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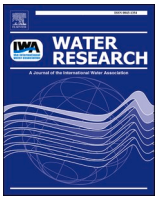

Temporal and spatial relationships of CrAssphage and enteric viral and bacterial pathogens in wastewater in North Carolina

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ABSTRACT

Enteric disease remains one of the most common concerns for public health, particularly when it results from human exposure to surface and recreational waters contaminated with wastewater. Characterizing the temporal and spatial variation of enteric pathogens prevalent in wastewater is critical to develop approaches to mitigate their distribution in the environment. In this study, we aim to characterize pathogen variability and test the applicability of the human-associated wastewater indicator crAssphage as an indicator of enteric viral and bacterial pathogens. We conducted weekly samplings for 14 months from four wastewater treatment plants in North Carolina, USA. Untreated wastewater samples were processed using hollow fiber ultrafiltration, followed by secondary concentration methods. Adenovirus, norovirus, enterovirus, *Salmonella*, Shiga toxin 2 (stx_2), *Campylobacter*, and crAssphage were measured by quantitative polymerase chain reaction (qPCR) and reverse transcriptase (rt)-qPCR. Our results revealed significant correlations between crAssphage and human adenovirus, enterovirus, norovirus, *Salmonella*, and *Campylobacter* ($p<0.01$). Pathogens and crAssphage concentrations in untreated wastewater showed distinct seasonal patterns, with peak concentrations of crAssphage and viral pathogens in fall and winter, while bacterial pathogens showed peaked concentrations in either winter (*Campylobacter*), fall (*Salmonella*), or summer (stx_2). This study enhances the understanding of crAssphage as an alternative molecular indicator for both bacterial and viral pathogens. The findings of this study can also inform microbial modeling efforts for the prediction of the impact of wastewater pathogens on surface waters due to increased flooding events and wastewater overflows associated with climate change.

1. Introduction

Waterborne and foodborne enteric disease outbreaks can lead to hospitalization, death, beach and school closures, and economic burdens (Buzby and Roberts, 2009). Enteric pathogens such as noroviruses, rotaviruses, *Salmonella*, pathogenic *Escherichia coli,* and *Cryptosporidium* remain one of the public health concerns following exposure to contaminated waters. The latest U.S. Environmental Protection Agency (EPA) Contaminant Candidate List (CCL4) contained 12 microbial contaminants, including human adenoviruses, enteroviruses, noroviruses, *Salmonella enterica, Campylobacter jejuni, E. coli* O157 and *Shigella sonnei*.

One of the challenges to detect enteric pathogens in environmental water samples is their low ambient concentrations, thus direct detection without multi-step sample processing may not be possible (McCall et al., 2020). Traditionally, culturable methods for fecal indicator, fecal coliforms, *E. coli, Enterococci,* and Coliphages have been used for water quality assessment (EPA, 2009a; b; 2018). Two drawbacks of these cultivation-based procedures are that most require 18 h or more to produce results and cannot track sources of fecal contamination, making it challenging to inform water safety managers on the same day.

Advances in molecular methods, particularly PCR-based methods offer an attractive alternative for improved and rapid detection of a diverse group of microbial targets in water. Gene markers assays including US EPA Method 1609.1 for *Enterococci,* EPA Method 1696 for the human fecal marker HF183, and coliphages phiX174 and MS2 assays have been applied in water remediation programs and provide rapid

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same-day water quality notification (EPA, 2019; Seurinck et al., 2005; Turgeon et al., 2014). However, an ideal pathogen and fecal indicator should be present in high concentration in source water and correlate with both viral and bacterial etiologic agents that cause enteric diseases.

Recently, crAssphage (cross-assembly bacteriophage) has been proposed as a human-associated marker for monitoring fecal contamination of surface water, lake water, stormwater, and wastewater treatments (Ahmed et al., 2018b; Bibby and Peccia, 2013; Crank et al., 2019; Farkas et al., 2019). Stachler et al. (2014) detected crAssphage in high abundance in the human gut by metagenomic analysis, and later found it is highly associated with human fecal contamination (Stachler and Bibby, 2014; Stachler et al., 2017). However, the correlations between the crAssphage marker and enteric pathogens in wastewater remain to be defined across geographic locations and seasons.

Studies have demonstrated that enteric diseases (e.g., gastroenteritis) exhibit seasonal patterns in the communities, with higher number of cases during winter and spring (O'Brien and Xagoraraki, 2019; Thwiny et al., 2022). However, the leading and dominant enteric pathogen in different seasons and the spatial variability (i.e., different treatment plants) have not been fully clarified. The objectives of this study were to (1) assess effectiveness of the emerging wastewater crAssphage marker as a microbial water quality indicator for both enteric viral and bacterial pathogens, and (2) identify the dynamics of the enteric pathogens throughout the seasons by characterizing the temporal and spatial variation of viral and bacterial pathogens in untreated wastewater.

2. Materials and methods

2.1. Wastewater sampling

Composited untreated wastewater samples (24-h flow-weighted) were collected biweekly from two wastewater treatment plants (WWTPs) located in the Raleigh-Durham-Chapel Hill Metropolitan Area (RDCMA), NC (RTP1 and RTP2). In addition, weekly samples were collected from two WWTPs located in Charlotte (Charlotte1 and Charlotte2), NC (Fig. 1). Five litres wastewater samples were processed in the first two months of the sampling events, and three litres of wastewater samples were processed for the rest of the sampling period due to the high turbidity of the samples. Samples were collected in sterile 1 L Nalgene bottles at each site. Following collection, the wastewater samples were transported to the laboratory in coolers packed with ice. Deionized water in a 1 L Nalgene sample collection bottle was used as field blanks. The collected samples were processed for primary concentration within 24 h after arriving at the laboratory.

