Adaptation of human enterovirus to warm environments leads to resistance against chlorine disinfection

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Abstract

Sunlight, temperature and microbial grazing are among the environmental factors promoting the inactivation of viral pathogens in surface waters. Globally, these factors vary across time and space. The persistence of viral pathogens, and ultimately their ecology and dispersion, hinges on their ability to withstand the environmental conditions encountered. To understand how virus populations evolve under changing environmental conditions, we experimentally adapted echovirus 11 (E11) to four climate regimes. Specifically, we incubated E11 in lake water at 10°C and 30°C and in the presence and absence of sunlight. Temperature was the main driver of adaptation, resulting in an increased thermotolerance of the 30°C adapted populations, whereas the 10°C adapted strains were rapidly inactivated at higher temperatures. This finding is consistent with a source-sink model in which strains emerging in warm climates can persist in temperate regions, but not vice versa. A microbial risk assessment revealed that the enhanced thermotolerance increases the length of time in which there is an elevated probability of illness associated with swimming in contaminated water. Notably, 30°C adapted viruses also exhibited an increased tolerance toward disinfection by free chlorine. Viruses adaptation to warm environments may thus become harder to eliminate by common disinfection strategies.

Keywords: QMRA, enterovirus, disinfection, experimental evolution, climate change

Introduction

Environmental and climatic factors have a major influence on the structure, composition and functioning of microbial communities that are essential for the ecological equilibrium of our planet.¹ The same factors also act on contaminant microorganisms, including human enteric viruses. Enteric viruses are continuously released into the environment, via the discharge of raw sewage, wastewater effluent, inadequate sanitation, or the land application of biosolids.² While circulating in the environment, enteric viruses are exposed to various environmental and climatic stressors, which modulate the composition and size of the infectious virus population. In surface waters, enteric viruses are inactivated by solar radiation, elevated temperature, and microbial grazing (which in turn is also influenced by temperature).³⁻⁸ Temperature and, to a minor extent, solar radiation were also found to inactivate viruses in fresh produce, such as lettuce and berries.^{9,10} Other environmental parameters including relative humidity and pH also play a role in the persistence of human enteric viruses in environmental samples and food.¹¹ Environmental and climatic factors thus may not only be important determinants affecting the dynamics of indigenous populations of microorganisms, but also those of infectious diseases.¹²

In a globalized world, humans and their goods (e.g., vegetables, shellfish, drinks) move across large distances. Human pathogens can therefore experience rapid changes in the environmental conditions they are exposed to. Changes in disease dynamics can also occur at a local scale across seasons or due to longer term trends like climate change, which includes global warming and extreme weather events.¹³ Globalization and climate change are expected to influence the occurrence of infectious diseases worldwide.¹⁴ However, it is not fully understood how the associated changes in environmental conditions influence the dynamics of infectious diseases. Particularly, little is known about how viruses adapted to a particular habitat or climate, persist when they are transported and exposed to a novel environment, and to what extent they are capable of replacing previously circulating strains.

In this study, we experimentally adapted a human enterovirus (echovirus 11, E11) to lake water incubated under four different regimes (10°C in the dark, 10°C under simulated sunlight, 30°C in the dark and 30°C under simulated sunlight). The resulting populations were exposed to different environmental stressors (heat, simulated sunlight and microbial grazing) and their phenotypic and genotypic differences were compared. Transplant experiments were also conducted to investigate the maintenance of environmental persistence when exposed to a different regime than that in which the population adapted. Finally, enteroviruses isolated from the environment have previously been found to be more resistant to chemical disinfection compared to their respective laboratory strains.^{15,16} This suggests that the different stressors that shape the viral population in the environment also select for

viruses with an increased disinfection resistance. We therefore exposed the adapted populations to a common water disinfectant (free chlorine) to determine if this treatment method differs in its efficacy depending on the conditions present in the adaptation history of a population. Our results expand the current understanding of the ability of viruses to move across the globe due to globalization and raise the awareness of the role of the environment on the evolution, dynamics, and control of environmentally transmitted viral pathogens infecting humans.

