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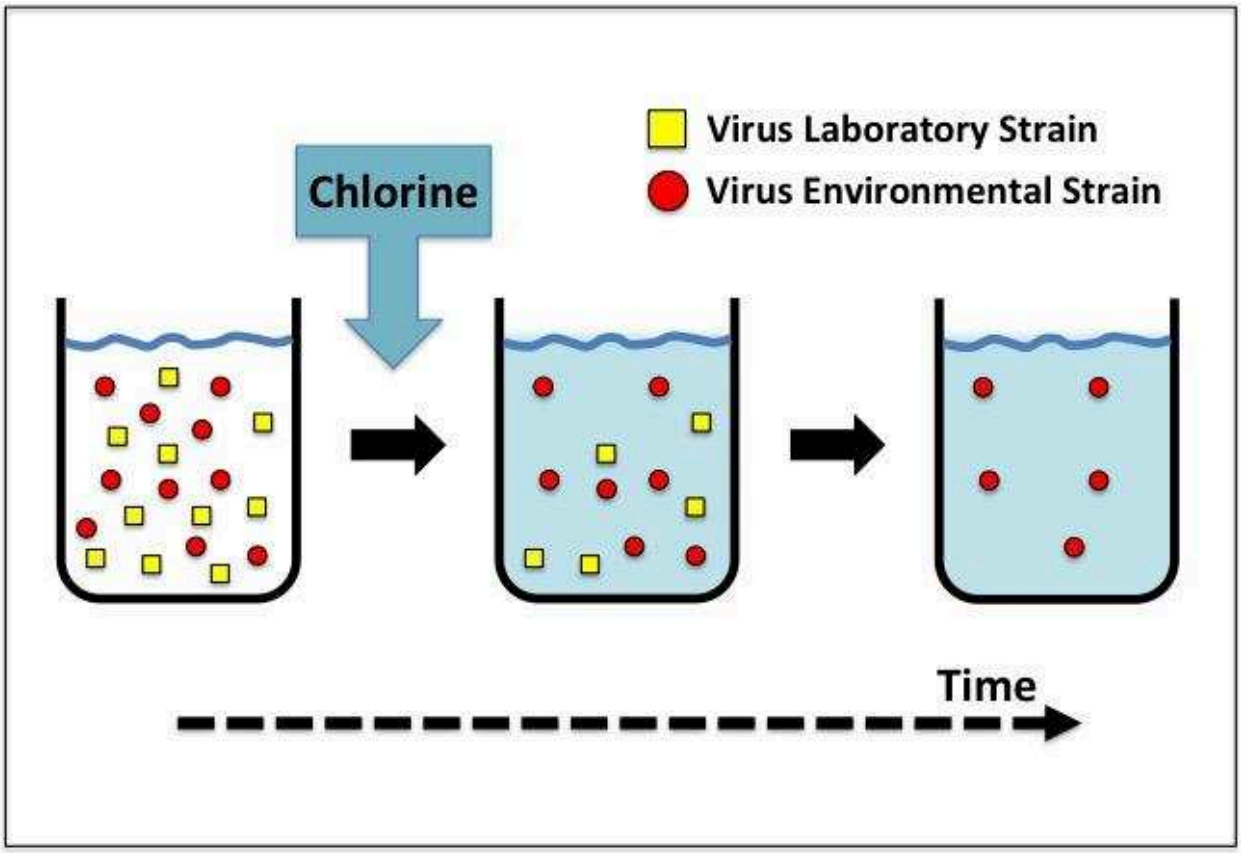
Virus Laboratory Strain
Virus Environmental Strain
Chlorine
Time

Variability in disinfection resistance within an between currently circulating *Enterovirus B* serotypes

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ABSTRACT

The susceptibility of waterborne viruses to disinfection is known to vary between viruses and even between closely related strains, yet the extent of this variation is not known. Here, different enteroviruses (six strains of coxsackievirus B5, two strains of coxsackievirus B4 and one strain of coxackievirus B1) were isolated from wastewater and inactivated by UV_{254}, sunlight, free chlorine (FC), chlorine dioxide (ClO_2), and heat. Inactivation kinetics of these isolates were compared with those of laboratory enterovirus strains (CVB5 Faulkner and echovirus 11 Gregory) and MS2 bacteriophage. FC exhibited the greatest (10-fold) variability in inactivation kinetics between different strains, whereas inactivation by UV_{254} differed only subtly. The variability in inactivation kinetics was greater between serotypes than it was among the seven strains of the CVB5 serotype. MS2 was a conservative surrogate of enterovirus inactivation by UV_{254}, sunlight or heat, but frequently underestimated the disinfection requirements for FC and ClO_2. Similarly, laboratory strains did not always reflect the inactivation behavior of the environmental isolates. Overall, there was considerable variability in inactivation kinetics among and within enteroviruses serotypes, as well as between laboratory and environmental isolates. We therefore recommend that future disinfection studies include a variety of serotypes and environmental isolates.

INTRODUCTION

Human enteric viruses are a leading cause of waterborne disease worldwide[1]. Their control remains problematic; compared to bacterial pathogens and fecal indicators, viruses are highly persistent in the environment[2] and have high resistance to disinfectants such as chlorine[3]. The *Enterovirus* genus of the *Picornaviridae* family in particular is a major source of the waterborne disease burden[4] and hence it is included on the EPA contaminant candidate list (EPA, CCL4[5]). Within this genus, species of concern include *Enterovirus A*, which contains coxsackievirus A serotypes, *Enterovirus B*, which includes serotypes of echovirus and coxsackievirus B, and *Enterovirus C*, which includes poliovirus and other coxsackievirus A serotypes[6,7].

Serotypes of the *Enterovirus* genus are generally more resistant than other enteric viruses to chlorine[8,9]. The different species and serotypes of this genus, however, have a wide range of susceptibilities to disinfection. For example, differences in chlorine resistance were observed between the serotypes coxsackievirus B4 (CVB4) and B5 (CVB5), as well as poliovirus serotypes (PV) 1, 2 and 3[10], with CVB5 displaying the highest resistance. Similarly, disinfection by monochloramine exhibited up to three-fold greater inactivation rates of serotypes coxsackievirus B3 (CVB3) compared to CVB5, and more than 100-fold greater rates for serotypes echovirus 1 compared to echovirus 11 (E11)[9]. Finally, disinfection of wastewater effluent by chlorine dioxide (ClO_2) revealed that CVB5 was more resistant than echovirus 1 and PV 1 serotypes[11]. More surprisingly, differences were also observed among strains of the same poliovirus serotype[12,13], revealing variability even among closely related viruses. Combined, these studies indicate that differences in resistance to oxidizing disinfectants exists between the different *Enterovirus* species, between serotypes and even within serotypes. In contrast, similar susceptibility was found among different enteroviruses to UV light and sunlight[14,15].

