Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts.

Supplementary Information
Temperature and particles interact to affect human norovirus and MS2 persistence in surface water

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Supplementary Methods

MS2 glycerol stock and inoculant preparation. US EPA method 1602[1] was followed with slight modifications to prepare hard agar plates, soft agar plates, and tryptic soy broth, each with 0.015 mg/mL of ampicillin and streptomycin. *Escherichia coli* host (American Type Culture Collection (ATCC), 700891, Manassas, VA, USA) culture broth was prepared using flame-sterilized inoculating loops to transfer culture throughout the process. First, a previously prepared glycerol stock of *E. coli* was streaked onto a hard agar plate (3% agar), and the plate was incubated at 37°C overnight. Next, one colony was transferred to 25 mL of tryptic soy broth, and the broth was incubated at 37°C overnight. Finally, the broth culture was transferred to 25 mL of fresh broth, and the broth was incubated at 37°C. The broth was monitored to determine when the *E. coli* reached logarithmic growth phase. The culture broth was used immediately in the preparation of MS2 glycerol stock and the remainder was aliquoted into cryotubes with 15% glycerol and stored at -80°C. 4.5 mL of soft agar (0.7% agar), was inoculated with 200 µl of *E. coli* culture broth and 300 µl of previously prepared MS2 glycerol stock and poured onto a hard agar plate. The plate was incubated at 37°C overnight, and then 5 mL of autoclave-sterilized phosphate buffered saline (PBS) was added to the plate. After 4 hours, the PBS was recovered, centrifuged at 3,000 RPM for 10 min, and passed through a 0.22 µm pore size filter. The filtrate was aliquoted into cryotubes with 15% glycerol and stored at -80°C. To prepare the MS2 inoculant for the persistence experiments, an aliquot of MS2 glycerol stock was diluted in autoclave-sterilized PBS and stored at -80°C until the experiments.

MS2 infectivity. The MS2 infectivity was measured using the double agar layer enumeration procedure, with slight modifications.[1] Briefly, each sample was serially diluted in increments of 10 in autoclave-sterilized PBS, and three to five dilutions were included per time point. 200 µL of *E. coli* host in logarithmic growth phase and 300 µL of diluted sample were combined in 4.5 mL of soft agar (0.7% agar), vortexed briefly, and then poured onto a hard agar plate (3% agar). The plates were incubated at 37°C overnight, and plaque-forming units (PFU) were counted within 16-24 hours. PFU were enumerated between 15 and 350 plaques, and plates above 350 PFU were considered too numerous to count. 50 PFU/mL was substituted for values that were not quantifiable.

RT-qPCR. A StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) was used as described previously,[2] where the fluorescence threshold was 0.02 ΔRn units and the baseline fluorescence cycle ranges were set by the instrument automatically (Table S1). RT-qPCR for experimental samples was completed in duplicate, including standard curves and no-template controls (NTCs). Quantities were calculated from cycle threshold (Ct) values using individual standard curves that were combined into master standard curves (Figure S1). Half of the minimum quantity in the standard curve in gc/mL was substituted for values that were not quantifiable (Table S4).

Samples were tested for inhibition by comparing the Ct values for a 1:2 dilution factor to a 1:5 dilution factor (ORF) or no dilution compared to a 1:5 dilution factor (MP1 and NR1) on the same qPCR plate. For each well that had no amplification, the Ct value was subbed

with the highest Ct value in the standard curve. The arithmetic mean of duplicate Ct values for each dilution was determined. If the mean Ct of the lowest dilution factor minus the expected difference in Ct with a 1 Ct buffer for machine error[3] was higher than the mean Ct of the 1:5 dilution, the sample was deemed inhibited. Out of 477 samples, 3 MP1 samples and 9 ORF samples were inhibited. For the 12 inhibited samples, the Ct value from the 1:5 dilution was used in subsequent data analysis.