Materials and methods

Virus preparation and quantification. Human echovirus 11 (E11) stocks (Gregory strain X80059.1, ATCC® VR737™) were produced by infecting sub-confluent monolayers of Buffalo Green Monkey Kidney (BGMK) cells (kindly provided by the Spiez Laboratory, Switzerland). The cells were maintained at 37°C in 5% CO₂ with modified Eagle medium (MEM; Gibco, Frederick, MD), supplemented with 1% Penicillin-Streptomycin (Gibco, Frederick, MD) and 10% (during the growth of the cells) or 2% (to maintain the cell monolayers and during viral infections) heat-inactivated fetal bovine serum (FBS; Gibco, Frederick, MD). The viruses were released from infected cells by freezing (-20°C) and thawing (at room temperature) the culture flasks for three times. To eliminate cell debris, the suspensions were centrifuged at 3,500 rpm for five minutes. Then, the supernatant was transferred to a new tube and the pellet was discarded. To quantify the virus samples, we used a most probable number (MPN) infectivity assay. In brief, BGMK cells were grown to 95% confluence in flat-bottom 96-well plates (Greiner CELLSTARVR; Sigma Aldrich). Then, virus samples were diluted in quintuplicates over a tenfold dilution series in MEM supplemented with 2% FBS. The growth medium in each well was discarded and replaced with 150 µl of the serially-diluted virus sample. Infected plates were incubated for five days, after which inverted microscopy was used to differentiate infected from non-infected wells. E11 concentrations in the samples were determined as the most probable number of cytopathic units per milliliter (MPNCU/ml). The resulting stocks of E11 ($\approx 10^8$ MPNCU/ml) were stored at 4°C until use.

Directed experimental evolution passages. Prior to exposure to variable environmental conditions, an E11 population was adapted to cell culturing in BGMK cells by conducting five passages in the presence of MEM supplemented with 2% FBS. In passage 1, 100 µl of virus solution (equivalent to 10^7 MPNCU) was inoculated in a flask containing BGMK cells in monolayer. After 3 days of incubation at 37°C and 5% of CO₂, 100 µl of the supernatant were transferred to a new flask of BGMK cells in monolayer, and this process was repeated up to passage five. The resulting cell-culture adapted population, hereafter named wild type (WT), served as the starting population for the following experimental evolution passages.

The WT population was adapted to four different environments over 20 cycles, which are equivalent to roughly 200 viral generations.¹⁷ Specifically, two populations were adapted to a cold habitat (10°C), and two were adapted to a warm habitat (30°C). For each cold/warm group, one population was adapted under dark conditions while the other was exposed to simulated sunlight radiation. Hereafter, the adapted populations will be referred to as 10D, 10L, 30D and 30L, where the number refers to the adaptation temperature, and D and L stand for the dark and light solar radiation regime, respectively. The adaptation was conducted in fresh lake water collected from Lake Geneva, which contains microorganisms capable of inactivating E11.⁷ Each evolution passage included two steps: first, the virus populations were inoculated in lake water and exposed to the corresponding temperature and light regime conditions during the time needed to achieve a 2-log₁₀ reduction in their initial titer. Because the inactivation at 10°C in dark conditions was too slow to achieve 2-log₁₀ decay within a feasible time frame, the 2 log₁₀ reduction in the titer of this population was completed by dilution with lake water and was measured by MPN as described in the previous section. Second, the survivors were regrown in BGMK cells for four days to expand the population size. After regrowth, the viruses were treated as detailed in the previous section and the experiment proceeded with a new round of exposure to temperature and light (inactivation phase) and subsequent regrowth. The 2-log₁₀ reduction in virus titer resulted in a low multiplicity of infection (MOI) for regrowth of approximately 0.01. An overall schematic representation of the experimental design is shown in Figure 1.

Replicative fitness of the adapted populations in host cells. To characterize the replicative fitness of the adapted populations, we compared their growth in BGMK cells over 72 hours. Specifically, sub-confluent monolayers of BGMK cells were infected at an MOI of 0.001 (initial virus concentration of 10³ MPN/ml approximately) and incubated at 37°C and 5% of CO₂. Aliquots of the culturing media were collected periodically at the beginning of the experiment and after 72 hours post-infection, and the increase in virus titer was quantified by MPN assay as described above. The experiment was conducted in triplicate.