The reason for variability in *Enterovirus* susceptibility to oxidants is not well understood, yet it may be driven through selection by the disinfectant for the most resistant variants. For example Bates et

al.[16] demonstrated that the repeated exposure of PV to chlorine led to increased resistance. Similarly, Shaffer et al.[17] reported that PV isolated from chlorinated drinking water were more resistant to chlorine than unexposed lab strains. More recently, our group has repeatedly exposed echovirus 11 to UV_{254} which resulted in the selection of UV_{254}-resistant strains. Similarly, repeated exposure of echovirus 11 to UV light at 254 nm (UV_{254}) led to the selection of UV_{254}-resistant strains[18]. Selection of disinfection-resistant viruses may also arise from exposure to environmental stressors. Specifically, Payment et al.[10] demonstrated that sewage isolates of CVB5 were more resistant to chlorine compared to the corresponding lab strains. Tree et al.[19] furthermore demonstrated that sewage-borne PV were more resistant to chlorination compared to lab strains.

To establish adequate disinfection practices, it is important that the diversity in disinfection kinetics of circulating viruses is taken into account. To this end, we isolated nine strains of *Enterovirus B* from untreated domestic sewage from three geographic locations (Lausanne, Switzerland, Minneapolis, MN and Tampa, FL), and compared their disinfection kinetics with those of two laboratory strains (echovirus 11 Gregory strain and coxsackievirus B5 Faulkner strain). Additionally, inactivation kinetic parameters were compared to those of MS2 bacteriophage, which has been proposed as a surrogate for enteric viruses for the assessment of household water treatment interventions (including chlorination, UV, solar disinfection, and heat treatment)[20]. All viruses were subjected to inactivation by five different treatments of which two mainly act by inducing genome damage (UV_{254} and simulated sunlight), two target both the viral genome and proteins (FC and ClO_2), and one (heat) induces non-oxidative protein denaturation[21]. The ultimate objective of this study was to quantify the extent of variability in disinfection resistance for different *Enterovirus B* serotypes and strains, as well as for different inactivation methods, and to assess if surrogate viruses can be used to represent the inactivation of circulating viruses.

EXPERIMENTAL SECTION

Chemicals, virus laboratory strains and cells

The sources of all chemicals, virus laboratory strains and their host cells is given in the Supporting Information.

Isolation of circulating viruses

Viruses were isolated from one liter of untreated domestic sewage from three wastewater treatment plants (Lausanne, Switzerland, Minneapolis, MS, and Tampa, FL), as described previously[22–25]. Insoluble contaminants were removed by adding 110 mL of glycine buffer (1M glycine, 3M NaCl, miliQ H_2O, pH9.5), stirring on ice for 20 min, and then centrifuging for 30 min at 6700×*g* at 4°C. The supernatant was transferred to a clean bottle, the pH was adjusted to 7.2 with HCl, 200 mL of polyethylene glycol (PEG) 8000 (40%) were added and samples were stirred overnight at 4°C. Samples were then centrifuged for 40 min at 4°C, then the supernatant discarded and the pellet was resuspended in 15 mL of phosphate-buffered saline (PBS; 5 mM NaH_2PO_4, 10 mM NaCl, pH7.4). To remove PEG and macromolecular inhibitors, the solution was vigorously mixed with 15 mL chloroform and centrifuged for 15 min at room temperature. The upper phase was harvested, filtered through a 0.45 µm filter (hydrophilic polyethersulfone filter; Millipore), and concentrated to 1.7 mL using an Amicon 100 kDa molecular weight cutoff column (Sigma-Aldrich, Germany). Buffalo Green Monkey Kidney (BGMK) cells were grown to confluence in T25 flasks (TPP Techno Plastic Products, Trasadingen, Switzerland) as described previously[26], were infected with the concentrated viruses diluted in cell culture media (see SI) at a 1:10 ratio, and the cytopathic effect (CPE) was checked daily. After full CPE was observed (around four days post-infection), the cell lysate was harvested and viruses were clarified by centrifugation. Finally, two successive plaque assays were performed to isolate individual virus strains (see SI for details).

92

93 **Virus identification, whole genome sequencing and alignment**

94 To identify the virus serotypes isolated, general enterovirus Inosine-degenerated primers targeting
95 the viral protein 1 (VP1) were used[27]. The PCR amplicon size was first checked by agarose gel, then
96 sequenced by Sanger technique using the same primers. The resulting sequences were identified by
97 the NCBI basic local alignment search tool (BLAST). Whole genome sequencing of the CVB5 isolates
98 was accomplished by aligning 86 CVB5 complete genomes listed in the ViPR[28] database. Primer
99 couples were designed along the consensus sequence and used to sequence the whole genome of
100 each isolate (Table S1). CVB4 isolates were sequenced with primers designed according to a single
101 CVB4 sequence (accession number: S76772[29]), and CVB1 was sequenced with CVB1-derived primers.
102 Based on the initial sequencing results, additional strain-specific primers were designed to complete
103 genome sequences not fully captured by these serotype-specific primers. Virus genome sequences
104 were checked, aligned, assembled, and annotated using the Geneious software version 8.1.8[30].

105 The whole sequence of VP1 was used to align the different viruses and calculate their pairwise
106 identity using multiple sequence comparison by log-expectation (MUSCLE)[31] with an iteration of 8, a
107 gap open penalty of 400, and a gap extension penalty of 0. An unrooted tree was built using the
108 neighbor-joining estimation method[32]. The protein pairwise identity was determined using the
109 translated region of the structural proteins (VP1 to VP4).

110 **Virus purification and enumeration**

111 To prepare viral stock solutions, each strain was individually amplified in BGMK cells, purified by PEG
112 precipitation and chloroform treatment[33], concentrated, and divided into aliquots of 100 µL. At least
113 two stock solutions from separate amplifications were produced for each virus. Viruses in all stock
114 solutions were analyzed by dynamic light scattering as described previously[34] and were found to be
115 monodispersed (data not shown). Infective virus concentrations were determined by endpoint
116 dilutions with Most Probable Number (MPN) statistics[35], and are reported as most probable number

of cytopathic units per mL (MPNCU·mL^{-1}). Endpoint dilutions were performed on BGMK cells in 96-well plates, with five replicates and eight dilutions per experimental sample. After inoculation, plates were incubated at 37°C with 5% CO_2[26], and the presence or absence of CPE in each well was determined five days post-infection through microscopy. MS2 bacteriophage was propagated, purified by PEG and chloroform, and enumerated using the double agar layer plaque assay method as described previously[33], and its infectivity was measured in plaque forming units per mL (PFU·mL^{-1}).