Supplementary Figures

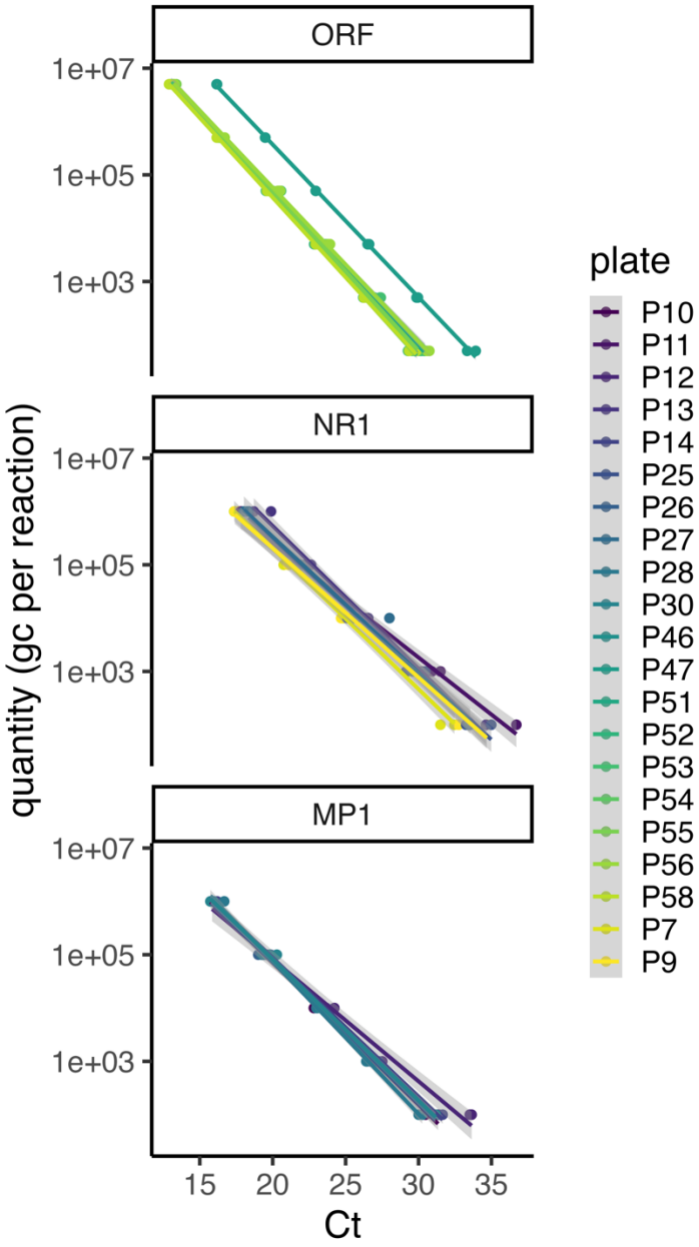

Figure S1

Compiled individual standard curves. Quantity in gene copies per reaction vs threshold cycle (Ct) for all qPCR plates.