Sanger sequencing. Nucleic acids were extracted using the PureLink™ Viral RNA/DNA kit (Invitrogen) following the manufacturer's instructions. The extracted RNA was reverse transcribed and amplified with a OneStep RT-PCR Kit (Qiagen) in a Trio thermal cycler (Biometra) as previously described.¹⁷ The obtained PCR amplicons were then purified using the MSB Spin PCRapace Kit (Strattec). The sequencing PCR reactions were conducted for 20 cycles as follows: 96 °C for 10 seconds, 50 °C for 10 seconds, 60 °C for four minutes with a final hold at 12 °C using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Lastly, the nucleic acids were sequenced in an ABI 3130x Genetic Analyzer (Applied Biosystems). Nearly the complete genome was sequenced for the 10D and 30D populations.

For the 10L and 30L populations we only sequenced the VP1 region in order to confirm the presence or absence of the mutations previously identified in the other populations.

Effect of microbial, thermal and sunlight stress on the adapted populations. To characterize the susceptibility of each adapted population to environmental stressors, we exposed them to microbial grazing, increasing temperatures and simulated solar radiation. Firstly, the susceptibility of the evolved populations to microbial grazing was investigated by conducting inactivation experiments in fresh lake water and in lake water that was filtered-sterilized through 0.22 μm filters (Millipore). The filtration step removed all autochthonous microbiota that may contribute to virus inactivation in lake water.⁷ In these experiments, 5-ml of water were placed in falcon tubes and inoculated with 100 μl of the stocks of each adapted population. Then, the tubes were incubated at room temperature (approximately at 20 °C) in the laboratory for 72 hours. During the experiment, aliquots were collected at different time points from the incubated tubes and frozen until the end of the experiment. Then, the virus titer in all samples was quantified by MPN as described above.

The thermal stability of the adapted population was tested with a temperature shift experiment using a PCR thermal cycler (PCR System 9700, GeneAmp). Ten microliters of virus solution were inoculated in PCR tubes containing 90 μL of phosphate-buffered saline (PBS; 5 mM phosphate, 10 mM NaCl [pH 7.5]) for each adapted population. One tube was prepared for every population and temperature, and one tube was retained for every population to determine the initial titer by MPN. Starting from 40°C, a thermal shift regime was applied by increasing 2°C per step, up to 50 °C. At each temperature, the PBS was preheated in the thermal cycler for 2 min before the virus solution was inoculated. The solution was kept at each target temperature for one minute and thereafter immediately transferred on ice until the enumeration by MPN was conducted, as described above.

Sunlight inactivation experiments were performed as previously described.^{18,19} Briefly, glass beakers containing two mL of filter-sterilized or unfiltered lake water inoculated with virus solutions at approximately 1×10^6 MPNCU/mL were placed under a solar simulator (Sun 2000, ABET Technologies) equipped with a 1,000 W Xenon lamp, an air mass 1.5 filter, and an atmospheric edge filter to simulate the solar radiation spectrum. The experiments were conducted in a thermostatic bath at 20°C and the glass beakers were continuously stirred by means of magnetic stir bars. Aliquots were taken at time intervals of one hour over the course of eight hours, and the virus titer in each aliquot was quantified by MPN as described above. The solar fluence rate was determined by a radiometer (ILT 900-R; International Light Technologies, Peabody, MA) for the wavelength range from 280 to 800 nm, and corresponded to $249 \pm 14 \text{ W/m}^2$.

All the experiments described in this section were conducted at least in two independent replicates.

Transplant experiments. We conducted transplant experiments to investigate the persistence of the 10D and 10L populations when exposed to 30°C, and the persistence of 30D and 30L populations when exposed to 10°C. Aliquots of the virus stocks of all four adapted populations were inoculated into tubes containing 900 µl of filter-sterilized lake water. The solutions were then incubated at 10 or 30°C in the dark for 56 and 7 days, respectively. During the experiment, aliquots were regularly collected and quantified by MPN as described above. The experiments conducted at 10°C were conducted in only one replicate, while at 30°C we analyzed two independent replicates.

Free chlorine inactivation experiments. Inactivation experiments with free chlorine (FC) were conducted as described elsewhere²⁰ using chlorine-demand free beakers prepared by soaking in concentrated FC solution overnight and washed with ultra-pure water thereafter. Initial chlorine concentrations were set to 1 mg/L and were achieved by diluting NaClO (13–14%, Reactorlab SA) in PBS (pH 7.4). Samples were taken periodically at time intervals of 10 or 15 s over 90 s, and were immediately diluted into a 9-fold volume of Tris buffer (10 mM Tris, pH 7.4) to quench the FC and stop the disinfection process. The FC concentration was monitored in every sample using the N,N-diethyl-p-phenylenediamine (Sigma-Aldrich) colorimetric method and was found to be stable during the experiment. Viral decay was monitored over time by MPN as described above. The experiments were conducted in duplicate.

