Enteric viruses in municipal wastewater effluent before and after disinfection with chlorine and ultraviolet light

Albert Simhon, Vince Pileggi, Cecily A. Flemming, José R. Bicudo, George Lai and Mano Manoharan

ABSTRACT

In Ontario, Canada, information is lacking on chlorine and ultraviolet (UV) light disinfection performance against enteric viruses in wastewater. We enumerated enteroviruses and noroviruses, coliphages, and *Escherichia coli* per USEPA methods 1615, 1602, and membrane filtration, respectively, in pre- and post-disinfection effluent at five wastewater treatment plants (WWTPs), with full-year monthly sampling, and calculated $\log_{10}$ reductions (LRs) while WWTPs complied with their monthly geometric mean limit of 200 *E. coli*/100 mL. Modeling of densities by left-censored estimation and Bayesian inference gave very similar results. Polymerase chain reaction (PCR)-detected enteroviruses and noroviruses were abundant in post-disinfection effluent (mean concentrations of $2.1 \times 10^{+4}$–$7.2 \times 10^{+5}$ and $2.7 \times 10^{+4}$–$3.6 \times 10^{+5}$ gene copies (GC)/L, respectively). Chlorine or UV disinfection produced modest LRs for culture- (0.3–0.9) and PCR-detected enteroviruses (0.3–1.3), as well as noroviruses GI + GII (0.5–0.8). Coliphages and *E. coli* were more susceptible, with LRs of 0.8–3.0 and 2.5, respectively. Sand-filtered effluent produced significantly higher enteric virus LRs (except cultured enteroviruses). Coliphage and human enteric virus densities gave significantly positive correlations using Kendall's Tau test. Enteric viruses are abundant in wastewater effluent following routine chlorine or UV disinfection processes that target *E. coli*. Coliphages appear to be good indicators for evaluating wastewater disinfection of enteric viruses.

Key words | coliphage, disinfection, enterovirus, norovirus, wastewater

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INTRODUCTION

In February 2018, there were approximately 290 disinfecting, mechanical wastewater treatment plants (WWTPs) in Ontario, Canada, of which 207 provided secondary treatment and 83 provided tertiary treatment with sand filtration. WWTPs are required to disinfect their effluent, particularly during times when recreational water activities occur (generally end of May to the beginning of September). Of the 290 WWTPs, 105 used chlorine-based products such as hypochlorite, while 185 used UV light. The Ontario Ministry of the Environment, Conservation and Parks (MECP) regulates effluent discharge quality at each facility through site-specific Environmental Compliance Approvals (ECA) that includes: (i) a variable microbiological compliance limit, which is typically 200 *Escherichia coli*/100 mL, as a monthly geometric mean (GM), based on a minimum of four weekly samples, before final effluent is discharged into a receiving water body and (ii) regulatory limits on chemical and physical parameters (Ontario 2008), such as 5-day carbonaceous biochemical oxygen demand ($cBOD_5$), total suspended solids (TSS), total phosphorus (TP), total ammonia nitrogen (TAN), unionized ammonia nitrogen (NH_3-N), as well as nitrates, nitrites, and pH, among others. As well, if chlorination is practiced, Ontario WWTPs must have a dechlorination step to comply with

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federal Wastewater Systems Effluent Regulations (WSER) stipulating that the average concentration of total residual chlorine cannot exceed 0.02 mg/L, where the averaging of data values for a given reporting period is dependent on plant size (Government of Canada 2012).

Chlorine and UV light kill/inactivate enteric bacteria and viruses including many waterborne viruses of public health importance. Because of concerns over chlorine toxicity (Government of Canada 2013) and the stringent WSER federal regulation cited above, several chlorinating WWTPs in Ontario have recently expressed interest in using alternative disinfection technologies, such as peracetic acid, besides UV light and ozone. However, before new disinfection technologies can be considered, MECP requires consistent evidence supporting that the new technology will perform in an equivalent or superior manner compared with established disinfection technologies such as chlorine, UV light, and/or ozone. MECP does not currently require testing for enteric viruses, but Ontario data from the 1970s indicate that culturable enteroviruses were detected in 5 of 102 secondary-treated effluent samples (Ontario 1975).

Although a number of studies in North America and other parts of the world have looked at the efficacy of chlorine and UV light for inactivation of enteric viruses in wastewater – reviewed by Crockett (2007) and Zhang *et al.* (2016), there is no specific information for Ontario conditions. Thus, between November 2014 and November 2017, MECP conducted a study to enumerate enteric viruses in pre- and post-disinfection secondary or tertiary effluents at five full-scale WWTPs that were routinely meeting their monthly GM limit of 200 *E. coli*/100 mL. Viruses of interest included human enteroviruses and noroviruses (GI and GII), as well as coliphage viral indicators of fecal contamination. These were investigated by both conventional culture and molecular methods, as appropriate.

METHODS

Wastewater treatment plants

Five municipal WWTPs (Plant A–Plant E) in West-Central Ontario were selected for sampling. Table 1 summarizes their characteristics and operational parameters. These WWTPs comply with Ontario's *Design Guidelines for Sewage Works 2008* (Ontario 2008).

Sampling of wastewater effluent

Each of the WWTPs was sampled monthly for 12 consecutive months. Sampling of the five plants was staggered, commencing in November 2014 and concluding in November 2017. Table 2 summarizes types (pre- and post-disinfection), location points and volumes obtained during sampling events. For enteric virus and coliphage enumeration, two large volume samples (31–150 L of pre-disinfection and 34–151 L of post-disinfection secondary or tertiary effluent) were filtered through electropositive Nanoceram® VS2.5-5 filters (Argonide Corp., Sanford, FL, USA). For *E. coli* enumeration, two grab samples in 250 mL plastic bottles with added thiosulfate were obtained (pre- and post-disinfection effluent); and for chemical analyses, three grab samples in 500 mL plastic bottles were taken (pre-disinfection only).

Flow rates through Nanoceram® filters were up to 4 L/min. When sampling effluents disinfected with chlorine, a DEMA 203B injector (DEMA Engineering, St. Louis, MO, USA) was used to quench any chlorine residual with 2% sodium thiosulfate at a rate of 2.4 mL/min, prior to effluent entering the Nanoceram® filter housing (USEPA 2014).