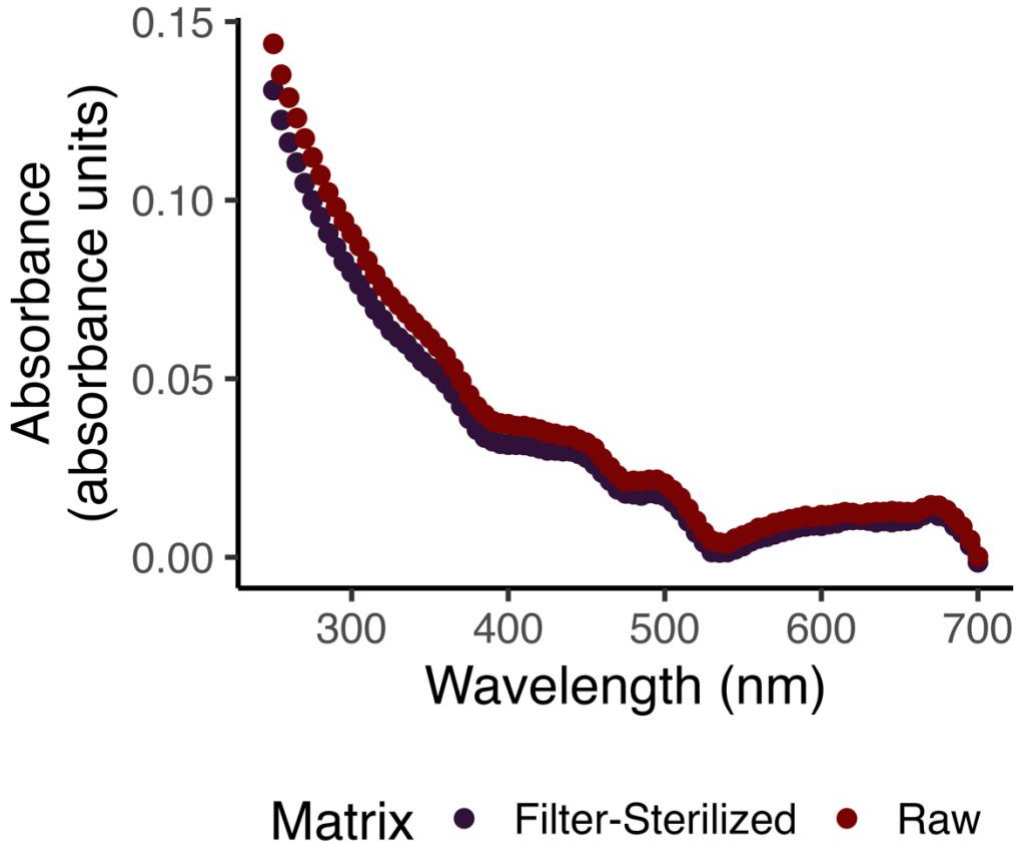

Figure S2

Absorbance of water samples from 250 to 700 nm for raw and 0.22-µm pore size filter-sterilized San Pedro Creek water. The filter-sterilized creek water is included here from a previous publication including experiments with the same creek water sample.[2]

Supplementary Tables

Table S1

qPCR assays used in this study.

Assay	Amplicon length (base pairs)	Genome Target	Forward Primer (5'-->3')	Reverse Primer (5'-->3')	Probe (5'-->3')	Ref.
ORF	89	HuNov (GII) ORF1 - ORF2 junction	ATGTTCA GRTGGAT GAGRTTC TCWGA	TCGACGCC ATCTTCAT TCACA	FAM-AGCACGTG GGAGGGC GATCG-TAMRA	[4]
NR1	506	HuNoV (MK762570)	CCGAGAG ACGAACC ACAAA	GGTCTAAC ACCCAGCA GCAA	-	[5]
MP1	313	MS2 (NC_001417)	TGTCTTTA GCGAGAC GCTACC	GATGACCC ACTTCGCT TGTAG	-	[5]

Table S2

Mastermix components and concentrations in each reaction and reaction volume by assay. Nuclease-free water was added to produce the final reaction volume.

	ORF	**NR1 & MP1**
Master mix kit (company)	Ag PathID (Applied Biosystems, Waltham, MA, USA)	Fast EvaGreen qPCR master mix (Biotium, Fremont, CA, USA)
Master mix kit components (added to 1x per reaction)	Ag PathID Buffer Ag PathID Enzyme Mix	Fast EvaGreen master mix ROX Reference Dye
Forward and Reverse Primer (µM)	0.2	0.5
Probe (µM)	0.2	-
BSA (mg/mL)	-	0.625
Template type	RNA	cDNA
Template Volume (µL)	5	2
Reaction Volume (µL)	25	20

Table S3

Thermal cycling conditions by assay.

Stage	ORF	NR1 & MP1
Reverse transcription	50°C (30 min)	-
Predenature	95°C (10 min)	95°C (5 min)
Cycle stage 1: Denature	95°C (15 sec)	95°C (20 sec)
Cycle stage 2: Anneal	60°C (1 min)	60°C (20 sec)
Cycle stage 3: Extension	-	72°C (20 sec)
Number of cycles	45	40
Final Extension	-	72°C (1 min)

Table S4

RT-qPCR assay details for assays used to quantify experimental samples, where “IDT” denotes that the standard was from Integrated DNA Technologies, Coralville, IA, USA.

RT-qPCR Assay	Standard Curve material	Minimum (gc/rxn)	Maximum (gc/rxn)
ORF	gBlocks Gene Fragments (IDT)	$5x10^1$	$5x10^6$
NR1	gBlocks Gene Fragments (IDT)	$1x10^2$	$1x10^6$
MP1	gBlocks Gene Fragments (IDT)	$1x10^2$	$1x10^6$

Table S5

Master standard curve parameters by RT-qPCR assay.

Assay	R^2	efficiency	slope	intercept
NR1	0.98	78.76	-3.96	41.70
MP1	0.99	82.90	-3.81	38.68
ORF	0.97	97.09	-3.39	36.17

Table S6

Physicochemical water quality and ATP data for water collected from San Pedro Creek on 11/8/21. The water collected was raw or filter-sterilized with a 0.22-µm pore size filter (previously published and repeated here for ease of comparison[2]). Data that were not collected are denoted "-." Temperature and salinity were assessed onsite during sample collection. Error denotes the standard deviation of triplicates and for intracellular ATP it is propagated error from total and extracellular ATP.