Quantitative microbial risk assessment. To demonstrate potential health impacts of increased environmental persistence of enterovirus in natural surface waters, a stochastic quantitative microbial risk assessment (QMRA) model was developed. The QMRA model estimated probability of illness from swimming in surface waters contaminated with wastewater effluent used using Monte Carlo methods (n = 10000 simulations).^{22,23} Probability of illness was estimated for a period of ten days following the contamination event, which was assumed to be a one-time point source contamination event.

Specifically, ingested dose from swimming was modeled as

$$D = VdC^{-kt} \quad \text{eq. (1)}$$

Where D is the dose or number of enterovirus ingested (in plaque forming units; PFU), V is the volume of water ingested while swimming (ml), d is the dilution of wastewater effluent in the water (unitless), C is the concentration of enterovirus in wastewater effluent (PFU/ml), k is the decay rate constant to model E11 inactivation in surface water since time t . The values of k were determined from first order fits of infectivity versus time from the transplant experiment at 30°C. Two separate QMRA models were used: one based on the decay rate constant for the 10D population (k_{10D}) and the other based on the decay rate constant for the adapted population; 30D (k_{30D}).

Probability of infection was modeled as:

$$P(\text{inf}) = 1 - e^{-rD} \quad \text{eq. (2)}$$

Where $P(\text{inf})$ is the probability of infection (unitless), estimated based on the best-fit exponential dose-response model for enterovirus²⁴ described by parameter r , which estimates the probability of infection per enterovirus ingested (1/PFU).

We assumed dose-response relationships and estimated parameters were the same for both the 10D and 30D population. However, adaptations to environmental persistence may also influence virus infectivity and the corresponding dose-response relationships. Enterovirus strains may differ in their infectivity, as shown by varying estimates of k for the two strains of enterovirus evaluated by Cliver (1981)^{24,25}. Observed similar in vitro growth rates of the 10D and 30D strains after 72 hours (ANOVA, $F(3, 8) = 1.2$, $p\text{-value} = 0.37$), supports the assumption of similar infectivity, though in vitro growth rates are not necessarily representative of infectivity. Nevertheless, the general trend of increased risk from increased environmental persistence is expected if the infectivity of the more persistent strain (30D) is equal to or greater than the infectivity of the less persistence strain (10D).

Because only a proportion of infections will lead to symptomatic illness, probability of illness is modeled as:

$$P(\text{ill}) = P(\text{ill}|\text{inf})P(\text{inf}) \quad \text{eq. (3)}$$

Where $P(\text{ill})$ is the probability of illness, and $P(\text{ill}|\text{inf})$ is the probability of illness given infection.

Parameter values are described by previously published distributions^{23,24,26–28} (Table 1). Additionally, decay rate constants were obtained from this study. Model interpretation is based on the time required since the contamination event for median probability of illness from swimming to fall below acceptable risk threshold (defined here as 8 out of every 1000 bathers, based on U.S. EPA).²⁸ Sensitivity Analysis was conducted using Spearman correlation coefficients between the input parameter values and the resulting probability of illness.²⁹

Data analyses. All statistical analyses were conducted using the Graph Pad Prism software (Prism 7 version 7.0a, 2016), with the exception of the QMRA model conducted in R (R Version 3.6.2).³⁰ Microbial and solar inactivation, as well as thermal inactivation in transplant experiments, were modeled as first-order processes. Thermal shift experiments were modeled by segmental linear regression. Disinfection experiments were fit to exponential decay models. To assess if inactivation kinetics differed among the populations, we determined if a single curve could fit the decay of all populations in a given experiment, by performing a F- test with $\alpha=0.05$. A two-way ANOVA was used to compare the replicative fitness among the adapted populations.

Results

Fitness of the adapted populations in host cells. We compared the replicative fitness of the adapted populations incubated with BGMK cells. As shown in Figure S1 there was no difference among the different adapted populations in the virus titer achieved 72-hours post infection (p-value=0.37). The maximum virus concentration of all populations was approximately 5×10^8 MPNCU/ml.