Samples from RTP1 and RTP2 were processed for primary concentration at the Environmental Protection Agency (EPA) laboratory in Research Triangle Park, while samples from Charlotte1 and Charlotte2 were processed at the University of North Carolina-Charlotte Environmental Water Laboratory. All secondary concentrations were performed at the EPA lab. A total of 147 raw wastewater samples were collected during 73 sampling events in 14 months (January 2021 to February 2022). Charlotte1 and RTP1 were sampled from January 2021 to February 2022. Sampling events for Charlotte2 were conducted from June 2021 to February 2022; sampling events for RTP 2 were conducted from February 2021 to February 2022. The characteristics of each WWTP are shown in Table 1.

2.2. Sample processing and concentrating

Due to the Covid-19 pandemic, wastewater samples have the potential of containing infectious SARS-CoV-2 virus, and therefore samples were processed in an enhanced biosafety laboratory level 2 (enhanced BSL-2) room as per CDC guidance (CDC, 2020). Sample processing workflow for bacteria and viruses is shown in Fig. 2. Wastewater samples were processed using a dead-end hollow fiber ultrafiltration (D-HFUF) procedure, followed by secondary concentration as described below. Wastewater (3 L to 5 L) was filtered through the dead-end hollow fiber ultrafilter (Rexeed 15S, Dial Medical Supply, Chester Springs, PA) using a peristaltic pump (Masterflex L/S Easy Load, Cole Parmer, Vernon Hills, IL, USA) set at 300 rpm. The Rexeed 15S filter was stabilized by a ring stand with utility clamp, and the filters were eluted by passing 200 ml of elution solution to obtain the first eluate (McMinn et al., 2021). The sample and filtration information were recorded on a Chain of Custody record form.

For bacterial pathogen analysis, 20 ml of the first eluate was filtered through a 0.45 mm filter (HAWP04700 Millipore, Bedford MA) in duplicate (Fig. 2). After filtration, the filter was transferred to the extraction tubes containing 0.1 mm glass beads provided in the Qiagen PowerLyzer PowerSoil extraction kit (Qiagen, Valencia, CA, USA), and saved at −80 °C. For enteric virus analysis, the rest of the first eluate (approximately 160 ml) was further processed using a beef-extract and celite method modified from EPA method 1615 (Cashdollar et al., 2013; McMinn et al., 2012). Briefly, 1.5% w/v beef extract powder was added to the eluate while slowly stirring on a stir plate. In the meantime, 0.1 g per 100 ml celite and 1 N HCl (dropwise) were added into the concentrate and stabilized for 10 min to reach a pellet to 4.0 to bind virus particles via organic flocculation. The first eluate was then centrifuged at 3800 x g for 10 min and pellet was saved. The pellet was resuspended in 8 ml of phosphate buffered saline (PBS) at a pH of 9 to 9.5 and centrifuged again at 6000 x g for 15 mins to release the virus into the liquid phase. The supernatant (secondary eluate) was saved into 500 ml aliquots and preserved at −80 °C for nucleic acid extraction after filtration through a 0.2 μm filter. Processed samples were analyzed for molecular assays within six months of sample collection.

2.3. Nucleic acid extraction and analysis

For enteric viruses, DNA and RNA were extracted from 200 μL secondary eluate using Qiagen All Prep PowerViral Kit (Qiagen, Valencia, CA, USA). For bacteria, DNA was extracted using the Qiagen PowerLyzer PowerSoil Kit (Qiagen, Valencia, CA, USA). The extractions followed manufacturer's instructions with an additional ethanol wash before nucleic acid elution to alleviate PCR inhibition. Each sample was extracted in duplicate, with a reagent blank (molecular-grade water) in

Table 1
Wastewater Treatment Plant Overview.

WWTP	RTP1	RTP2	Charlotte1	Charlotte2
Permitted Flow	14.5 MGD	12.5 MGD	20 MGD	12 MGD
Average Daily Flow	9 MGD	9 MGD	14.6 MGD	9.6 MGD
Estimated Serviced Population	83,000	90,000	182,500	120,000
Service Area	University campus, hospitals, suburban Development	University Campus, hospital, urban development	14 Permitted Significant Industrial Users, Major Hospitals, urban and suburban developments	3 Permitted Significant Industrial Users, Major Hospital, University Campus
Number of Samples	38	30	50	29

MGD: million gallon per day; WWTP: wastewater treatment plant; RTP: Research Triangle Park.