During 60 monthly sampling events, we experienced two individual filter malfunctions, one each at secondary Plants B and E. Thus, the total number of paired (pre- and post-disinfection) samples for three secondary plants was 34 out of a possible 36 (12×3). For tertiary treatment plants, the number of paired samples was 24 out of 24 (12×2).

Filtration was performed according to USEPA Method 1615 (USEPA 2014). Enteric viruses and coliphage from Plants A–D were tested and enumerated at the laboratories of SMI – Scientific Methods Inc., Granger, IN, USA, while those of Plant E were tested and enumerated at BCS Laboratories Inc., Gainesville, FL, USA. All filtered samples were shipped on ice by overnight air transport. Grab (liquid) samples were shipped by overnight ground transport at ambient temperature, except those destined for BCS, which were shipped by overnight air. At both SMI and BCS laboratories, samples were processed within 48 h of collection. Samples for *E. coli* enumeration and chemical analyses were

Table 1 | Characteristics and operational performance of selected WWTPs, Ontario, 2014–2017

Plant code and parameter	Plant A	Plant B	Plant C	Plant D	Plant E
Types of treatment	Conventional + SF	Conventional	Conventional + SF	Conventional	Conventional (EA)
Effluent quality	Tertiary	Secondary	Tertiary	Secondary	Secondary
Disinfection type	UV	UV	Chlorine	Chlorine	Chlorine
Intensity/dosage	30–40 mJ/cm^2	30–40 mJ/cm^2	3.1 mg/L	5.8 mg/L	7.3 mg/L
Contact time	0.23 s	0.23 s	15 min	10 min	13 min
Receiver	Creek	Great Lake	River	River	River
Study period	November 2014–October 2015	November 2014–October 2015	May 2015–April 2016	July 2015–June 2016	December 2016–November 2017
Rated capacity, m^3/day	22,727	24,548	7,200	81,800	9,320
Mean daily flow, m^3	16,900	15,040	3,040	42,610	6,750
$cBOD_5$ mean ± SD, mg/L	1.2 ± 0.4	4.2 ± 2.8	2.1 ± 0.1	3.9 ± 2.3	3.5 ± 1.4
Range	1.0–2.0	2.0–11.7	2.0–2.3	2.2–10.4	2.0–5.8
Regulatory effluent limit	5	25	25	25	25
TSS mean ± SD, mg/L	1.7 ± 0.5	6.5 ± 5.8	2.4 ± 0.4	6.9 ± 3.7	5.9 ± 1.4
Range	1.0–2.6	3.0–20.5	2.0–3.5	2.3–14	3.9–8.2
Regulatory effluent limit	5	25	25	25	25
TP mean ± SD, mg/L	0.11 ± 0.04	0.3 ± 0.18	0.08 ± 0.02	0.42 ± 0.14	0.39 ± 0.06
Range	0.05–0.15	0.10–0.75	0.05–0.12	0.2–0.60	0.30–0.49
Regulatory effluent limit	0.3	1	0.3	1	1
TAN mean ± SD, mg/L	0.10 ± 0.03	8.36 ± 4.92	0.08 ± 0.05	2.06 ± 1.78	1.68 ± 1.60
Range	0.08–0.14	0.34–12.0	0.05–0.2	0.18–5.23	0.12–4.86
Regulatory effluent limit			1.0, 2.0*		
UAN, mean ± SD, mg/L	0.001 ± 0.0				
Regulatory effluent limit	0.02				
pH mean ± SD	7.65 ± 0.06	7.20 ± 0.08	7.17 ± 0.12	7.55 ± 0.18	7.49 ± 0.19
Range	7.52–7.71	7.10–7.35	7.00–7.36	7.24–7.89	7.15–7.69
Regulatory effluent limit	6.0–9.5	6.0–9.5	6.0–9.5	6.0–9.5	6.0–9.5
E. coli monthly GM ± SD	1.3 ± 0.5	59.8 ± 75.3	2.4 ± 2.9	17.0 ± 19.2	48.3 ± 21.4
Range, CFU/100 mL	1.0–2.0	7.0–285	1.0–11	3.0–75.0	11.2–79.0
Regulatory effluent limit	200	200	200	200	200

SF, sand filtration; EA, extended aeration, i.e., no screening and primary clarification; $CBOD_5$, 5-day carbonaceous biochemical oxygen demand; TSS, total suspended solids; TP, total phosphorus; TAN, total ammonia nitrogen; UAN, unionized ammonia nitrogen; SD, standard deviation; GM, geometric mean; CFU, colony forming units.
*May–November, 1 mg/L; December–April, 2 mg/L.

transported on ice and processed at MECP laboratories in Toronto, Ontario, within 24 h of sample collection.

Recovery of poliovirus (matrix spike)

Details of pre- and post-disinfection samples for matrix spikes and recovery calculations for each of the five WWTPs are listed in Table 2. Briefly, for example, if 100 L of (pre- or post-disinfection) effluent was filtered through a Nanoceram® filter, then the corresponding matrix spike sample would also consist of 100 L (90 L of filtered effluent + 10 L of effluent grab-sample, the latter in a cubitainer or jerry can). Upon arrival at the virus testing laboratory, the 10 L liquid grab samples were spiked with 1,000 ± 100

Table 2 | Sample types, locations and volumes at five Ontario WWTPs

	Monthly sampling, $n = 12$				Matrix spike, $n = 1$	
	Volume filtered, L					
Plant	Pre-disinfection	Location	Post-disinfection	Location	Filtered, L Pre- and post-disinfection	Grab, L Pre- and post-disinfection
A	100–150	PSF	102–151	Post-UV	110	10
B	50–120	PSC	61–120	Post-UV	80	10
C	105–120	PSF	112–120	Post-Cl_2 CT	110	10
D	31–100	PSC	34–100	Post-Cl_2 CT	45	10
E	50	PSC	36–50	Post-Cl_2 quenching	40, 26*	10

PSF, post-sand filtration; PSC, post-secondary clarifier; Post-Cl_2 CT, post-Cl_2 contact tank (sodium thiosulfate injected prior to entering filter housing – see the text).
*Pre-disinfection volume = 40 L; post-disinfection volume = 26 L.