Genetic characterization of the adapted populations. Sanger sequencing analyses revealed differences in the consensus sequences of the different populations obtained in the study (Table 2). For the 10D and 30D populations we sequenced 95.1% of the genome. For the 10L and 30L populations, we only confirmed the presence or absence of the mutations previously identified in the genome region coding for the VP1 protein, one of the structural proteins building the viral capsid. In total, we identified eight mutations that were present in the consensus sequence of at least one of the sequenced populations. Out of these, three mutations are synonymous, while five are non-synonymous. Three of the non-synonymous mutations were located in the VP1 region (Ile-219-Val, Thr-235-Ile and Val-236-Ile), one was located in the 2A (Tyr-5-His) and one in the 3C region (Ala-97-Val). The consensus sequence of the 30D population showed four non-synonymous mutations; two in the VP1 region (Ile-219-Val and Thr-235-Ile), one in the 2A region (Tyr-5-His) and one in the 3C region (Ala-97-Val). These mutations were absent in the consensus sequence of the 10D population, and the mutations in the VP1 were also absent in the 10L and 30L populations. Instead, the 10L and 30L populations had a non-synonymous mutation in the VP1 region (Val-236-Ile), which was not detected in the 10D and 30D populations. It should be noted that the absence of a mutation in the consensus sequence does not imply that these mutations are absent from the entire population, but only that their frequency was too low to be detected by Sanger sequencing.

Effect of environmental stressors on E11 populations adapted to different simulated habitats. The susceptibility of each adapted population of E11 to different environmental stressors (microbial grazing, heat and simulated sunlight) was investigated. No difference was observed between the inactivation curves of all adapted populations when they were incubated in fresh lake water (ANOVA test, p-value=0.99; Figure 2A) or in filter-sterilized lake water (p-value=0.72; Figure S2) for 3 days. As expected the inactivation was faster in the biologically active lake water than in the filter-sterilized water. Specifically, while an approximate decay of 3-log_{10} was observed in all adapted E11 populations incubated in microbially active lake water at 20°C for three days, only 1-log_{10} of inactivation was achieved in sterilized water under equivalent conditions (Figure S2).

Figure 2B shows the decay in viral titer of each adapted E11 population in thermal shift experiments. The decay of the four populations could not be fit with a single curve (ANOVA test, p-value<0.0001),

indicating differences in the thermotolerance among the populations. While the E11 populations adapted to a warm environment (30L and 30D) tolerate temperatures up to 47°C, the populations passaged in cold conditions (10L and 10D) start decaying at approximately 45°C. In addition, the overall inactivation in the 10L and 10D populations was higher. For example, a 2-log decay was observed after one minute of exposure to 50°C for the 30L and 30D populations. Comparatively, the decay observed in the 10L and 10D populations was greater than 3-logs. These results suggest that the populations of E11 adapted at warmer temperatures increased their tolerance to heat as compared to those passaged at 10°C.

Finally, experiments were conducted in filter-sterilized lake water at 20°C exposed to simulated solar radiation to explore the adaptation of the evolved populations to sunlight. Under our experimental conditions we did not observe any statistically significant difference between the inactivation curves of the adapted populations (p -value=0.71), indicating a lack of adaptation to sunlight. The inactivation achieved for all virus populations was 6- $\log_{10}$ in 8 hours.

Persistence of the adapted populations in transplant experiments. Once microbial inactivation and sunlight radiation were ruled out as relevant selective pressures in our experimental evolution passages, we performed transplant experiments at 10°C and 30°C in natural lake water and under dark conditions to confirm the adaptation to warming under environmentally relevant temperatures (Figure S3). The differences in the inactivation curves among the adapted populations at a given temperature were statistically significant (p -value <0.0001 at 30°C, p -value=0.0154 at 10°C). As expected, decay was faster at 30°C than 10°C, yielding first order decay constants (k) ranging from 0.7 to 1.3 day^{-1} at 30°C and from 0.1 to 0.2 day^{-1} at 10°C. Based on these values of k , the time to achieve a 2-log of viral decay (t_{99}) were determined for every population (Figure 3). Our results indicate that t_{99} in populations that were adapted to 30°C (30D and 30L) is greater than that of the populations adapted to colder environments. In particular, at 10°C the t_{99} was approximately 30 and 52 days for the 30D and 30L populations, while it was 28 and 34 days for the 10D and 10L populations. Similarly, at 30°C we could observe the same trend. T_{99} was 6.7 and 6.8 days for the 30D and 30L populations, while it was 3.5 and 5 days for the 10D and 10L populations.