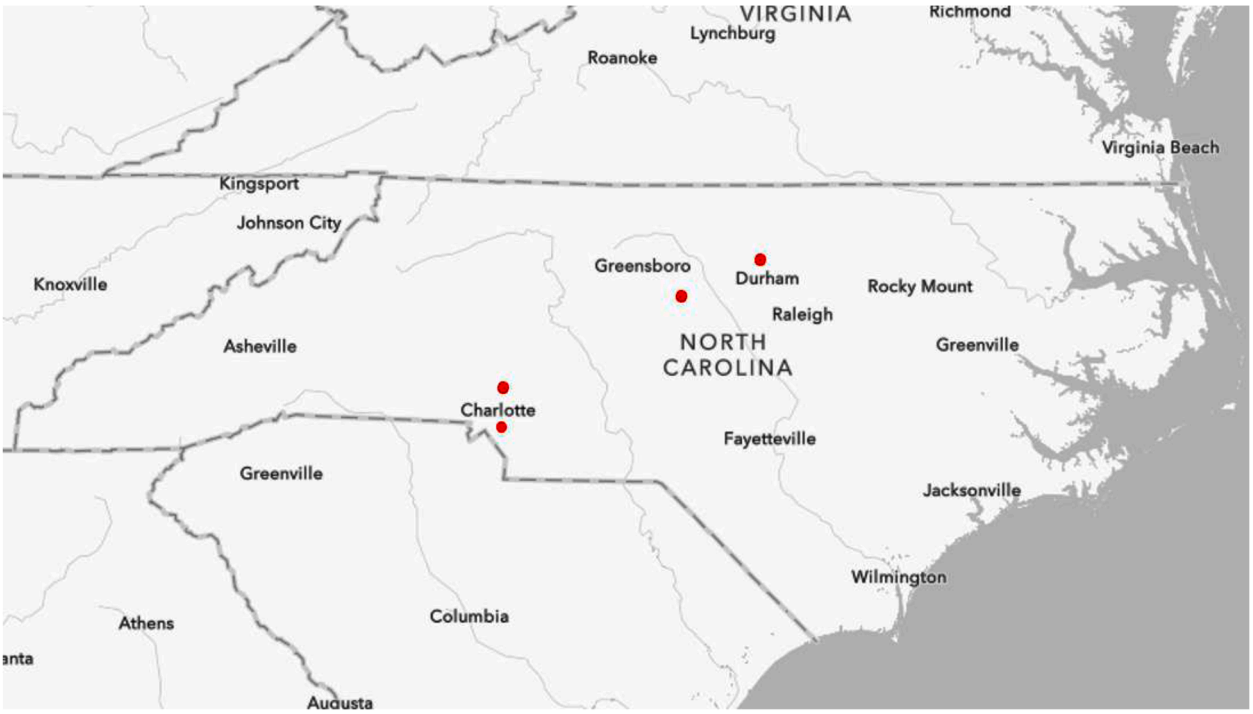

Fig. 1. Sampling locations for the Raleigh-Durham-Chapel Hill Metropolitan Area RDCMA (RTP1 and RTP2) and Charlotte (Charlotte1, Charlotte2) wastewater treatment plants.

each extraction batch. The final elution volume for each extraction was 100 μL, which was then divided into 30 μL aliquots and stored at −80 °C.

2.4. Detection and quantification using qPCR and RT-qPCR

For human adenoviruses and bacterial pathogen targets stx2, Campylobacter, and Salmonella, extracted DNA samples were diluted five-fold and quantified by qPCR. Enterovirus and norovirus were quantified using a two-step RT-qPCR. Detailed method descriptions are included in the supplementary material. A five-point standard curve ranging from 10^4 to 3 gene copy (gc)/reaction was included with samples in each reaction plate for each pathogen assay. IDT gblocks were used for standard curves and positive controls (IDT, Coralville, IA, USA). For crAssphage, the standard curve ranged from 10^7 to 10^3 gc/reaction due to its abundance in untreated wastewater. The assays efficiencies were 90–110% and linearities (R-squared values) were above 0.98 based on standard curves. Detection limit was three copies per reaction or 1000 gc/L (lower measurement limit). Inhibition of the samples was determined by performing HF183 internal amplification control (IAC) multiplex assay as described in EPA Method 1696. The inhibition of the samples was evaluated by comparing the IAC threshold as previously described (EPA, 2019). Three no template controls (NTCs) using molecular-grade water were included with each instrument run. Untreated wastewater has high organic content, and it often leads to inhibition in qPCR analysis (Uchii et al., 2019). We found inhibition in all the undiluted samples. Therefore, after performing a range of dilutions in the extracted samples, a fivefold dilution was determined to provide the best balance between the detection of the target and dilution of the inhibition.

2.5. Statistical analyses

All statistics were computed using RStudio (version 2022.2.0.443). qPCR data was $\log_{10}$ transformed and its normality was tested by the QQ-plot function. The microbial targets were grouped into four different seasons, spring (March to May 2021), summer (June to August 2021), fall (September to November 2021), and winter (December 2021 to February 2022). For spatial variation, samples were grouped into four different sites (RTP1, RTP2, Charlotte1, Charlotte2). A one-way analysis of variance (ANOVA) was used to investigate significance between mean concentrations of select pathogens in wastewater samples. Tukey's honestly significant difference test (Tukey's HSD) was used to find means that are significantly different from each other for seasonal and spatial variation comparisons. Correlation structure between the wastewater indicator crAssphage and enteric pathogens (pair by pair) was evaluated using Spearman r coefficient as a non-parametric measure for its robustness toward influential data points and outliers (Singh et al., 2004) with P-value cutoffs of 0.05. Finally, for measurements reported as non-detects or below the lower measurement limit, the non-detected (ND) designation was substituted with half of the square root of the detection limit (15.8 gc/L). Non-Metric multidimensional scaling (NMDS) analysis was performed on analytical composites of the enteric pathogen assays (human adenovirus, enterovirus, norovirus, *stx*$_2$, *Salmonella, Campylobacter*). A confidence interval of 0.7 was used to delineate data ellipses.

3. Results and discussion

The objectives of this study were to characterize the temporal and spatial dynamics of enteric pathogens in untreated wastewater in North Carolina, and to evaluate the correlations between crAssphage and enteric pathogens in wastewater throughout different seasons. No-template controls were negative in each assay suggesting no cross-contamination. The limit of detection (LOD) was 3 gene copies (gc)/reaction for pathogen assays and samples below this concentration were considered non-detects. Since crAssphage was always detected in high abundance, LOD was not applied to crAssphage.

3.1. Concentrations and prevalence of crAssphage and enteric pathogens

The sampling sites in North Carolina are in the humid sub-tropical climate zone, with very warm summers and moderately cold winters.