MPN (most probable number of infectious units) per mL of Sabin poliovirus type 3 (USEPA 2014) and the spiked 10 L volume passed through the respective Nanoceram® filter for matrix spike recovery. Elution, concentration, cultivation, and enumeration of spiked poliovirus were done as described below. Recovery for each pre- and post-disinfection matrix was calculated as described in USEPA Method 1615 (USEPA 2014).

Enumeration of enteric microorganisms

Enteric viruses

Enteric viruses were eluted from Nanoceram® filters with beef extract at pH 9.0 as described in USEPA Method 1615 (USEPA 2014). Out of a total of 1,000 mL of eluent, 900 mL were concentrated 30-fold to 30 mL by organic flocculation and divided as follows: 10 mL for enterovirus cell culture assay; 10 mL for reverse transcription and quantitative polymerase chain reaction (RT-qPCR); and 10 mL were archived at −70 °C. The remaining 100 mL of unconcentrated beef extract eluent was used for coliphage culture.

Culturable enteroviruses were grown on Buffalo Green Monkey (BGM) kidney cells (USEPA 2014). Briefly, 10 mL of concentrated eluent was used to inoculate 10 replicate flasks of BGM cells and the monolayers examined for the development of cytopathic effect for 2 weeks and then passaged again for confirmation with additional four dilutions. Virus concentration in each test sample was calculated as the MPN of infectious units per liter using EPA's MPN calculator (USEPA 2013), i.e., MPN/L equals MPN/mL times the assay sample volume (mL), divided by the volume (L) of the original water sample assayed. Non-detects were reported as less than '1' MPN/mL times the assay sample volume (mL), divided by the volume (L) of the original water sample assayed.

Molecular quantification of enteric viruses included a tertiary, centrifugal concentration step, followed by RT-qPCR (USEPA 2014). Briefly, of the 400 µL of final concentrate, 200 µL were used for RNA extraction and the remaining 200 µL were archived at −70 °C. RT-qPCR for enterovirus, norovirus GI, norovirus GII, and hepatitis G were assayed in triplicate RT and qPCRs using the primers/probes indicated in USEPA Method 1615. A synthetic hepatitis G Armored RNA® (Asuragen, Austin, TX, USA) was used to identify samples that are inhibitory to RT-qPCRs. To minimize false-positives, the average of cycle threshold (CT) values of the RT-qPCR containing sample cannot be greater than 1 CT value of the average control replicates containing nuclease-free water. If inhibition is detected, dilutions of the sample are reanalyzed until the less than 1 CT value criterion is met. When our two laboratories were asked about hepatitis G results, SMI indicated that some samples from Plants A–D showed inhibitory effects, but this was diluted out and the respective dilution factor entered in the calculations. BCS indicated that none of the samples from Plant E showed inhibitory effects and therefore did not require dilution. Gene copies per liter (GC/L) were calculated per Method 1615 taking into account the number of detected GC, total dilution factor for volume reductions and inhibitory effects (if required), as well as the original volume sample that was

assayed. Non-detects were reported as less than GC/L where the number of GC was set to '1', while keeping in the formula the dilution factors and original volume sample were assayed.

Coliphages

For coliphage enumeration, a modification of USEPA Method 1602 (USEPA 2001) was used. One hundred milliliters of the original unconcentrated beef extract eluent (see above) was adjusted to pH 7.0 and duplicate serial 10-fold dilutions made. Magnesium chloride, antibiotics, log-phase host bacteria (*E. coli* F_{amp} for F^+ (male-specific)) coliphages, i.e., both RNA and DNA bacterial viruses that infect via the F-pilus of male strains of *E. coli*.

E. coli CN-13 for somatic coliphages, i.e., DNA bacterial viruses that infect host cells via the outer cell membrane of *E. coli*) and an equal volume of double-strength molten tryptic soy agar were added. After overnight incubation, circular plaques were counted and summed for all plates from a single sample. The number of coliphages in a sample was expressed as plaque forming units per liter (PFU/L), where the total plate count is divided by the total volume analyzed (mL), and this ratio is multiplied by the total volume of eluent (mL) divided by the total volume filtered. Non-detects are reported as less than PFU/L where the total plate count is set to '1', while the rest of the factors are kept in the calculation. For quality control purposes, both a coliphage positive and a negative (method blank) reagent water samples were analyzed for each type of coliphage with each sample batch.

E. coli

E. coli in the original pre- and post-disinfection effluent was enumerated by a standard membrane filter procedure on m-FC agar containing BCIG (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) (APHA 2017).

Statistical analysis of measured microorganisms

Raw data for this study are available in Supplementary Material (available with the online version of this paper), including a detailed description of non-detect modeling using two different approaches: left-censored data and Bayesian inference. These two approaches above allowed the calculation of pre- and post-disinfection mean densities for chlorine or UV light-disinfected effluent. Finally, microorganism-specific mean $\log_{10}$ reductions (LRs) were calculated using the following equation:

$$\mathrm{LR} = \log_{10}(M_i/M_o) \quad (1)$$

where M_i and M_o are the pre- and post-disinfection mean densities, respectively.

In addition, we queried whether this study's concentrations of F^+ (male-specific) and somatic coliphages correlated with those of human enteric viruses and *E. coli*. This was tested in 'R' using the Kendall's Tau non-parametric correlation analysis in the NADA package, based on concordant and discordant pairs of observations in pre- and post-disinfection effluent.

RESULTS

For each microorganism, measured densities and non-detects were modeled as left-censored data and are reported here throughout. In addition, the datasets were modeled by Bayesian inference. Both approaches yielded very similar results (see comparison tables in Supplementary Material, available with the online version of this paper). LRs of human enteric viruses and coliphage viral indicators at each of the five WWTPs showed marked variability (including a negative LR for cultured enterovirus at Plant C), irrespective of whether chlorine or UV light was used for disinfection of wastewater effluent (Figure 1(a) and 1(b)).