	Raw	Filter-Sterilized
Temperature (°C)	11.3	-
pH	7.44	7.59
Salinity (ppt)	0.3	-
Turbidity (NTU)	<1	<1
Intracellular ATP (nM)	$4.71 \times 10^{-2} \pm 3.15 \times 10^{-3}$	$8.97 \times 10^{-4} \pm 3.69 \times 10^{-4}$
Total ATP (nM)	$6.79 \times 10^{-2} \pm 3.02 \times 10^{-3}$	$2.70 \times 10^{-2} \pm 3.54 \times 10^{-4}$

Table S7

First-order decay rate constants (k; day^{-1}) for each experiment and RT-qPCR assay. The microorganism, virus target, Enumeration method ("Assay"), incubation temperature ("Temperature"), and matrix that HuNoV and MS2 were seeded into (i.e., raw ("R") or filter-sterilized ("F")) are the first four columns. The next three columns are k, its standard error, the number of points used to calculate it ("n"), and its p value ("p"). The final column indicates where the p value is <0.0001 (***), <0.001 (**), <0.01 (*), or <0.05 (.) ("Significance level").

Virus	Virus Target	Assay	Temperature (°C)	Matrix	*k*	Standard error	n	p	Significance level
MS2	long genome segment	MP1	10	F	0.026	0.011	10	0.040	.
MS2	long genome segment	MP1	10	R	0.089	0.0094	10	<0.0001	* * *
MS2	intact capsid	MP1	10	R	0.13	0.0096	10	<0.0001	* * *
MS2	long genome segment	MP1	15	F	0.079	0.0098	10	<0.0001	* * *
MS2	intact capsid	MP1	15	F	0.10	0.0095	10	<0.0001	* * *
MS2	long genome segment	MP1	15	R	0.37	0.016	9	<0.0001	* * *
MS2	intact capsid	MP1	15	R	0.40	0.020	8	<0.0001	* * *
MS2	long genome segment	MP1	20	F	0.276	0.014	10	<0.0001	* * *
MS2	intact capsid	MP1	20	F	0.2	0.024	9	<0.0001	* * *
MS2	long genome segment	MP1	20	R	0.55	0.047	7	<0.0001	* * *
MS2	infectious	Culture	10	F	0.14	0.032	10	0.0022	*
MS2	infectious	Culture	10	R	0.20	0.042	10	0.0013	*
MS2	infectious	Culture	15	F	0.09	0.040	10	0.047	.
MS2	infectious	Culture	15	R	0.71	0.16	6	0.011	.

MS2	infectious	Culture	20	F	0.40	0.063	8	0.0007	* *
MS2	infectious	Culture	20	R	1.5	0.37	4	0.055	
HuNoV	long genome segment	NR1	10	F	-0.076	0.017	10	0.0018	*
HuNoV	intact capsid	NR1	10	F	-0.018	0.0090	10	0.076	
HuNoV	long genome segment	NR1	10	R	0.17	0.020	8	0.0002	* *
HuNoV	intact capsid	NR1	10	R	0.29	0.052	7	0.0024	*
HuNoV	long genome segment	NR1	15	F	-0.016	0.0065	10	0.036	.
HuNoV	intact capsid	NR1	15	F	-0.0083	0.0095	10	0.41	
HuNoV	long genome segment	NR1	15	R	0.51	0.082	5	0.0084	*
HuNoV	intact capsid	NR1	15	R	0.69	0.080	5	0.0033	*
HuNoV	long genome segment	NR1	20	F	0.1	0.029	9	0.0018	*
HuNoV	intact capsid	NR1	20	F	0.14	0.025	9	0.0009	* *
HuNoV	long genome segment	NR1	20	R	0.57	0.63	3	0.53	
HuNoV	short genome segment	ORF	10	F	-0.0023	0.0043	10	0.61	
HuNoV	short genome segment	ORF	10	R	0.19	0.024	10	<0.0001	* * *
HuNoV	short genome segment	ORF	15	F	-0.0051	0.0057	10	0.40	
HuNoV	short genome segment	ORF	15	R	0.48	0.050	7	0.0002	* *

HuNoV	short genome segment	ORF	20	F	0.16	0.015	10	<0.0001	* * *
HuNoV	short genome segment	ORF	20	R	0.56	0.024	7	<0.0001	* * *

Table S8

Multiple linear regression coefficients for equation 2, which was used to assess the effect of incubation temperature, particle content, and their interaction on the persistence of HuNoV short genome segments. The residual standard error was 0.74 on 46 degrees of freedom (adjusted $R^2 = 0.88$; $p < 0.0001$).