Inactivation experiments

Inactivation experiments were performed two to four times per virus and disinfectant, and eight samples were taken to construct each inactivation curve. All experiments were performed in PBS at an initial virus concentrations of 10^7 to 10^8 MPNCU·mL^{-1} or PFU·mL^{-1}.

UV_{254}. A low-pressure monochromatic UVC lamp was used to test the virus inactivation at a wavelength of 254 nm (UV_{254}). A bench scale device containing a 17 W mercury UV lamp (Philips, TUV F17T8) with a manual shutter was used. Two mL of PBS were added to a darkened glass beaker, were spiked with viruses, and were exposed to UV_{254} for up to four minutes under constant stirring. Aliquots of 100 µL were harvested every thirty seconds. The UV_{254} fluence rate was measured by iodide/iodate actinometry[36,37], and corresponded to 1.398 W·m^{-2}.

Simulated sunlight. Sunlight was simulated using Sun 2000 (Abet Technologies) equipped with a 1000W Xenon lamp, an AirMass 1.5 filter, and a 2 mm atmospheric edge filter. The irradiance spectrum was determined using a radiometer (ILT 900-R; International Light Technologies, Peabody, MA). The average UVB fluence rate was calculated by integrating the irradiance from 280 to 320 nm and corresponded to 0.563 W·m^{-2}. For typical 12-hour day exposure, this corresponds to four times the equatorial UVB fluence determined elsewhere[38]. 100 µL of virus concentrate were added to 10 mL of PBS in a glass beaker immersed in a 22°C temperature-controlled water bath, and were exposed to simulated sunlight under constant stirring. Samples of 100 µL were taken each 2-3 hours over the course of up to 24 hours.

142 *Free chlorine*. Prior to experiments, 10 mL glass beakers were incubated overnight in a concentrated
143 FC solution to quench any chlorine demand. The FC working solution was prepared by diluting bleach
144 solution (15% HOCl) in PBS to a final concentration between 0.8 and 3.1 $mg \cdot L^{-1}$. The FC concentration
145 was measured by the N,N-diethyl-p-phenylenediamine colorimetric method[39] at the beginning and
146 end of each experiment, and typically varied by less than 20% throughout the experiment. Therefore
147 the average of the initial and final FC concentration in each experiment was considered as the
148 working concentration. Prior to each experiment, beakers were rinsed twice with the working
149 solution. Then 50 µL of virus stock solution were spiked into a 2 mL working solution under constant
150 stirring. 10 µL aliquots were harvested every 10-30 seconds over the course of 3 minutes, and were
151 directly mixed with 90 µL PBS containing 1.4 M sodium thiosulfate to quench the residual FC. The
152 initial virus concentration was sampled from a 2 mL PBS solution without FC, spiked with 50 µL virus
153 stock.

154 *Chlorine dioxide.* A concentrated ClO_2 solution was obtained by mixing 100 mL 4% $K_2S_2O_8$ with 100 mL
155 2% $NaClO_2$ as described elsewhere[40], and was kept at 4 °C. ClO_2 concentrations were measured by
156 spectrophotometer (UV-2550; Shimadzu) at 358 nm. The concentrated solution was mixed with PBS
157 in order to obtain a supply solution (4-7 $mg \cdot L^{-1}$) and a working solution (0.25-1 $mg \cdot L^{-1}$). All beakers
158 were rinsed three times with the working solution. Then 2 mL of working solution were amended
159 with 50 µL of virus stock solution under constant stirring. Throughout the experiment, the ClO_2
160 concentration was maintained approximately constant (± 7 %) by continuously adding the supply
161 solution with a syringe pump at a flow rate of 5-20 $\mu L \cdot min^{-1}$. The sampling procedure, ClO_2
162 quenching, and measurement of the initial virus concentration were performed as described for FC.

163 *Heat*. Inactivation experiments by heat were performed in a PCR thermocycler (Applied Biosystems,
164 GeneAmp PCR system 9700). PCR tubes containing 90 µL of PBS were heated to 55°C, then 10 µL of
165 virus stock solution was spiked into each tube. At each time point, a sample was removed and quickly

placed in an aluminium PCR cooling block on ice. The initial virus concentration was measured by spiking 10 µL of viruses in 90 µL of PBS at room temperature.

Inactivation Rate Modelling

The rates of infectivity loss for all viruses by UV_{254}, sunlight and FC were modeled by first-order kinetics according to the Chick-Watson model[41,42], where k is the decay rate constant, C is the concentration (or fluence rate) of the disinfectant, N is the concentration of viruses at time t, and η is the coefficient of dilution, assumed to be equal to one:

$$\frac{\partial N}{\partial t} = -kC^{\eta}N \qquad \text{(equation 1)}$$

Given that C was approximately constant in our experimental systems, the integration of Equation 1 gives the following,

$$N = N_o e^{-kCt} \qquad \text{(equation 2)}$$

where Ct is the dose, and the inactivation rate constants (k) have the units $mJ^{-1}\cdot cm^2$ (for UV_{254} and sunlight), and $mg^{-1}\cdot min^{-1}\cdot L$ (for FC).

Infectivity loss by $ClO_2$and heat were modeled by segmental regression, according to the following equation,

$$N = N_o e^{-k1Ct} + N_1 e^{-k2Ct} \qquad \text{(equation 3)}$$

where N_1 is the breakpoint (the virus concentration at which kinetics deviate from the initial exponential decay), and k_1 (hereafter denoted as k_{ClO2} or k_{heat}) and k_2 are the rate constants associated with inactivation before and after the breakpoint. Here, Ct is the dose in $mg\cdot min\cdot L^{-1}$ for ClO_2 and seconds at 55 °C for heat. The inactivation rate constants k_{ClO2} and k_{heat} have units of $mg^{-1}\cdot min^{-1}\cdot L$ and s^{-1}, respectively.