We combined detected/modeled concentrations of enteric viruses by treatment type: secondary treatment (Plants B, D, and E) and tertiary treatment (Plants A and C). Descriptive statistics and LRs by treatment type for combined concentrations of human enteric viruses, as well as coliphage and *E. coli* indicators in pre- and post-disinfection effluent are presented in Tables 3 and 4, respectively. Differences in LR means of secondary and tertiary plants were significantly different for all microorganisms, except for NoV GII and *E. coli*, by Welch two-sample *t*-tests. Tertiary treatment plants had higher LRs against all microorganisms (except cultured enterovirus and *E. coli*) compared with

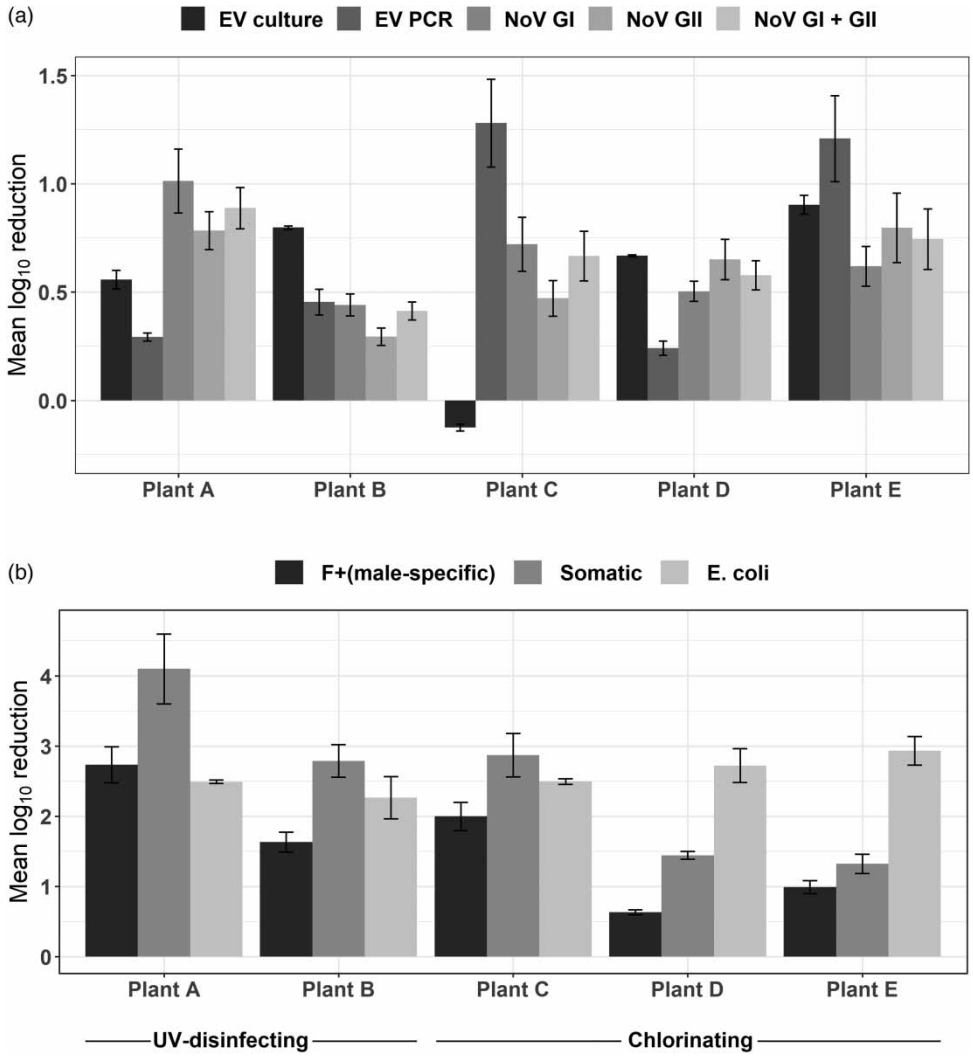

Figure 1 | Mean LRs by disinfection of human enteric viruses (a) and of coliphage (viral) and *E. coli* indicators (b) at five Ontario WWTPs, from 2014 to 2017. Error bars represent standard errors. EV, enterovirus; NoV GI, norovirus Group I; NoV GII, norovirus Group II; NoV GI + GII, norovirus Group I + Group II combined; F^+ (male-specific), F^+ (male-specific) coliphage; Somatic, somatic coliphage.

secondary treatment plants (Figure 2), likely because sand filtration in the former produced a higher quality effluent with less suspended solids.

Enteroviruses cultured on BGM cell monolayers were detected at mean densities of <1 infectious MPN/L in pre-disinfection effluent; however, they were still detectable, albeit at low concentrations, in 20 (59%) of 34 and 12 (50%) of 24 secondary post-disinfection, and tertiary post-disinfection effluent samples, respectively. Their mean LRs were considered poor, 0.9 and 0.3, respectively (Table 3). The method detection limit (MDL) for cultured enterovirus was 0.02–1.0 infectious MPN/L.

Enteroviruses by RT-qPCR were present in high densities in secondary- and tertiary-treated, pre-disinfection effluent, $1.4 \times 10^{+6}$ and $4.3 \times 10^{+5}$ GC/L, respectively, and were still detectable in 20 (59%) of 34 and 5 (21%) of 24 post-disinfection effluent samples, respectively, at mean concentrations of $7.2 \times 10^{+5}$ and $2.1 \times 10^{+4}$ GC/L. LRs were poor-to-modest, 0.3 and 1.3 (Table 3). The MDL ranged widely from 0.4 to 1,950 GC/L.

Combined norovirus GI + GII were present in high densities, $1.1 \times 10^{+6}$ and $1.7 \times 10^{+5}$ GC/L, and detectable in 31 (91%) of 34 and 12 (50%) of 24 secondary post-disinfection, and tertiary post-disinfection effluent samples, respectively.