Coefficient	Estimate	Standard error	T Statistic	p value
β_0	0.30	0.24	1.3	0.21
β_1	-0.39	0.028	-14	<0.0001
β_2	-0.044	0.31	-0.14	0.89
β_3	0.024	0.055	0.43	0.67
β_{12}	0.34	0.030	11	<0.0001
β_{13}	-0.042	0.0059	-7.2	<0.0001
β_{23}	0.048	0.074	0.65	0.52
β_{123}	0.026	0.0065	4.0	0.00024

Table S9

Multiple linear regression coefficients for equation 2, which was used to assess the effect of incubation temperature, particle content, and their interaction on the persistence of infectious MS2. The residual standard error was 1.5 on 40 degrees of freedom (adjusted $R^2 = 0.68$; $p < 0.0001$).

Coefficient	Estimate	Standard error	T Statistic	p value
β_0	-0.77	0.55	-1.4	0.17
β_1	-0.79	0.12	-6.3	<0.0001
β_2	-1.56	0.69	-2.3	0.028
β_3	0.063	0.12	0.52	0.60
β_{12}	0.62	0.13	4.8	<0.0001
β_{13}	-0.12	0.025	-4.8	<0.0001
β_{23}	-0.23	0.16	-1.5	0.15
β_{123}	0.10	0.026	4.0	0.00029

Table S10

Multiple linear regression coefficients for equation 3, which was used to assess potential inactivation mechanisms of HuNoV incubated in raw and filter-sterilized water at 15°C. The residual standard error was 0.54 on 39 degrees of freedom (adjusted $R^2 = 0.90$; $p < 0.0001$).

term	estimate	std.error	statistic	p.value
β_0	0.18	0.25	0.69	0.49
β_1	-0.51	0.036	-14	<0.0001
β_2	-0.026	0.24	-0.11	0.92
β_3	-0.040	0.26	-0.15	0.88
β_4	0.23	0.26	0.88	0.38
β_{12}	0.51	0.036	14	<0.0001
β_{13}	0.0038	0.018	0.21	0.84
β_{14}	0.020	0.018	1.1	0.28

Table S11

Multiple linear regression coefficients for equation 3, which was used to assess potential inactivation mechanisms of MS2 incubated in raw and filter-sterilized water at 15°C. The residual standard error was 0.92 on 45 degrees of freedom (adjusted $R^2 = 0.87$; $p < 0.0001$).

term	estimate	std.error	statistic	p.value
β_0	-2.37	0.38	-6.3	<0.0001
β_1	-0.40	0.035	-12	<0.0001
β_2	-1.0	0.37	-2.8	0.0068
β_3	2.8	0.44	6.5	<0.0001
β_4	0.018	0.44	0.041	0.97
β_{12}	0.30	0.030	10	<0.0001
β_{13}	0.025	0.030	0.83	0.41
β_{14}	-0.024	0.029	-0.81	0.42

References

1 Environmental Protection Agency, *Method 1602: Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure*, 2001.

2 L. C. Kennedy, V. P. Costantini, K. A. Huynh, S. K. Loeb, W. C. Jennings, S. Lowry, M. C. Mattioli, J. Vinjé and A. B. Boehm, Persistence of Human Norovirus (GII) in Surface Water: Decay Rate Constants and Inactivation Mechanisms, *Environ. Sci. Technol.*, 2023, **57**, 3671–3679.

3 Y. Cao, J. F. Griffith, S. Dorevitch and S. B. Weisberg, Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring Enterococcus in environmental waters, *J. Appl. Microbiol.*, 2012, **113**, 66–75.

4 F. Loisy, R. L. Atmar, P. Guillon, P. Le Cann, M. Pommepuy and F. S. Le Guyader, Real-time RT-PCR for norovirus screening in shellfish, *J. Virol. Methods*, 2005, **123**, 1–7.

5 S. K. Loeb, W. C. Jennings, K. R. Wigginton and A. B. Boehm, Sunlight Inactivation of Human Norovirus and Bacteriophage MS2 Using a Genome-Wide PCR-Based Approach and Enzyme Pretreatment, *Environ. Sci. Technol.*, 2021, **55**, 8783–8792.