Bayesian analysis of rate constants

The probabilities associated with the values of *k* were estimated using Bayesian inference (see Supporting Information for details). The probability of one virus strain or serotype being more or less resistant than another was calculated as the difference between the posterior distributions of the two inactivation rate constants. Bayesian inference was used instead of conventional hypothesis testing (*e.g.,* with p-values and confidence intervals) to provide a more intuitive assessment of the probabilities that a given virus has an inactivation rate constant, and hence a disinfection resistance, that differs from a reference virus considered (*e.g.*, CVB5 Faulkner strain). Furthermore, the use of Bayesian inference allows for the reduction of uncertainty in the rate constant by utilizing raw endpoint dilution data (number of positive wells in a given dilution sample) directly in a likelihood model[43], instead of using MPN estimates at different dose levels as inputs to fit a log-linear inactivation curve using the least squares method. For the comparison of serotypes, *k* distributions of all viruses of a given serotype were combined and 30'000 values were randomly sampled from the resulting mixture distribution. These values were then used to calculate posterior distribution differences.

Data analysis

All computations of kinetic parameters and pairwise identities were performed in R[44], supplemented with JAGS for Bayesian analysis[45]. The following CRAN packages were used: ggplot2[46], gridExtra[47], rjags[48], segmented[49], seqinr[50], sjPlot[51], bbmle[52], coda[53], msa[54], ape[55], ggtree[56].

Accession Numbers

Genome sequences of all virus isolates have been deposited in GenBank with the accession numbers MG845887 to MG845895.

RESULTS AND DISCUSSION

Virus Isolation

A total of nine virus strains were isolated from untreated domestic sewage. By sequencing their whole genomes, six isolates were identified as serotype coxsackievirus B5, two as serotype coxsackievirus B4 and one as serotype coxsackievirus B1. All CVB5 isolates belong to genotype IV, which mostly contains viruses isolated after 1984, whereas the Faulkner strain, which was isolated in 1952, belongs to genotype I[57]. The different isolates were named according to the isolation date and the first letter of the city they were isolated from (Table 1).

The genetic distance between isolates, determined by comparison of their VP1 genes, is illustrated in a neighbor-joining tree (Figure 1). All CVB5 isolates exhibited 79.1-99.4% pairwise identity, whereas they shared 76.8-81.7% with the CVB5 Faulkner strain (Table S2). This discrepancy can be explained by the different year of isolation of the CVB5 isolates and the Faulkner strain (2015 versus 1952 respectively), and their different genotypes (IV versus I, respectively). The CVB4 isolates had 88.1% identity among each other, and shared 63.4-66.1% with CVB5 isolates. The single CVB1 isolate (CVB1-L071615) shared 61.3-67.6% identity with all other viruses. Finally, E11 exhibited the greatest genetic distance, sharing only 59.3-62.2% of its VP1 gene with the other viruses considered. At the protein level, the pairwise identity among CVB5 environmental isolates corresponded to 99%, but was only 92 and 90% when compared to CVB4 isolates and E11, respectively (Table S3). The distribution of isolated serotypes is consistent with literature reporting that CVB5 is the most recurrent enterovirus, with high isolation frequencies[58] and high annual prevalence[59,60].

Inactivation kinetics

The distributions of the inactivation rate constants for all viruses and treatment methods studied is shown in Figure 2. The values of the inactivation rate constants and associated statistics, along with the inactivation curves, are shown in the Supporting Information (Table S4 and Figures S1-S5). From these data it is evident that variability exists among different viruses in their susceptibility to disinfectants, and that the extent of this variability differs between the inactivation methods tested. An ANCOVA analysis furthermore confirmed that the rate constants differed between virus strains,

237 but were mostly consistent between experimental replicates of a single virus, even if stock solutions
238 produced by different amplifications were used (see Supporting Information).

239 *UV and Sunlight inactivation.* Inactivation by UV_{254} and sunlight were first-order with respect to
240 fluence (Figures S1 and S2). The mean UV_{254} inactivation rate constants (k_{UV}) for all enteroviruses
241 tested ranged from 0.28 to 0.38 $mJ^{-1}\cdot cm^2$ (Figure 2A). These rate constants are consistent with those
242 previously reported for different enteroviruses (Hijnen et al. 2006, and references therein[61]). If only
243 strains of the CVB5 serotype are considered, the variability is smaller, with mean values of k_{UV}
244 ranging from 0.32 to 0.37 $mJ^{-1}\cdot cm^2$. MS2 was more resistant to UV_{254} (0.15 $mJ^{-1}\cdot cm^2$) than all
245 enteroviruses.

246 Inactivation by sunlight led to a wider distribution of inactivation rate constants. Specifically, the
247 mean k_{sun} values for the different enteroviruses spanned a range from $1.3\cdot10^{-3}$ to $9.0\cdot10^{-3}$ $mJ^{-1}\cdot cm^2$
248 (Figure 2C). These values correspond well to those previously reported for the inactivation of PV3 by
249 simulated sunlight in PBS[62]. The variability of inactivation by sunlight was slightly reduced if only
250 CVB5 strains were considered, with the most resistant strain (Faulkner) exhibiting a mean rate
251 constant of $3.2\cdot10^{-3}$ $mJ^{-1}\cdot cm^2$. As for UV_{254}, MS2 was more resistant than any of the enteroviruses
252 tested.

253 The differences in susceptibility of the different viruses to UV_{254} and sunlight can be partly explained
254 by the difference in the genome length. Given that all viruses tested have the same genome type
255 (ssRNA), and assuming a constant rate of genome lesion formation during exposure to radiation, a
256 longer genome will result in a higher number of lesions per genome[63]. Correspondingly, if *k* of each
257 virus is normalized by its respective genome length, the variability in *k* decreases, though does not
258 disappear (Figure S6).

259 *Free chlorine and chlorine dioxide.* Inactivation by FC was first-order with respect to dose (Figure S3),
260 and yielded enterovirus inactivation rate constants (k_{FC}) ranging from 0.8 to 8.0 $mg^{-1}\cdot min^{-1}\cdot L$ (Figure
261 2E). As such, inactivation by FC exhibited the greatest variability among the disinfectants tested.

Variability was also observed within the different CVB5 strains, for which the k_{FC} ranged from 0.8 to 4.9 mg^{-1}·min^{-1}·L. This range also includes FC inactivation rates constants of CVB5 Faulkner determined by others under similar experimental conditions[64]. The k_{FC} of MS2, which corresponded to 5.9 mg^{-1}·min^{-1}·L, fell within the upper range of enteroviruses.