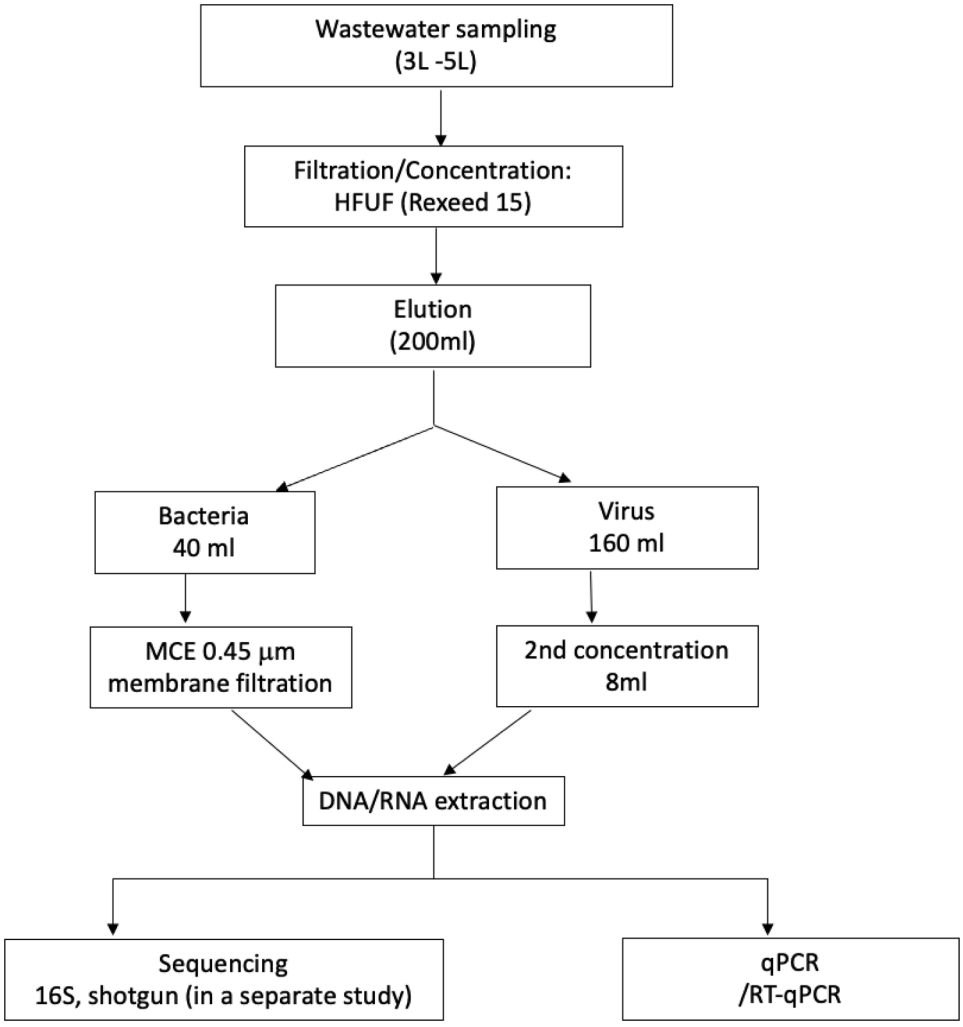

Fig. 2. Wastewater sample processing workflow.

We collected 24-h composite samples. Based on previous studies, virus abundance in composite samples is more representative of viral concentrations than grab samples (Ahmed et al., 2021). All untreated wastewater samples were positive for the crAssphage marker (Table 2; Fig. 3.). We observed the lowest crAssphage concentrations at Charlotte1 on 5/5/2021, and the highest concentration at Charlotte2 on 8/9/2021 (Tables 1 and 2). The crAssphage concentrations reported in our study (10^7–10^{10} gc/L in untreated wastewater) aligns with concentrations reported by others, including studies in Florida, Italy, and Japan (Ahmed et al., 2018a; Crank et al., 2020; Malla et al., 2019). High abundance of crAssphage marker in untreated wastewater suggests that crAssphage could serve as an indicator for wastewater contamination in subtropical climate regions (Sabar et al., 2022).

The concentrations of viral and bacterial pathogen targets in wastewater are shown in Table 2, and enteric viruses showed high detection frequency (above 85%). The observed concentrations agreed with previous studies (McCall et al., 2021; O'Brien et al., 2017). Human adenoviruses are known to cause gastroenteritis and respiratory diseases (Ganesh and Lin, 2013; Maunula et al., 2008; Papapetropoulou and Vantarakis, 1998). The highest adenovirus concentration was observed on 8/9/2021 at Charlotte 2. Enterovirus can cause an array of afflictions depending on the virus type, including viral sinusitis (common cold), meningitis, and poliomyelitis, and have been linked to outbreaks of these diseases (Maunula et al., 2008). The highest enterovirus concentration was observed on 10/25/2021 at Charlotte 1. Noroviruses are one of the most significant gastroenteritis-causing viral agents, considered to

Table 2
Average Enteric Pathogen Concentrations in Untreated Wastewater.

	Mean (gc/L)	± SD (N)	Min (gc/L)	Max (gc/L)	Below quantification	Detection Frequency	LOD (gc/L)
CrAssphage	2.46E+08	1.84E+08	1.60E+07	9.05E+08	0	100%	1000
HAdV	2.16E+06	3.63E+06	0	2.73E+07	8	95%	1000
Enterovirus	5.57E+04	7.67E+04	0	3.98E+05	22	85%	1000
Norovirus	6.58E+04	8.86E+04	0	5.28E+05	8	95%	1000
stx$_2$	3.90E+04	1.30E+05	0	1.35E+06	8	95%	1000
Campylobacter	2.73E+03	2.21E+03	0	1.13E+04	51	65%	1000
Salmonella	5.31E+04	1.78E+05	0	2.02E+06	12	92%	1000

Gc/L: genome copy per liter.
LOD: lower detection limit.