Table 3 | Descriptive statistics using uncensored and modeled left-censored values for concentrations of human enteric viruses in pre- and post-disinfection effluent, Ontario, Canada, 2014–2017

	Plants B, D, and E combined, $n = 34$ (providing secondary treatment)			Plants A and C combined, $n = 24$ (providing tertiary treatment)			Secondary vs. tertiary plant differences in LR means; p-value; 0.95 CI Welch two-sample t-test
Human enteric virus	***d*** **(%)**	**nd (%)**	**mean (±SE)**	***d*** **(%)**	**nd (%)**	**mean (±SE)**	
Enterovirus culture, MPN/L							
Pre-disinfection	27 (79)	7 (21)	8.3×10^{-1} (2.4×10^{-1})	10 (42)	14 (58)	9.6×10^{-2} (3.2×10^{-2})	
Post-disinfection	20 (59)	14 (41)	1.1×10^{-1} (2.5×10^{-2})	12 (50)	12 (50)	5.3×10^{-2} (2.2×10^{-2})	
Mean LR			0.9 (0.05)			0.3 (0.03)	$p < 0.001$; 0.5–0.7
Enterovirus RT-PCR, GC/L							
Pre-disinfection	29 (85)	5 (15)	$1.4 \times 10^{+6}$ ($5.1 \times 10^{+5}$)	13 (54)	11 (46)	$4.3 \times 10^{+5}$ ($1.6 \times 10^{+5}$)	
Post-disinfection	20 (59)	14 (41)	$7.2 \times 10^{+5}$ ($4.1 \times 10^{+5}$)	5 (21)	19 (79)	$2.1 \times 10^{+4}$ ($1.3 \times 10^{+4}$)	
Mean LR			0.3 (0.03)			1.3 (0.2)	$p < 0.001$; 0.7–1.3
Norovirus GI, GC/L							
Pre-disinfection	32 (94)	2 (6)	$7.2 \times 10^{+5}$ ($2.2 \times 10^{+5}$)	17 (71)	7 (29)	$1.4 \times 10^{+5}$ ($5.4 \times 10^{+4}$)	
Post-disinfection	28 (82)	6 (18)	$2.5 \times 10^{+5}$ ($1.2 \times 10^{+5}$)	11 (46)	13 (54)	$2.0 \times 10^{+4}$ ($1.4 \times 10^{+4}$)	
Mean LR			0.5 (0.05)			0.8 (0.1)	$p = 0.002$; 0.2–0.6
Norovirus GII, GC/L							
Pre-disinfection	33 (97)	1 (3)	$3.8 \times 10^{+5}$ ($1.8 \times 10^{+5}$)	15 (62)	9 (38)	$3.0 \times 10^{+4}$ ($1.2 \times 10^{+4}$)	
Post-disinfection	30 (88)	4 (12)	$1.1 \times 10^{+5}$ ($4.6 \times 10^{+4}$)	10 (42)	14 (58)	$7.5 \times 10^{+3}$ ($5.0 \times 10^{+3}$)	
Mean LR			0.5 (0.1)			0.6 (0.1)	$p = 0.4$ (NS); (–)0.1–0.2
Norovirus GI + GII, GC/L							
Pre-disinfection	34 (100)	0	$1.1 \times 10^{+6}$ ($3.3 \times 10^{+5}$)	17 (71)	7 (29)	$1.7 \times 10^{+5}$ ($6.3 \times 10^{+4}$)	
Post-disinfection	31 (91)	3 (9)	$3.6 \times 10^{+5}$ ($1.4 \times 10^{+5}$)	12 (50)	12 (50)	$2.7 \times 10^{+4}$ ($1.9 \times 10^{+4}$)	
Mean LR			0.5 (0.04)			0.8 (0.1)	$p = 0.006$; 0.1–0.5

MPN, most probable number; GC, gene copies; n, number tested; d, number detected (percent); nd, number of non-detects (percent) that were modeled; SE, standard error; LR, $\log_{10}$ reduction; CI, confidence interval; NS, not significant ($p > 0.05$); counts are unadjusted for recovery – see the text.

Their respective means were $3.6 \times 10^{+5}$ and $2.7 \times 10^{+4}$ GC/L, with poor-to-modest mean LRs of 0.5–0.8 (Table 3). Tertiary treatment plants had higher norovirus LRs than secondary treatment plants. The reported MDL varied widely from 0.4 to 1,540 GC/L.

Table 4 summarizes results for coliphage viral indicator and *E. coli* bacterial indicator. F^+ (male-specific) and somatic coliphage at secondary treatment plants had mean LRs of 0.8 and 1.8, compared with mean LRs of 2.3 and 3.0, respectively, at tertiary treatment plants. The coliphage MDL was 0.2–9.0 PFU/L. *E. coli* bacterial indicator had a mean LR of 2.5, irrespective of treatment type, with an MDL of 1–4 CFU/100 mL.

Recovery of poliovirus (matrix spike)

Recoveries of poliovirus type 3 matrix spike grown on BGM cells after background deduction in pre- and post-disinfection effluent at each of the five plants were Plant A, 50% and 58%; Plant B, 50% and 52%; Plant C, 99.7% and 80%; Plant D, 39% and 25%; Plant E, 36% and 53%. Disinfection did not seem to exert a generalized inhibitory effect on recovery. Rather, there may have been filter-specific or manufacturing issues affecting recovery. For instance, throughout the three-year sampling period, there were several occasions when defective Nanoceram® filters were noticed, e.g., filters allowing very fast flow indicative of breaches, or filters failing to

Table 4 | Descriptive statistics using uncensored and modeled left-censored values for concentrations of coliphage and *E. coli* indicators in pre- and post-disinfection effluent, Ontario, Canada, 2014–2017