In contrast to FC, inactivation by ClO_2 deviated from first-order and exhibited a tail at higher ClO_2 doses (Figure S4). The earliest onset of a tail occurred at a ClO_2 dose of approximately 0.5 mg·min·L^{-1}. Tailing could not be attributed to ClO_2 depletion, as the ClO_2 concentration was approximately constant throughout the experiment (see Experimental Section). Tailing during ClO_2 disinfection has frequently been reported and has been attributed to various causes including virus aggregation[34], heterogeneity of the virus population[65], or the accumulation of oxidation products that form a protective layer around the residual infective viruses[66]. Aggregation was not observed among the viruses studied herein (see Experimental Section), which rules out this feature as a cause of tailing. To determine k_{ClO2}, only the rapid, initial portion of the inactivation curve was considered (k_1 in equation 3). Values of k_{ClO2} for all viruses tested ranged from 16.4 to 46.1 mg^{-1}·min^{-1}·L (Figure 2G). A slightly smaller range in mean k_{ClO2} values was observed among strains of CVB5, which ranged from 16.4 to 35.1 mg^{-1}·min^{-1}·L. The k_{ClO2} for MS2 (37.9 mg^{-1}·min^{-1}·L) fell within the upper range of the enteroviruses.

For E11, it was previously found that FC and ClO_2 act on both the viral proteins and genome[67]. Differences in the chemical reactivity of the viral proteins or genome toward FC and ClO_2 may thus explain some of the variability in the observed inactivation rate constants of the viruses considered in the present study. The abundance of readily oxidizable, solvent-exposed amino acids on the structural proteins was strongly correlated with k_{FC} (Pearson's r=0.79), but only weakly and inversly with k_{ClO2} (Pearson's r=-0.22; Figure S7). At the genome level, guanosine is the most reactive nucleotide toward both FC and ClO_2[68,69], and the degradation of the 5' non-coding region was previously found to correlate with inactivation by ClO_2[70]. Here, we therefore explored if the

guanosine content of the 5' non-coding region could be used as an indicator of a virus susceptibility to FC or ClO_2. A moderate correlation with the respective inactivation rate constants was observed for FC (Pearson's r=0.51), and a weak one with ClO_2 (Pearson's r=0.31; Figure S8). To improve these correlations, further information on the RNA and protein secondary and tertiary structure may be needed. This analysis indicates that kinetics of inactivation by FC may be influenced by the chemical virus composition, whereas inactivation by ClO_2 is mainly linked to biological factors, such as the use of different host cell receptor sites or different recombination efficiencies.

Heat. Similar to inactivation by ClO_2, exposing the different enteroviruses to a temperature of 55 °C resulted in tailing inactivation curves (Figure S5). The onset of the tail varied greatly between the different viruses, ranging from 15 seconds of heat exposure (CVB5-Faulkner, CVB5-L030315, CVB5-L061815, CVB4-M063015, and E11, Figure S5) to no observable tail throughout the experimental time considered. For the early tailing viruses, only the linear part of the inactivation curve was considered to determine the inactivation rate constant k_{heat} (k_1 in equation 3). The mean values of k_{heat} for the enteroviruses considered ranged from 0.15 to 2.11 sec^{-1}. The corresponding range for only CVB5 strains was slightly narrower, reaching from mean values of 0.16 to 0.63 sec^{-1}, whereas MS2 displayed a much lower mean value of k_{heat} of 0.006 sec^{-1} (Figure 2I).

Changes in capsid amino acid residues have been previously linked to increases in the thermal stability of foot-and-mouth disease virus, another virus in the *Picornaviridae* family[71]. A similar effect may be caused by small differences in the amino acid content of the structural virus proteins (Table S2), which may cause the observed differences in k_{heat} among the different enteroviruses considered. Furthermore, at the treatment temperature used (55°C), capsid disruption followed by RNA escape is a probable reason for the inactivation of enteroviruses[72,73]. Heat resistance is thus likely linked to the strength of the interaction between the virus capsid subunits[73], which may differ among the different serotypes and strains. A corresponding analysis is the subject of an ongoing study in our laboratory.

Inter-serotype comparison of inactivation kinetics

312 The different enterovirus strains considered herein exhibit similar genomic and protein features
313 (Tables S2 and S3), yet the different serotypes are still genetically distant from one another (Figure
314 1). To determine how this genetic diversity is reflected in disinfection susceptibility, the viruses were
315 grouped by serotype; for CVB4 and CVB5, which contained more than one strain, a mixture
316 distribution of *k* values was established (Figures 2B, D, F, H, and J). Bayesian analysis was then
317 applied to quantify the probability that a given serotype is more or less resistant than any of the
318 other serotypes tested. Because the different disinfectants inactivate viruses via different
319 mechanisms[74], these analyses were carried out for each disinfectant individually, to capture
320 mechanism- or disinfectant-specific variability in virus resistance.

321 *UV_{254} and Sunlight inactivation.* Comparisons between the four different serotypes revealed that
322 despite their similarity in genome length and composition, their sensitivities to UV_{254} nevertheless
323 differed (Figures 2B). Specifically, CVB1 exhibited a >99% probability of being more resistant than the
324 other serotypes tested, whereas the rate constants for the other serotypes grouped more closely. .
325 However, while the observed differences in k_{UV} between CVB1 and the other enterovirus serotypes
326 were quantifiable, they are of little practical significance: to achieve a 4-$\log_{10}$ inactivation, CVB1
327 required a UV_{254} dose of 32.5 mJ·cm^{-2}, whereas the most susceptible serotype (CVB4) required a
328 similar dose of 25.3 mJ·cm^{-2} (Table S4).

329 A very different resistance pattern was observed for inactivation by sunlight (Figures 2D). CVB5 was
330 the least resistant virus, with a >85% probability of being less resistant than any other serotype,
331 whereas E11 had a >99% probability of being the most resistant. In contrast to UV_{254} inactivation, this
332 variability translates into a substantial differences in the environmental persistence in sunlit waters,
333 or the inactivation performance by devices relying on disinfection by solar UVB: to achieve a 4-$\log_{10}$
334 inactivation, CVB5 required a UVB dose of 1369 mJ·cm^{-2} , which is the equivalent of 2.3 days of solar
335 UVB exposure at the equator. To achieve the same $\log_{10}$ reduction, E11 required a dose of 5430
336 mJ·cm^{-2}, which corresponds to approximately 9.1 days of solar UVB exposure at the equator.