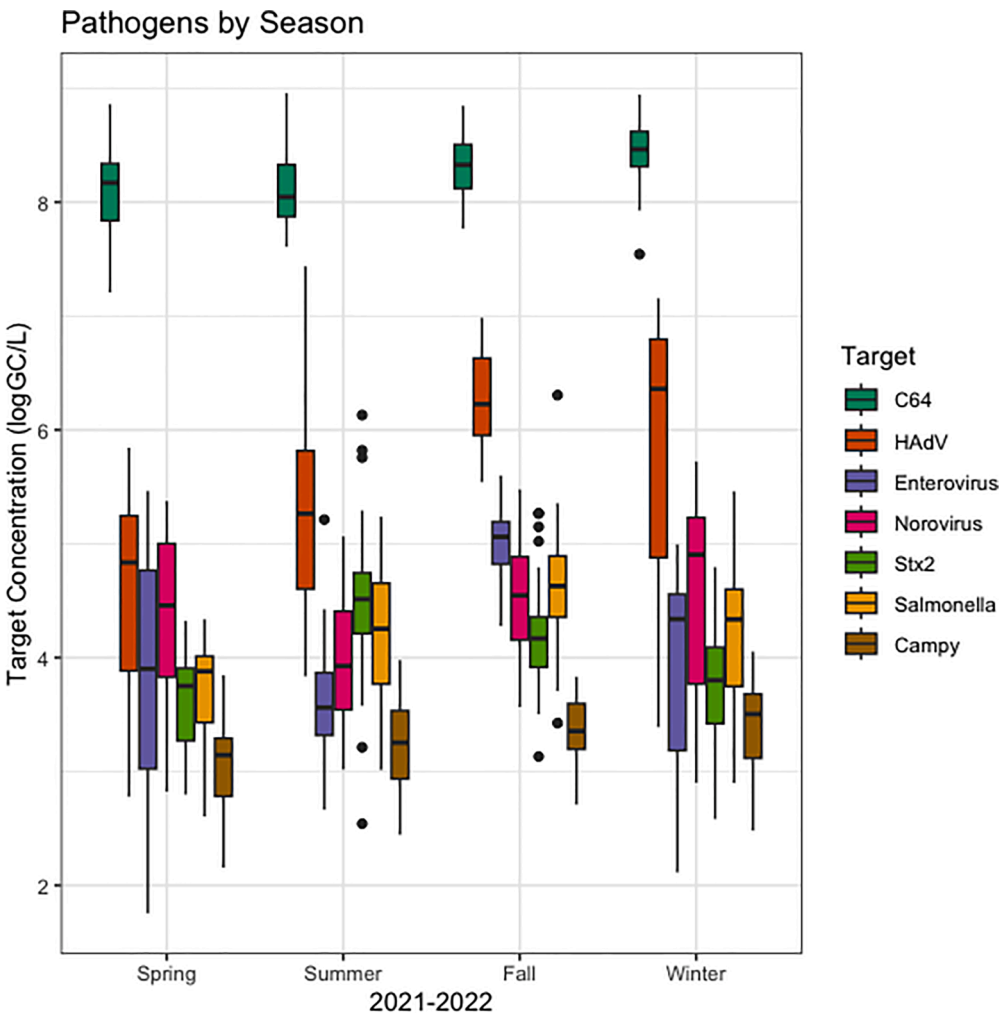

Fig. 3. Pathogen concentrations grouped by seasons. Box plot showing the median concentration (min-max) of crAssphage (C64, green), human adenovirus (HAdV, orange), enterovirus (purple), norovirus (pink), stx_2 (green), *Salmonella* (yellow), and *Campylobacter* (Campy, brown). Samples from the four treatment plants were combined and plotted for the same target. Sample concentration below the lower limit of quantification was considered 1.5 log gc/L. Circles indicate outliers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

be a leading cause of food borne and waterborne disease outbreaks (Kukkula et al., 1999; Maunula et al., 2008). Noroviruses have been commonly investigated and widely detected in wastewater (Cashdollar et al., 2013; Katayama et al., 2008). The highest concentration of norovirus was observed at RTP2 on 12/22/2021.

Bacterial pathogen signals stx_2, *Campylobacter,* and *Salmonella* were also prevalent in untreated wastewater (Table 2). The observed concentrations agreed with previous studies (Diemert and Yan, 2020; Ginn et al., 2021; Yan et al., 2018). Shiga Toxin genes stx_2 are involved in the pathogenicity of some enteric bacteria such as *E. coli* O157:H7, O104:H4 and *Shigella dysenteriae* (McLellan et al., 2007; Spears et al., 2006). The highest stx_2 concentration was observed on 8/9/2021 at Charlotte2. *Campylobacter spp*. are gram-negative bacteria that can cause gastroenteritis and may lead to Guillain-Barré syndrome, as well as reactive arthritis and irritable bowel syndrome (Farhadkhani et al., 2020). The highest *Campylobacter* concentration was observed on 1/24/2022 at Charlotte1. *Salmonella enterica* consists of a diverse group of serotypes that are ubiquitous in nature and is a common cause of human salmonellosis, which leads to considerable morbidity and mortality (Hoorfar et al., 2000). The highest concentration of *Salmonella* was observed on 11/10/2021 at RTP2.