	Plants B, D, and E combined, $n = 34$ (providing secondary treatment)			Plants A and C combined, $n = 24$ (providing tertiary treatment)			Secondary vs. tertiary plant differences in LR means; *p*-value; 0.95 CI Welch two-sample *t*-test
Indicator organism	***d* (%)**	**nd (%)**	**mean (±SE)**	***d* (%)**	**nd (%)**	**mean (±SE)**	
F_+ (male-specific), coliphage, PFU/L							
Pre-disinfection	34 (100)	0	$2.6 \times 10^{+3}$ ($4.6 \times 10^{+2}$)	24 (100)	0	$2.9 \times 10^{+2}$ ($4.4 \times 10^{+1}$)	
Post-disinfection	27 (79)	7 (21)	$3.9 \times 10^{+2}$ ($1.3 \times 10^{+2}$)	11 (46)	13 (54)	1.5×10^{0} (6.8×10^{-1})	
Mean LR			0.8 (0.05)			2.3 (0.2)	$p < 0.001$; 1.1–1.9
Somatic coliphage, PFU/L							
Pre-disinfection	34 (100)	0	$2.9 \times 10^{+4}$ ($5.2 \times 10^{+3}$)	24 (100)	0	$9.2 \times 10^{+3}$ ($1.6 \times 10^{+3}$)	
Post-disinfection	29 (85)	5 (15)	$5.2 \times 10^{+2}$ ($1.9 \times 10^{+2}$)	12 (50)	12 (50)	8.8×10^{0} (5.4×10^{0})	
Mean LR			1.8 (0.1)			3.0 (0.4)	$p < 0.001$; 0.6–2.0
E. coli, CFU/100 mL							
Pre-disinfection	33 (100)	0	$4.7 \times 10^{+4}$ ($1.3 \times 10^{+4}$)	24 (100)	0	$1.0 \times 10^{+3}$ ($2.0 \times 10^{+2}$)	
Post-disinfection	29 (88)	4 (12)	$1.6 \times 10^{+2}$ ($8.4 \times 10^{+1}$)	18 (75)	6 (25)	3.0×10^{0} (3.0×10^{-1})	
Mean LR			2.5 (0.3)			2.5 (0.1)	$p = 0.8$ (NS); (–)0.4–0.5

PFU, plaque forming units; CFU, colony forming units; *d*, number detected (percent); nd, number of non-detects (percent) that were modeled; SE, standard error; LR, $\log_{10}$ reduction; CI, confidence interval; NS, not significant ($p > 0.05$); counts are unadjusted for recovery – see the text.

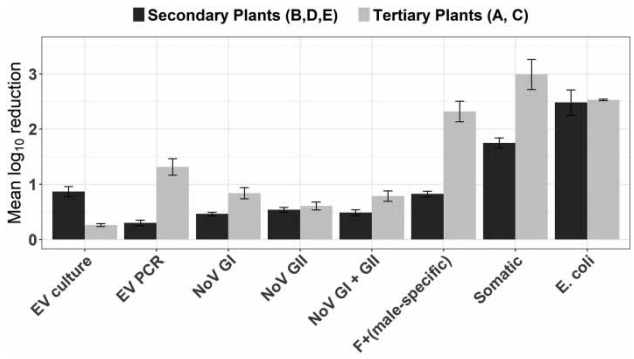

Figure 2 | Mean LRs of human enteric viruses and indicators by treatment type (secondary – Plants B, D, and E, or tertiary – Plants A and C). EV, enterovirus; NoV GI, norovirus Group I; NoV GII, norovirus Group II; NoV GI + GII, norovirus Group I + Group II combined; F^+ (male-specific), F^+ (male-specific) coliphage; Somatic, somatic coliphage; *E. coli* bacterial indicator; differences in LR means of secondary and tertiary plants are significantly different for all microorganisms, except for NoV GII and *E. coli*, Welch two-sample *t*-tests (see Tables 3 and 4).

swell during elution at the laboratory. Thus, it was decided to report enteric virus densities unadjusted for recovery, with the caveat that densities of both human enteric viruses and coliphages are probably underestimates (Dr. Shay Fout, USEPA, personal communication).

Correlations between coliphage indicators and human enteric viruses

Concentrations of coliphages and human target viruses were analyzed using Kendall's Tau non-parametric rank correlation, based on concordant and discordant pairs of observations, where pre- and post-disinfection virus densities for secondary and tertiary plants were lumped together (34 secondary + 24 tertiary = 58 sets of sample measurements). In each sampling event, the concentration of F^+ (male-specific) or somatic coliphage was compared with the concentration of each of the other microorganisms. Thus, for each microorganism in pre- or post-disinfection samples, there were 58 observation pairs that were evaluated as concordant or discordant. The 'cenken' NADA macro was used to measure the strength of association between microorganisms when censored observations and multiple detection limits are present (Helsel 2012). Most human enteric viruses in both pre- and post-disinfection effluent gave statistically significant correlations, $p \leq 0.05$, with F^+ (male-specific) and somatic coliphages (Table 5). See Figure 3 for an example where norovirus

Table 5 | Kendall's Tau (KT) non-parametric correlation analysis based on concordant and discordant pairs of observations[a] in pre- and post-disinfection effluent, five WWTPs, Ontario, 2014–2017

	F^+ (male-specific) coliphage			Somatic coliphage		
	KT value	***p*-value**	**Significance****	**KT value**	***p*-value**	**Significance****
Pre-disinfection						
Norovirus GI	0.28	0.002	Yes	0.19	0.03	Yes
Norovirus GII	0.32	0.0004	Yes	0.28	0.002	Yes
Norovirus GI + GII	0.33	0.0003	Yes	0.26	0.004	Yes
Enterovirus culture	0.13	0.14	No	−0.05	0.5	No
Enterovirus RT-PCR	0.22	0.01	Yes	0.33	0.0003	Yes
Somatic coliphage	0.28	0.002	Yes			
E. coli	0.47	2.7×10^{-7}	Yes	0.095	0.3	No
Post-disinfection						
Norovirus GI	0.26	0.004	Yes	0.22	0.01	Yes
Norovirus GII	0.18	0.04	Yes	0.13	0.1	No
Norovirus GI + GII	0.24	0.008	Yes	0.19	0.03	Yes
Enterovirus culture	0.29	0.001	Yes	0.32	0.0003	Yes
Enterovirus RT-PCR	0.22	0.01	Yes	0.22	0.01	Yes
Somatic coliphage	0.58	1×10^{-10}	Yes			
E. coli	0.43	1.6×10^{-6}	Yes	0.3	0.0008	Yes

[a]In each sampling event, the concentration of F^+ (male-specific) or somatic coliphage was compared with the concentration of each of the other microorganisms. Thus, for each microorganism in pre- or post-disinfection samples, there were 58 observation pairs (34 for secondary-treated + 24 tertiary-treated effluent) that were evaluated as concordant or discordant.
**Statistical significance given by *p*-value ≤ 0.05.