337 The discrepant resistance patterns of viruses toward UV_{254} and sunlight indicates that the mechanism
338 of action of these two inactivating treatments differ. This can be rationalized by a number of causes.
339 First, the wavelength spectrum and fluence rates of these two methods are different, and thus the
340 type and yield of lesions to the viral genome likely differs[75]. Second, differences in thermal stability of
341 the viruses may influence the observed inactivation rates, in particular if thermal inactivation during
342 the lengthy sunlight experiments synergistically promoted inactivation by sunlight. And finally,
343 inactivation by sunlight may involve a greater portion of protein damage compared to UV_{254}, which
344 may contribute to inactivation[76].
345 *Free chlorine and chlorine dioxide.* FC and ClO_2 treatment varied in their effect on the different
346 enterovirus serotypes (Figures 2F, 2H). For inactivation by FC, E11 had a >98% probability of being
347 less resistant than the other serotypes, whereas CVB1 was the most resistant serotype (>85%
348 probability). The susceptibility of the different serotypes results in considerable differences in the FC
349 dose to achieve a 4-log_{10} inactivation: E11 required a dose of 1.1 mg·min·L^{-1} while CVB1 required a
350 dose of 6.4 mg·min·L^{-1}.

351 For disinfection by ClO_2, CVB4 and CVB5 had high probabilities (>99% and >92%, respectively) of
352 being more resistant than CVB1 or E11. The latter two serotypes exhibited comparable
353 susceptibilities to ClO_2. This latter finding is surprising, as CVB1 was the most resistant serotype
354 toward FC. The observed extent of variability in disinfection kinetics among serotypes did not lead to
355 significant differences in the ClO_2 requirements at low levels of inactivation. Specifically, the most
356 resistant serotype (CVB4) required a dose of 0.11 mg·min·L^{-1} for a 1-log_{10} inactivation, and the most
357 susceptible serotype (CVB1) a dose of 0.05 mg·min·L^{-1}. These dose requirements, however, differ
358 more dramatically if higher levels of inactivation are considered, as for some serotypes and
359 environmental isolates, a 4-log_{10} inactivation could not be achieved due to tailing of the disinfection
360 curve (Figure S4 and Table S3).

Heat. Inactivation by heat was the least effective against CVB1 and CVB5, which were more thermally stable than the other two serotype tested with >99% probability (Figure 2J). The most heat sensitive serotype was CVB4, though the two strains contained in this serotype exhibited vastly different susceptibilities to heat.

In summary, the results of this study demonstrate pronounced variability in disinfection susceptibility among four different enterovirus serotypes. While disinfection requirements were fairly homogeneous across serotypes and strains for UV_{254}, the requirements for sunlight, FC, ClO_2 and heat for a given enterovirus serotype were not predictive of that of other serotypes.

Assessment of enterovirus lab strains or MS2 as surrogates for the disinfection of environmental isolates

Many virus disinfection studies to date rely on laboratory strains[9,64,77–81], which are easy to obtain because they are commercially available. Results from such studies should be interpreted with caution, since we showed here that a single laboratory strain of a single serotype may not accurately reflect the inactivation kinetics of other serotypes (Figure 2). Additionally, as illustrated below, we here show that even the lab strain of a specific serotype (here CVB5 Faulkner strain) may not be a suitable representative for environmental isolates of the same serotype. Specifically, inactivation kinetics of different isolates of CVB5 by sunlight and heat were only poorly represented by the corresponding laboratory strain. Bayesian analysis demonstrated that the Faulkner strain had a > 99% probability of being more resistant to solar disinfection and less resistant to heat compared to the environmental isolates (Figure 3). This indicates that the lab strain is not a good surrogate to assess the environmental stability of isolates.

The Faulkner strain was also not a suitable surrogate for the inactivation of most environmental CVB5 isolates by oxidants and UV_{254}. Bayesian analysis confirmed that all but one of the CVB5 isolates (CVB5-L060815) had a high (>99%) probability of being more resistant to FC than the Faulkner strain (Figure 3), and exhibited inactivation rate constants that were up to five times lower than the

386 Faulkner strain (Table S4). Similarly, all but two isolates (CVB5-L061815 and CVB5-L070215) had a
387 high (90%) probability of being more resistant to ClO_2 compared to the Faulkner strain (Figure 3),
388 though the differences in inactivation rate constants were less pronounced compared to FC (Table
389 S4). Finally, three of the environmental isolates (CVB5-L061815, CVB5-L070215 and CVB5-L030315)
390 were more resistant to UV_{254} than the Faulkner strain, while others (CVB5-L060815, CVB5-L070915
391 and CVB5-M063015) had similar probabilities of being more or less resistant (Figure 3).

392 Our data thus imply that disinfection studies based on lab strains may not be representative of many
393 viruses circulating in the environment. Reliance on laboratory strains may therefore lead to the
394 underestimation of actual treatment requirements. This latter point is emphasized in Figure 4, which
395 compares our data with the US EPA's recommended *Ct* value for a 4 log_{10} inactivation of viruses by FC
396 at 20°C[82]. While both laboratory strains (E11 Gregory and CVB5 Faulkner) tested fall well below the
397 EPA *Ct* requirement, several of the environmental isolates exceed it, such that the EPA
398 recommendation would not guarantee a 4-log_{10} reduction for these viruses.

399 Bacteriophages such as MS2 have also been proposed as surrogates for the disinfection of enteric
400 viruses[20], since they have similar properties as enteric viruses[83]. This approach is popular because
401 phages are easier and cheaper to handle than actual human viruses. However, as is evident from
402 Figure 2 and confirmed by Bayesian analysis (Figure S9), MS2 is not always a good surrogate for the
403 inactivation kinetics of enteroviruses present in the treatment systems. Specifically, MS2 was less
404 resistant to inactivation by FC and ClO_2 than many of the coxsackieviruses studied, though it was
405 representative of, or even more resistant than, E11. If MS2 is used as an indicator for virus
406 inactivation by FC or ClO_2, the disinfection requirements of many *Enterovirus B* strains would thus be
407 underestimated. In contrast, MS2 can be considered a good conservative surrogate for *Enterovirus B*
408 inactivation by heat, UV_{254} and sunlight, as it was significantly more resistant to these treatments
409 than any enterovirus studied. As discussed above, the greater resistance of MS2 to radiation can be
410 partly explained by the difference in the genome length of MS2 (3569 bases) compared to

411 enteroviruses (ca. 7400 bases). However, even if corrected for genome length (Figure S6), MS2 still
412 mostly underestimated enterovirus inactivation by UV_{254} and sunlight.