Wastewater monitoring of enteric pathogens could provide a snapshot of the community's health status (Chen et al., 2014; O'Brien et al., 2017). Enteric infections are highly contagious and enteric viruses require low infectious doses to cause infection (Yezli and Otter, 2011). Globally, viruses have been found as the most abundant and hazardous pathogens in wastewater, and viruses have been identified to cause more outbreaks than bacteria (Adegoke et al., 2018; Eftim et al., 2017; Xagoraraki and O'Brien, 2020). In this study, the detection rates and concentrations of viral and bacterial pathogens were high as measured by qPCR (Table 2). More specifically, human adenoviruses were one or more magnitudes higher than stx_2, *Campylobacter, and Salmonella* in the same WWTP; enterovirus and norovirus were also detected in high concentrations (Fig. S1). This patten indicates that viral loads could be more prevalent than bacterial loads as causes for enteric diseases in the studied area. A number of factors may influence the difference between the observed viral and bacterial loads, including number of infected individuals within the community, differences in the fate and transport of each target, and differences in the shedding loads of bacteria and viruses (Brouwer et al., 2022; Gopinath et al., 2012; Mukhopadhya et al., 2013). The variations in target amplicon genomic copies per organisms could also alter the prevalence of the pathogen detection. Viruses and bacteria have different mechanisms of genome replications, and the number of target amplicons does not always equate the number of organisms (Bernabeu et al., 2019; Challberg and Kelly, 1989; Reyes-Lamothe et al., 2012).

3.2. Correlations between crAssphage, viral, and bacterial pathogens

Concentrations of crAssphage in untreated wastewater samples were approximately two to four magnitudes higher than concentrations of enteric pathogens (Fig. 3). Significant correlations were observed between crAssphage and enteric viruses ($p<0.0001$). Spearman's rank correlations were 0.51, 0.24, and 0.44 between crAssphage and human

adenovirus, enterovirus, and norovirus, respectively (Table 3). Similar correlation patterns between crAssphage and human enteric viral pathogens were reported in a study conducted in river water and estuarine environments in U.K. (Farkas et al., 2019), however our paper studied enterovirus and bacteria pathogens as well. A strong, significant positive correlation was also noted between human adenovirus and norovirus (Rho=0.57), enterovirus (Rho=0.41), and *Salmonella* (Rho=0.54); as well as between norovirus, enterovirus and *Salmonella* (Table 3).

Enteric bacteria were also positively associated with crAssphage as determined by Spearman correlation analysis (Table 3). The correlation of enteric viruses and enteric bacteria has been studied in clinical samples via metagenomic approach. For example, Gao et al., 2021 conducted a gut microbiome analysis with clinical samples and showed that gut bacteria were negatively associated with viruses in colorectal cancer patients and health control groups (Gao et al., 2021). In this study, crAssphage and bacterial pathogens in wastewater were measured in the same sample by qPCR, which offers a quantitative approach that metagenomic analysis does not offer. Identifying an organism that can be used as an indicator for both bacterial and viral contaminants is a cost-effective approach for monitoring efforts saving both time and resources. The samples in this study were collected from municipal WWTPs which provided a comprehensive view of the pathogen dynamics of the serviced community, rather than from individual clinical patients. Further studies are needed to validate the crAssphage marker as an effective indicator for bacterial pathogens using various types of environmental samples, such as wastewater impacted surface waters and groundwater.

CrAssphage did not show significant correlation with stx_2 ($P = 0.77$). Since stx_2 is a toxic gene, it could be present in a variety of hosts (i.e., *E. coli, Shigella and* coliphages) and thus will have different fate and transport pathways in comparison to individual bacterial pathogens (Garcia-Aljaro et al., 2004).

3.3. Temporal variation of crAssphage and enteric pathogens in wastewater

The majority of the acute gastroenteritis among adults and children are caused by viruses and bacteria and have a substantial impact on public health (Victoria et al., 2009). The concentrations of human enteric pathogens in environmental water samples and clinical samples have been shown to have seasonal variation, suggesting that infections resulting from enteric etiologic agents are more prevalent at certain times of the year (Hata et al., 2021; Li et al., 2011; Thwiny et al., 2022). In this study, crAssphage, human adenovirus, enterovirus, norovirus, stx_2, *Campylobacter, and Salmonella* showed strong seasonal variation ($p<0.01$) and significant differences among seasons depending on the targets (Table 4). Concentrations of crAssphage in wastewater during the fall and winter months were significantly higher than those during the spring and summer months ($p<0.05$). This contrasts with a fecal indicator evaluation study from river and estuarine water in the U.K. that showed crAssphage had no distinct seasonal pattern (Farkas et al., 2019). The difference between the two studies might be due to differences between the climate zones and sampling schedule frequency. North Carolina is in a subtropical climate zone while UK is in a temperate climate zone. The UK study sampled once per month which was less intensive compared to the sampling frequency conducted in this study (once per week or every other week), which provided us with higher temporal resolution.

Table 4
One way ANOVA on seasonal and spatial variation probability values and dominant seasons.

	WWTPs	Seasons	Dominant Seasons
CrAssphage	0.084	0.000009*	winter and fall
HAdV	0.144	0.000000*	winter and fall
enterovirus	0.817	0.000000*	fall, winter, and spring
norovirus	0.080	0.000031*	spring, fall and winter
stx_2	0.027*	0.000000*	summer
Campylobacter	0.184	0.027400*	winter
Salmonella	0.095	0.000000*	fall

Star.
* Indicate p value less than 0.01.