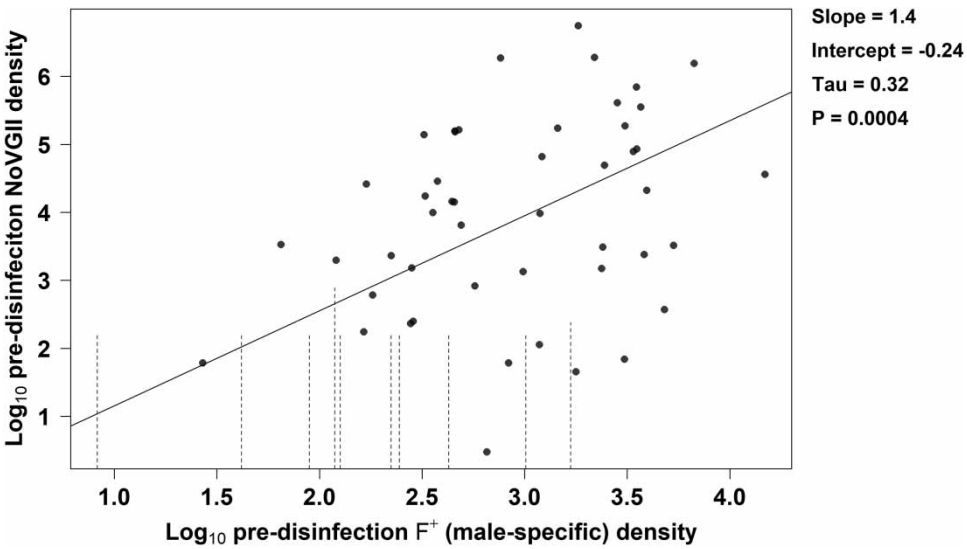

Figure 3 | Kendall's Tau (KT) non-parametric rank correlation between densities of norovirus GII (GC/L) and F^+ (male-specific) coliphage (PFU/L) in pre-disinfection effluent, $n = 58$ observation pairs, five Ontario WWTPs providing secondary and tertiary treatment. The correlation yielded a KT value of 0.32 and was statistically significant, a *p*-value of 0.0004. See Table 5 for details on other comparisons.

GII densities are compared against F^+ (male-specific) coliphage, giving a Kendall's Tau coefficient of 0.32, a *p*-value of 0.0004, and a statistically significant positive correlation.

DISCUSSION

The aim of this study was to quantify enteric viruses before and after routine chlorine or UV disinfection processes at five Ontario WWTPs and calculate their respective LRs as they complied with their *E. coli* fecal indicator limit in the final effluent. In Ontario, this information is critical for evaluating new wastewater disinfection technologies such as peracetic acid (Kitis 2004) or performic acid (Karpova *et al.* 2013; Ragazzo *et al.* 2013), because, from a regulatory point of view, a new disinfection technology must be shown to perform in an equivalent or superior manner as the approved technology that it intends to replace.

In many jurisdictions, including Canada and the United States, municipal WWTPs are required to disinfect their effluent, particularly during times when recreational water activities occur. Additionally, in many jurisdictions, when chlorine is used in the disinfection process, final discharged effluent must be devoid of acutely lethal effects on organisms in the aquatic environment. Microbiological water quality of WWTP effluent is generally regulated in terms of fecal indicator bacteria, e.g., fecal coliforms, *E. coli*, or *Enterococcus*, by requiring routine sampling of the post-disinfection effluent quality prior to its final discharge into a receiving body of water. *E. coli* and fecal coliforms are the most common indicator organisms. In Ontario, the *E. coli* regulatory limit in the final effluent is usually a monthly GM of 200 colony forming units/100 mL, based on weekly sampling. Chlorinating secondary WWTPs can generally meet this limit operationally by ensuring a total residual chlorine of 0.5 mg/L after 30 min contact time, i.e., a CT value of 15 mg-min/L, at the design average daily flow (Ontario 2008). UV-disinfecting, secondary WWTPs can similarly meet the *E. coli* limit by providing a dosage of 30–40 mJ/cm^2 (Metcalf & Eddy 2003). Neither the presence nor the density of enteric viruses, including coliphage viral indicator, is currently regulated in municipal post-disinfection effluent in Ontario. However, it is known that human pathogenic viruses are more resistant to wastewater disinfection than bacterial indicators such as *E. coli* and fecal coliforms, so it is expected that discharged effluents that meet bacterial indicator limits will contain enteric viruses (Rose *et al.* 2004; Simmons & Xagoraraki 2011; Gerba *et al.* 2013; Wong *et al.* 2013).

In this study, enteroviruses and noroviruses were abundant in post-disinfection effluent at mean concentrations of $2.1\times10^{+4}$–$7.2\times10^{+5}$ and $2.7\times10^{+4}$–$3.6\times10^{+5}$ GC/L, respectively. Chlorine or UV disinfection produced poor-to-modest LRs for enteroviruses and noroviruses, 0.3–1.3 and 0.5–0.8, respectively. Coliphages were more susceptible, with LRs of 0.8–3.0. In a recent study, Kingsley *et al.* (2017) used a receptor binding assay to assess chlorine inactivation of human norovirus and reported that chlorine is not effective for inactivation of human norovirus at levels normally used for wastewater disinfection. Nevertheless, our results indicate that, except for cultured enterovirus, norovirus GII and *E. coli*, tertiary treatment with sand filtration produced statistically significantly higher enteric virus LR than secondary treatment, likely because of reduced shielding by suspended solids in the former, resulting in unhindered disinfecting activity of chlorine or UV light (USEPA 2003).

Our results are consistent with those reported in the literature. Rose *et al.* (1996) studied the removal of pathogenic and indicator microorganisms at a tertiary, chlorinating, full-scale water reclamation facility in St. Petersburg, FL, USA, where the final effluent is used for golf course and residential landscape irrigation. These authors showed that the chlorination of filtered-effluent step resulted in enterovirus and coliphage LRs of 1.5 and 1.0, respectively. However, infectious enteroviruses were detected in 25% of post-chlorination samples and in 8% of storage (16–24 h) tank samples, albeit in low numbers (mean 1.0×10^{-4} PFU/L), including an isolate of Echovirus-7 from the storage tank site, which indicates enterovirus persistence. Furthermore, in a monitoring study of six full-scale water reclamation facilities in Arizona, California, and Florida, Rose *et al.* (2004) found cultivable enteric viruses in 31% of final effluents.