413

414 Overall, this analysis reveals that neither the lab strains nor MS2 bacteriophage can satisfactorily
415 model inactivation behavior of all *Enterovirus B* species. Given the significant variability of
416 inactivation kinetics among commonly occurring enteroviruses, we therefore recommend that future
417 disinfection studies be conducted based on a range of viruses that include environmental isolates, as
418 well as different serotypes.

419 **SUPPORTING INFORMATION**

420 The Supporting Information is available free of charge on the ACS Publications website at
421 http://pubs.acs.org. Additional information on chemicals, laboratory virus strains, plaque assays,
422 Bayesian analysis and ANCOVA analysis; tables with primer information, nucleotide and protein
423 pairwise identities of all viruses, *k* values and *Ct* values for 1 to 4-$\log_{10}$ inactivation; plots showing the
424 inactivation curves for all virus-disinfectant combinations; k_{UV} and k_{sun} normalized by genome length;
425 a correlation analysis of oxidizable amino acids or guanosine content with k_{FC} and k_{ClO2}; and the
426 Bayesian comparison between enteroviruses and MS2.

427

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432

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Table 1. Serotype, genus, strain, and isolation location of viruses used in this work. The environmental isolates are named according to first letter of the city (Minneapolis, Tampa, or Lausanne) and the isolation date (month/day/year).

Name	Serotype	Genus	Location	Strain	Access.No
MS2		Levivirus	Lab strain	ATCC 15597-B1	NC001417
Echovirus 11	Echovirus 11	Enterovirus	Lab strain	ATCC-Gregory	X80059
CVB1-L071615	Coxsackievirus B1	Enterovirus	Lausanne	Env. isolate	MG845887
CVB4-M063015	Coxsackievirus B4	Enterovirus	Minneapolis	Env. isolate	MG845888
CVB4-T051217	Coxsackievirus B4	Enterovirus	Tampa	Env. isolate	MG845889
CVB5-Faulkner	Coxsackievirus B5	Enterovirus	Lab strain	ATCC-Faulkner	AF114383
CVB5-L030315	Coxsackievirus B5	Enterovirus	Lausanne	Env. isolate	MG845890
CVB5-L060815	Coxsackievirus B5	Enterovirus	Lausanne	Env. isolate	MG845891
CVB5-L061815	Coxsackievirus B5	Enterovirus	Lausanne	Env. isolate	MG845892
CVB5-L070215	Coxsackievirus B5	Enterovirus	Lausanne	Env. isolate	MG845893
CVB5-L070915	Coxsackievirus B5	Enterovirus	Lausanne	Env. isolate	MG845894
CVB5-M063015	Coxsackievirus B5	Enterovirus	Minneapolis	Env. isolate	MG845895

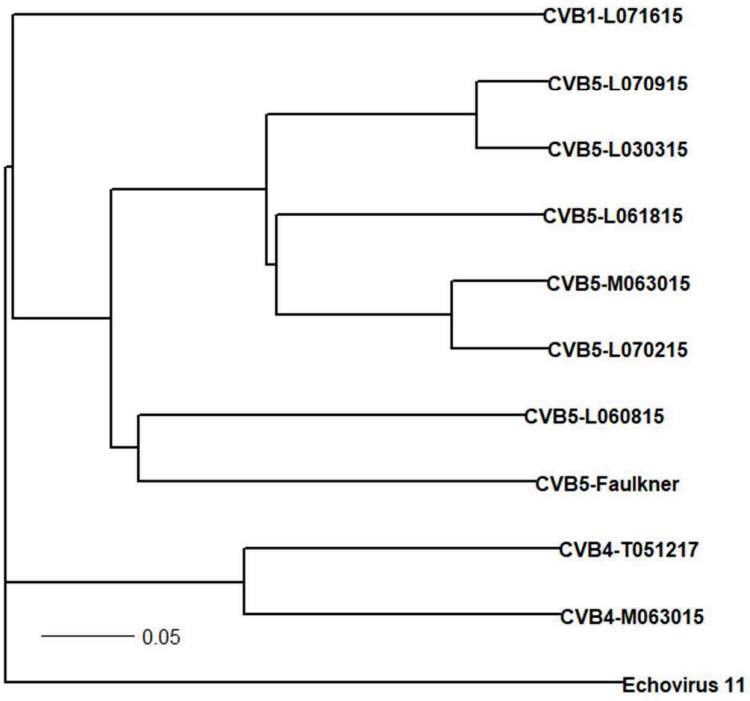

Figure 1. Unrooted neighbor joining tree of the viruses studied, built based on the virus VP1 coding region. The horizontal lines lengths are proportional to the genetic distance (see Table S2). The four serotypes represented can be clearly differentiated by their genetic distance.