A study conducted in Japan found that norovirus concentrations in wastewater were highest during the months of November to April, while enterovirus and adenovirus concentrations were largely consistent throughout the year (Katayama et al., 2008). A 9-year study in source water for potable use, and untreated and treated wastewater in Milwaukee, Wisconsin, found that enteroviruses and adenoviruses were highest during the months of July to December (Sedmak et al., 2005). In our study, viral pathogens and bacterial pathogens in untreated wastewater showed strong seasonality (Table 4), with peak concentrations of human adenovirus in fall and winter, while enterovirus and norovirus were highest in fall, winter, and spring, *Campylobacter* in winter, and *Salmonella* in fall. Shiga Toxin gene stx_2 increased during the warmer seasons with peak concentrations during Summer (Table 4). Elevated concentrations in human adenovirus, enterovirus, norovirus, and *Salmonella* were observed at the onset of the fall season. Lower temperatures and beginning of the school season in the fall may have an influence on the pathogen concentration dynamics and need to be further investigated. A better characterization of parameters associated with human social behaviors and transmission routes might help explain the seasonality of these pathogens. In addition, environmental factors such as predation, temperature, turbidity in various built and natural environments may also impact the seasonal variation observed in these pathogens (Jagai et al., 2017; Sommerfeld and Kroeger, 2012).

The high prevalence and concentration of stx_2 gene identified in our samples suggest that *shiga toxin-producing organisms (e.g. enterohemorrhagic E. coli, Shigella, Aeromonas, etc.)* could be associated with a higher risk of gastroenteritis disease during the summer (García-Aljaro et al., 2006; 2009). Climate change is leading to more extreme weather events that, in turn, may trigger a higher frequency of combined sewage overflows (CSO) and sanitary sewage overflows (SSO). As a result, climate change may be considered a driver for deteriorating water quality, along with the effects of increased global temperatures which have been found to produce a shift in virulence and die off rates of viral

Table 3
Spearman's rank correlation of pathogens in untreated wastewater (number of samples).

		crAssphage	HAdV	enterovirus	norovirus	stx_2	*Campylobacter*	*Salmonella*	Influent flow rate
Wastewater indicator	crAssphage	1	0.51 (139)*	0.24 (125)*	0.44 (139)*	−0.02 (139)	0.25 (96)*	0.36 (135)*	−0.01 (147)
Viral pathogen	HAdV		1	0.41 (121)*	0.57 (135)*	0.17 (133)*	0.27 (95)*	0.54 (130)*	−0.21 (139)*
	enterovirus			1	0.48 (121)*	0.05 (120)	0.14 (86)	0.38 (117)*	−0.12 (125)
	norovirus				1	−0.02 (132)	0.3 (92)*	0.4 (130)*	0.06 (139)
	stx_2					1	0.05 (95)	0.29 (131)*	0.07 (139)
Bacterial pathogen	*Campylobacter*						1	0.36 (92)*	0.09 (96)
	Salmonella							1	−0.11 (135)
	Influent flow rate								1

* Denotes a significant Spearman's rank correlation coefficient $p < 0.05$.

and bacterial pathogens (Hutchins et al., 2019). The pathogen seasonality identified in our samples is important for beach and recreational water quality monitoring programs, and it can be particularly useful at locations that could be at risk of contamination by wastewater inputs due to extreme weather events, such as tropical beaches. Awareness of significantly higher seasonal concentrations of enteric pathogen markers, can inform water quality monitoring programs to ensure a safe recreational water environment for the public. The information from our study may assist water managers with making evidence-based decision to control risk associated with microbial contamination and minimize incidence of human waterborne diseases based on seasonal trends. Our findings can also advise health care workers to adjust precautions and preventative measurements throughout the year for viral and bacterial infections.

The NMDS analysis showed that wastewater samples cluster together based on seasons (Fig. 4). The NMDS analysis offers a map that spatially conveys the relationships between the collected wastewater samples in a reduced two-dimensional space with objective insights into the samples' dissimilarity. In this study, pathogen assays (human adenovirus, enterovirus, norovirus, *stx$_2$, Campylobacter, Salmonella*) are used as variants and the Bray-Curtis distance between the samples are calculated. The stress level is 0.155 from the NMDS test, which indicates that two dimensions are appropriate for the pathogen dataset. Results show overlapping pathogen communities during fall and winter, with spring and summer samples starting to separate, and summer and fall samples distinctly separating from each other. This finding illustrates how seasonal changes alter the dynamics and drive transitions in the community of enteric pathogens, thus offering beach managers and public health officers with decision support regarding the modification or implementation of advisories when surface waters become contaminated with untreated wastewater.

3.4. Spatial variation of crAssphage and enteric pathogens in wastewater

There was no significant difference in crAssphage marker concentrations among the four WWTPs in North Carolina ($p = 0.084$), even though the service population and daily flowrates in the two WWTPs in Charlotte are larger than at the RTP ones (Table 1). Also, data collected did not show significant difference among the four WWTPs for human adenovirus, enterovirus, norovirus, *Salmonella, and Campylobacter* (Table 4). Although the RDCHMA has been developing rapidly in the past decades, the city of Charlotte is more urbanized and industrialized. Our results indicate that the differences in WWTP size, daily flowrate, and level of urbanization did not influence the abundance and prevalence of crAssphage and did not produce significant spatial variability among markers. Similarly, a study that screened a public human gut metagenomic database found that crAssphage was similarly associated with industrialized and rural lifestyles, and there was no strong biogeographic diversity within global crAssphage strains (Honap et al., 2020). Our results suggest that untreated wastewater shares common microbial components across regional locations (i.e., statewide) and water quality data retrieved from one wastewater treatment plant can be applied to another wastewater treatment plant. Because the size of the studied WWTPs and service population in this study are relatively similar (Table 1), a study including a higher geographic diversity with different climate zones and a larger range of WWTP service populations would be essential to offer a more comprehensive spatial assessment.