Katayama *et al.* (2008) reported that noroviruses GI and GII peaked during November through March and were detected in post-chlorination effluent from six Japanese WWTPs at GM concentrations of $2.9\times10^{+3}$ and $2.6\times10^{+3}$ GC/L, respectively. Enteroviruses were detected more

uniformly during the year at a post-chlorination GM value of 44 GC/L.

Kitajima *et al.* (2014) studied enteric viruses at two chlorinating WWTPs in Arizona and reported that norovirus GI and GII were detected by RT-qPCR in 9 (75%) of 12 final effluent samples at each of the two plants. Enteroviruses were also detected by RT-qPCR in 7 (58%) of 12 and 11 (92%) of 12 final effluent samples at each of the two plants. Chlorination unit process LRs were not given.

Using RT-qPCR, Qiu *et al.* (2015) assessed human enteric virus LRs during municipal wastewater treatment in Edmonton, Alberta, Canada, at a plant providing secondary treatment and UV disinfection before discharging the effluent to a river. Among a suite of human viruses, noroviruses were detected in 16 (100%) and enteroviruses in 10 (63%) of 16 post-UV disinfection samples, at average concentrations of $2.3 \times 10^{+4}$ and $7.4 \times 10^{+2}$ GC/L, respectively. At this WWTP, the UV disinfection process accounted for a norovirus LR of 0.1 ± 0.4 SD and an enterovirus LR of 0.6 ± 1.0 SD. Similarly, Qiu *et al.* (2018) reported poor inactivation of norovirus GI and GII nucleic acid at two UV-disinfecting WWTPs in Calgary Alberta, Canada, where mean LRs ranged from 0.1 to 0.2, and mean densities in post-disinfection effluent, from 10^{+4} to $1.3 \times 10^{+5}$ GC/L. Mean LRs of enteroviruses by RT-qPCR ranged from 0.2 to 0.3 and mean densities in post-disinfection effluent from $3.0 \times 10^{+3}$ to $6.6 \times 10^{+3}$ GC/L. LRs of infectious enteroviruses by integrated cell culture PCR were not informative.

By contrast, Seto *et al.* (2018) reported efficient norovirus inactivation at a secondary treatment, chlorine-disinfecting plant in Vacaville, California, where genogroups GI and GII were reduced from a median density of $5.3 \times 10^{+3}$ and $6.0 \times 10^{+3}$ GC/L in raw sewage, respectively, to below the MDL of 2 GC/L (0 of 11 samples were positive) in the final post-disinfection effluent, although authors did not provide details about the chlorine disinfection process.

Human enteric viruses in environmental samples are usually tested in highly specialized laboratories, at a considerable cost. However, coliphage testing is more readily available and costs are much lower. Even though coliphages were more sensitive to disinfection (had higher LRs) than human enteric viruses, we explored the possibility of using coliphages in the future evaluation of alternative wastewater disinfection technologies. Thus, we tested for possible correlations between coliphage indicators and human enteric viruses by Kendall's Tau nonparametric test of concordant and discordant pairs of observations, and found that, for the most part, F^+ (male-specific) and somatic coliphages significantly correlated with human enteric viruses, as others have reported (Purnell *et al.* 2016; Dias *et al.* 2018; Lee *et al.* 2018). However, Rose *et al.* (2004) found no correlation between the number of coliphages and enteric viruses.

We aimed to begin collecting data to establish enteric virus LR benchmarks in Ontario for existing wastewater disinfection technologies such as chlorine and UV light, against which new disinfection technologies, e.g., peracetic acid or performic acid, can be compared. A definitive benchmark cannot be established until additional data are collected from more WWTPs with varying disinfection requirements in terms of a suitable fecal indicator. Until then, we would recommend that a WWTP wishing to replace an existing disinfection technology, e.g., chlorine or UV light, with a new disinfection technology, e.g., peracetic acid or performic acid, would have to match or improve on the LRs for combined F^+ (male-specific) and/or somatic coliphages currently achieved by the existing technology.

Limitations of the present study include generalizations made about wastewater effluent quality based on limited (monthly) sampling; filtration volumes for wastewater samples that many times fell below the recommended 120 L (USEPA 2014), due to premature filter clogging, particularly when sampling secondary effluent; microbial concentrations that were unadjusted for recovery and which probably represent an underestimate; potential lack of analytical testing uniformity inasmuch as Plant E samples were tested at a laboratory different from the laboratory that was used to test Plants A–D samples; and isolation rates of culturable enteroviruses that may have been higher had we used other cell culture lines in addition to our standard BGM kidney cells. Finally, Chik *et al.* (2018) have recently argued that microbial nondetects are not left-censored values and should not be handled as such. To this point, we counter that our data are rigorous given that (i) our two reporting labs duly adjusted detected as well as non-detected values for assayed volumes and other correction factors and (ii) this study's enumeration data were then modeled by two approaches yielding comparable results: censored data estimation and Bayesian inference. As summarized

in Supplementary Material (available with the online version of this paper), these two methods gave very similar results.

CONCLUSIONS

Human enteric viruses, as well as coliphages, were abundant in the final post-disinfection effluent at five Ontario WWTPs, as they complied with their monthly GM regulatory limit of 200 *E. coli*/100 mL. Since *E. coli* is the compliance target organism in wastewater disinfection processes in Ontario and since *E. coli* is more susceptible to disinfection than human enteric viruses, it follows that LRs of human viruses would be lower than that of *E. coli* indicator. LRs in the disinfection treatment process at five Ontario WWTPs were poor-to-modest for enterovirus and norovirus, 0.3–1.3 and 0.5–0.8, respectively, whereas coliphage was more susceptible with LRs of 0.8–3.0. *E. coli* had an LR of 2.5 irrespective of whether the effluent was sand-filtered or not. Because of their statistically significant positive correlation with human enteric viruses, a coliphage viral indicator may potentially be used to gauge the efficacy of new wastewater disinfection technologies. Further studies are needed to evaluate the potential impact of discharged viruses on microbiological water quality downstream.

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