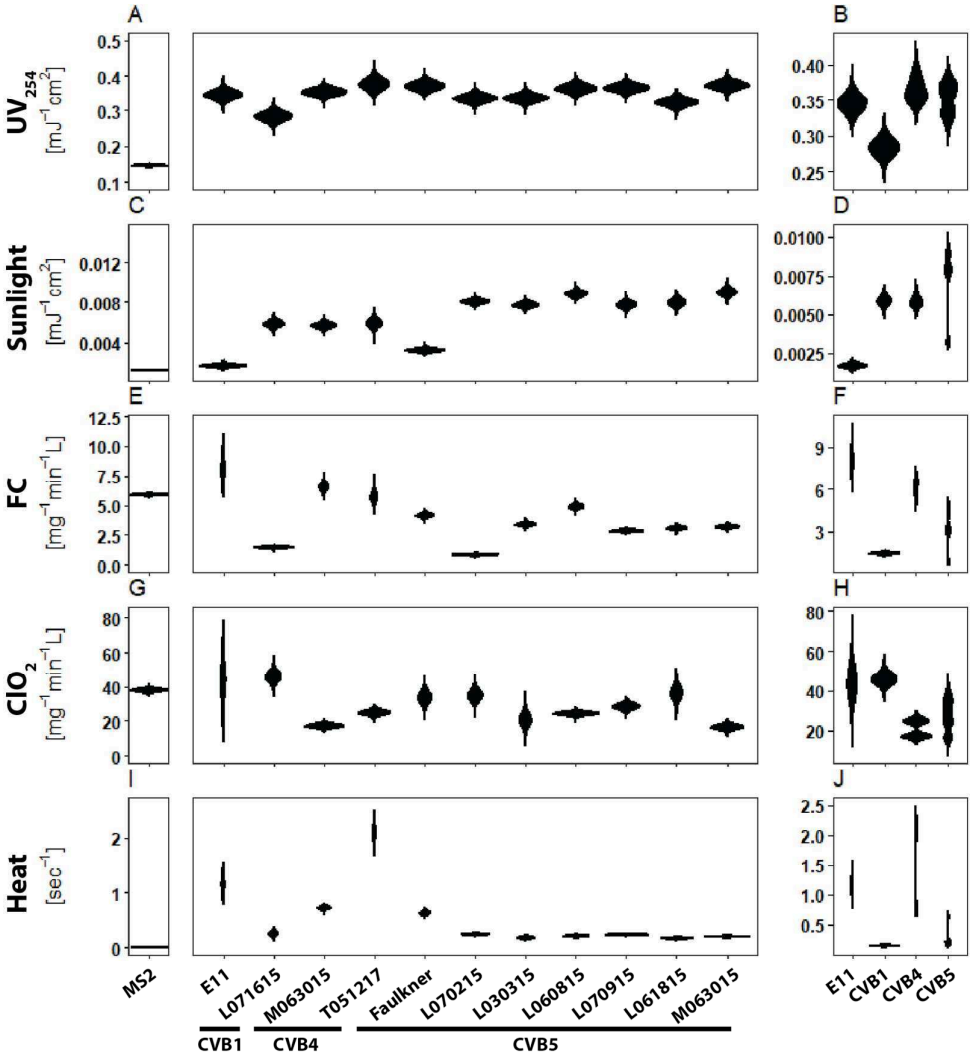

Figure 2. Violin plots of showing the distribution and probability density of rate constants *k* associated with inactivation by UV_{254} (panels A and B), sunlight (C and D), FC (E and F), ClO_2 (G and H) and heat (I and J). The values of *k* for individual viruses (MS2 and all enteroviruses) are shown in the left panels. The right panels show the mixture probability distribution of each serotype (E11, CVB1, CVB4, and CVB5), which results from the combination of the individual *k* distributions of all viruses of a given serotype. The exact values of *k* are given in Table S4.

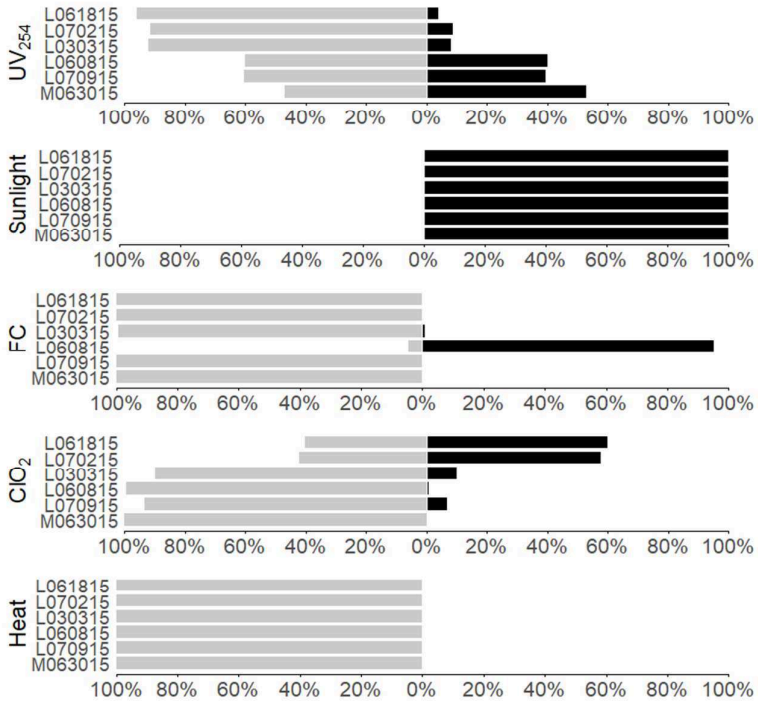

Figure 3. Bar plots showing a comparison of the probabilities of inactivation rate constants (*k* values) for all environmental isolates of CVB5 with the CVB5-Faulkner laboratory strain. Grey bars indicate the probability that the environmental isolate is more resistant than the Faulkner strain; black bars indicate the probability that the environmental isolate is less resistant than the Faulkner strain.

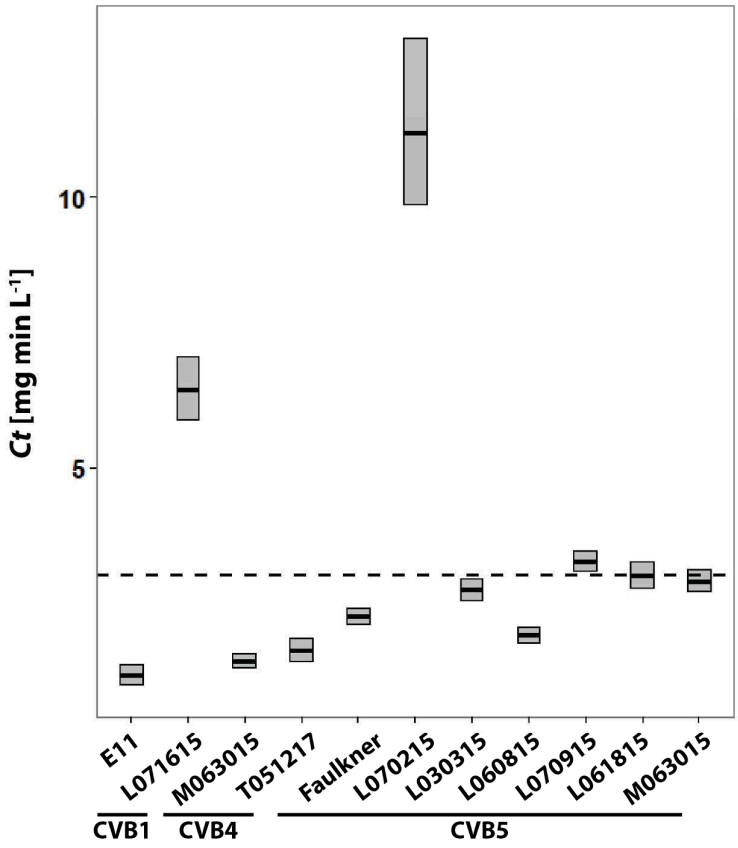

Figure 4. *Ct* values to achieve a 4-$\log_{10}$ virus inactivation by FC. The dashed line corresponds to the US EPA's recommendation for *Ct* value at 20°C[82] (3 mg·min·L^{-1}). The bar plots indicate the mean *Ct* (black line) calculated based on the k_{FC} measured herein, along with the upper and lower 95% confidence intervals (top and bottom of box).