Shiga toxin gene *stx$_2$* concentrations were the only ones that showed a significant difference among WWTPs ($p = 0.027$), with p-values of 0.003 for RTP2 and Charlotte2 and 0.005 for RTP2 and Charlotte1 from the post-hoc Tukey test. The spatial differences among plants could be because the *stx$_2$* gene can be present in a variety of pathogens sources, such as *E. coli* O157:H7, O104:H4 and *Shigella dysenteriae* (McLellan et al., 2007; Spears et al., 2006). The multi-host feature of *stx$_2$* may result in different fate and transport pathways and a larger concentration range compared to other pathogens in the sewer system.

3.5. Correlations between microbial targets and influent flowrates

We found that WWTP influent flowrates had significant correlations with human adenovirus ($p = 0.0128$). Wastewater influent flowrate is one of the key components to characterize the dynamic of pollutant transport in urban sewersheds (Ogidan and Giacomoni, 2015; Sansalone and Buchberger, 1997). Hydrological conditions such as flowrates have been heavily investigated in stormwater monitoring systems (Wu et al., 2018), and they could be useful in wastewater flow driven systems to explain trends and relationships, and to interpret and normalize the levels of diverse pathogens in wastewater. For example, concentration levels of pathogens may increase because the flow brings higher viral and bacterial loads. In contrast, the concentrations may decrease due to the dilution created by a higher flow during hydraulic transport (Goettle and Krauth, 1981). The correlation coefficient between flowrate and human adenovirus was −0.21 (Table 3). The negative correlation indicates a dilution in the source of human adenovirus, meaning that there are not enough viral particles available in the sewershed to maintain the same concentration when the water in the system increases (e.g., morning and evening domestic rush hour usage) during the sampling period. The limitation of this flow and transport analysis is that we did not capture the tail end of the occurrence because our study was designed to capture only 24hr composited samples. Establishment of a regular and long-term monitoring program with more frequent sampling periods would be helpful to capture the fate and transport of pathogens in the built environment and understand better environmental impacts (e.g., stormwater), breaches in the sanitary system, or disease outbreaks

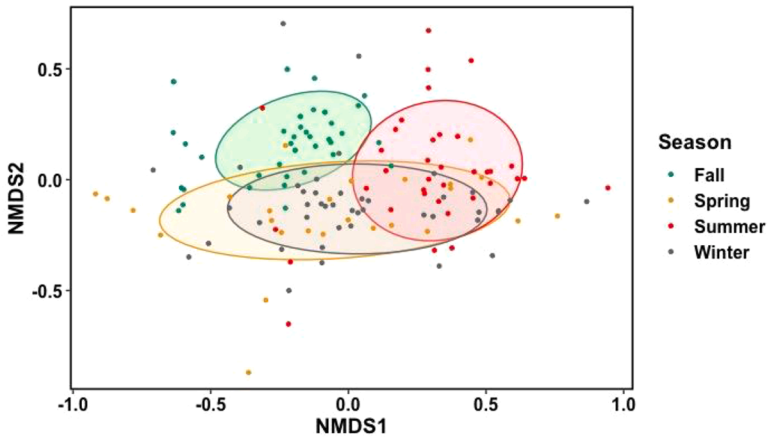

Fig. 4. Pathogen assays in raw wastewater (human adenovirus, enterovirus, norovirus, *Campylobacter, Salmonella*, and *stx$_2$*) composition among four seasons visualized by Non-Metric Multidimensional Scaling (NMDS). The plot is based on Bray-Curtis dissimilarity matrices of log 10 transformed qPCR results. Samples below detection limit were transformed to 1.5 log gc/L. Ellipsoids represent a 70% confidence interval surrounding the data point of each season group. NMDS ordination stress value is 0.155. CrAssphage assay is not included into the distance score calculation since it is not considered a waterborne pathogen.

in the population.

4. Conclusions

- This study presents evidence for the presence of crAssphage in untreated wastewater samples in an understudied region of US, the humid subtropical climate region (North Carolina, USA). We found crAssphage concentrations two to four orders of magnitude higher than other microbial contaminants, supporting the application of crAssphage as a microbial water quality indicator.
- CrAssphage and microbial pathogen concentrations displayed distinct seasonal patterns in untreated wastewater samples, but no distinct spatial patterns. Fall, winter, and spring had significantly higher pathogen concentrations than summer ($p<0.05$). Regardless of the variability identified, crAssphage had significant correlations with human adenovirus, enterovirus, norovirus, *Campylobacter*, and *Salmonella*, demonstrating that crAssphage could be a water quality indicator for surface waters contaminated with untreated wastewater across seasons.
- *Stx*$_2$ did not show correlations with crAssphage, which requires further investigation of other fecal indicators for the monitoring of outbreaks associated with s*tx*$_2$ and underlines one of the possible limitations of crAssphage as a water quality indicator of broad application.
- The findings of this research can provide baseline information for the development of new approaches for bacterial and viral pathogen monitoring efforts, quantitative microbial risk assessment, and strategies for pathogen removal during treatment processes. These results can also inform microbial modeling efforts and enhance prediction of the impact of wastewater pathogens on surface waters due to increased flooding events associated with climate change in urban scenarios.

CRediT authorship contribution statement

Huiyun Wu: Sample collection, processing, and extraction, qPCR, conceptualization, writing-original draft. **Md Ariful Islam Juel:** Sample collection, processing, and editing. **Stephanie Eytcheson:** Sample extraction and qPCR. **Marirosa Molina:** Project administration, supervision, conceptualization, writing and editing. **Mariya Munir:** Conceptualization, supervision, and editing. **Tiong Gim Aw:** Review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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