of human norovirus and bacterial pathogens in water samples from an agricultural region in central California coast. Front Microbiol 8:1560. https://doi.org/10.3389/fmicb.2017.01560.
- 53. He X, Wei Y, Cheng L, Zhang D, Wang Z. 2012. Molecular detection of three gastroenteritis viruses in urban surface waters in Beijing and

correlation with levels of fecal indicator bacteria. Environ Monit Assess 184:5563–5570. https://doi.org/10.1007/s10661-011-2362-6.

- Miagostovich MP, Ferreira FFM, Guimarães FR, Fumian TM, Diniz-Mendes L, Luz SLB, Silva LA, Leite JPG. 2008. Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream water of Manaus, Central Amazonia, Brazil. Appl Environ Microbiol 74:375–382. https://doi.org/10.1128/AEM.00944-07.
- Sima LC, Schaeffer J, Le Saux J, Parnaudeau S, Elimelech M, Le Guyader S. 2011. Calicivirus removal in a membrane bioreactor wastewater treatment plant. Appl Environ Microbiol 77:5170–5177. https://doi.org/10 .1128/AEM.00583-11.
- 56. Grondahl-Rosado RC, Yarovitsyna E, Trettenes E, Myrmel M, Robertson LJ. 2014. A one year study on the concentrations of norovirus and enteric adenoviruses in wastewater and a surface drinking water source in Norway. Food Environ Virol 6:232–245. https://doi.org/10.1007/s12560-014 -9161-5.
- 57. Debels P, Figueroa R, Urrutia R, Barra R, Niell X. 2005. Evaluation of water quality in the Chillan River (central Chile) using physicochemical parameters and a modified water quality index. Environ Monit Assess 110:301–322. https://doi.org/10.1007/s10661-005-8064-1.
- Abowei JFN. 2009. Salinity, dissolved oxygen, pH and surface water temperature conditions in Nkoro River, Niger Delta, Nigeria. Adv J Food Sci Technol 2:36–40.
- 59. Gerba CP. 2007. Virus occurrence and survival in the environmental waters. Perspect Med Virol 17:91–108. https://doi.org/10.1016/S0168 -7069(07)17005-1.
- Curriero FC, Patz JA, Rose JB, Lele S. 2001. The association between extreme precipitation and waterborne disease outbreaks in the United States, 1948–1994. Am J Public Health 91:1194–1199. https://doi.org/10 .2105/AJPH.91.8.1194.
- LaBelle RL, Gerba CP. 1979. Influence of pH, salinity, and organic matter on the adsorption of enteric viruses to estuarine sediment. Appl Environ Microbiol 38:93–101. https://doi.org/10.1128/AEM.38.1.93-101.1979.
- 62. Green MO, Coco G. 2007. Sediment transport on an estuarine intertidal flat: measurements and conceptual model of waves, rainfall and exchanges with a tidal creek. Estuar Coast Shelf Sci 72:553–569. https:// doi.org/10.1016/j.ecss.2006.11.006.
- Sobsey MD, Glass JS. 1984. Influence of water quality on enteric virus concentration by microporous filter methods. Appl Environ Microbiol 47:956–960. https://doi.org/10.1128/AEM.47.5.956-960.1984.

- 64. Haymaker J, Sharma M, Parveen S, Hashem F, May EB, Handy ET, White C, East C, Bradshaw R, Micallef SA, Callahan MT, Allard S, Anderson B, Craighead S, Gartley S, Vanore A, Kniel KE, Solaiman S, Bui A, Murray R, Craddock HA, Kulkarni P, Foust D, Duncan R, Taabodi M, Sapkota AR. 2019. Prevalence of Shiga-toxigenic and atypical enteropathogenic *Escherichia coli* in untreated surface water and reclaimed water in the Mid-Atlantic U.S. Environ Res 172:630–636. https://doi.org/10.1016/j.envres.2019 .02.019.
- 65. Anderson-Coughlin BL, Kniel KE. 2019. Recovery and detection of enteric viruses from non-traditional irrigation water sources. Methods Protoc 2:55–58. https://doi.org/10.3390/mps2030055.
- 66. Kitajima M, Hata A, Yamashita T, Haramoto E, Minagawa H, Katayama H. 2013. Development of a reverse transcription-quantitative PCR system for detection and genotyping of Aichi viruses in clinical and environmental samples. Appl Environ Microbiol 79:3952–3958. https://doi.org/10.1128/ AEM.00820-13.
- Jothikumar N, Cromeans TL, Sobsey MD, Robertson BH. 2005. Development and evaluation of a broadly reactive Taqman assay for rapid detection of hepatitis A virus. Appl Environ Microbiol 71:3359–3363. https://doi.org/10.1128/AEM.71.6.3359-3363.2005.
- Kageyama T, Kojima S, Schinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K. 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol 41:1548–1557. https://doi.org/10.1128/jcm.41.4 .1548-1557.2003.
- Zhang T, Breitbart M, Lee WH, Run JQ, Wei CL, Soh SW, Hibberd ML, Liu ET, Rohwer F, Ruan Y. 2005. RNA viral community in human feces: prevalence of plant pathogenic viruses. PLoS Biol 4:e3. https://doi.org/10.1371/ journal.pbio.0040003.
- Haramoto E, Kitajima M, Kishida N, Konno Y, Katayama H, Asami M, Akiba M. 2013. Occurrence of pepper mild mottle virus in drinking water sources in Japan. Appl Environ Microbiol 79:7413–7418. https://doi.org/10 .1128/AEM.02354-13.
- Wang Q, Hirneisen KA, Markland SM, Kniel KE. 2013. Survival of murine norovirus, Tulane virus, and hepatitis A virus on alfalfa seeds and sprouts during storage and germination. Appl Environ Microbiol 79:7021–7027. https://doi.org/10.1128/AEM.01704-13.
- Tian P, Yang D, Quigley C, Chou M, Jiang X. 2013. Inactivation of Tulane virus, a novel surrogate for the human norovirus. J Food Prot 76:712–718. https://doi.org/10.4315/0362-028X.JFP-12-361.