Disinfection of the adapted populations by chlorine. To investigate if the increase in stability observed in the E11 populations adapted to warm conditions coincides with an increased stability against oxidizing disinfectants, we measured the inactivation of all the adapted E11 populations by free chlorine (Figure 4). Statistically significant differences among the decay curves of the four populations were observed (p <0.0001). The 30D and the 30L populations were inactivated by 2 and 3- $\log_{10}$ respectively, after an exposure of one minute to a stable chlorine concentration of 1 mg/L. Conversely,

in the 10D and 10L populations, the inactivation under the same experimental conditions was approximately 4-log₁₀. These data illustrate that the E11 populations evolved under warm conditions are indeed disinfected less efficiently than those adapted under cold conditions.

Effect of temperature adaptation on risk to swimmers. To assess the public health relevance of environmental adaptation of E11, we created a hypothetical scenario of a one-time wastewater contamination event in water used for swimming. We then used QMRA to estimate the time span required for the probability of illness arising from the swimming in the contaminated water to drop below the acceptable risk threshold of 8:1000.²⁸ To this end, we assumed a water temperature of 30°C, and we considered viral populations adapted to dark conditions at different temperatures ($k_{10D}=1.3$ day⁻¹ and $k_{30D}=0.7$ day⁻¹). Based on the QMRA model, the probability of illness was median [95% range] 0.066 [2.8×10^{-4} , 0.38] immediately after the contamination event. A little more than one day later, the median simulation probability of illness decreased below the acceptable risk threshold for the 10D population (Figure 5). In contrast, the median probability of illness exceeded the risk threshold for almost an additional two days for the persistent virus population (30D). Sensitivity analysis demonstrated that the decay rate constants were the second and fourth most influential parameter (Spearman's rho = -0.10 and -0.061), with wastewater effluent concentration as the most influential (Spearman's rho = 0.42) (Supplemental Material Figure S4).

Discussion

While it is well known that viruses can become resistant to single stressors or disinfectants, less is known about the potential evolution of viruses exposed to multiple stressors simultaneously. Yet, in the environment the survival of viruses is challenged by a diverse combination of factors acting together such as temperature, relative humidity, pH, microbial grazing and others.^{4,31} In the present study, we used experimental evolution to obtain different populations of E11 adapted to distinct environmental regimes. Each of these regimes represented a specific combination of factors (microbial grazing, sunlight radiation and temperature) which are known to be a source of natural inactivation of human enteric pathogens in the environment.³ Among the environmental stressors tested, the sole selective force in the experimental evolution passages was temperature.

The dominant effect of temperature on E11 adaptation can be rationalized by considering the mechanisms of action of the different stressors tested. Small changes in VP1 composition, the most surface-exposed structural protein in enteroviruses, have been found to greatly enhance their thermotolerance³²⁻³⁴, and the VP1 region was found to exhibit the greatest sequence divergence among the adapted populations considered in this study. Temperature may therefore be considered a

selective stressor that leads to the accumulation of thermotolerant E11 variants in a population. In contrast, sunlight exerts a less discriminate selection pressure. The dominant process leading to sunlight inactivation of viruses is the absorption of solar light in the UVB range (290 to 320 nm) by viral nucleic acids, which leads to the generation of photoproducts inhibiting the replication of the genome.^{35,36} We have previously shown that E11 can adapt to UV radiation, but that the selective force needs to be greater than the one used herein.¹⁷ Finally, not much information is available on the mechanisms leading to the inactivation of viruses by microbial grazing.⁸ However, the efficiency of this process may depend mostly on the size, rather than the composition, of a given virus species.^{7,37} Because E11 variants are not expected to differ in size, microbial grazing is thus unlikely to exert a selective pressure on the population.

Adaptation to temperature furthermore had implications on the disinfection susceptibility of E11. The population exhibiting the highest thermostability (30D) accumulated four non-synonymous mutations, two of which were in the genome region coding for the structural protein VP1. An earlier study investigating the experimental adaptation of E11 to chlorine dioxide showed that mutations in the VP1 region coincided with an increased resistance to the disinfectant.³⁸ The same study also found that E11 populations with increased resistance to chlorine dioxide were cross-resistant to temperature. This suggests that mutations in VP1 are involved in both an increased resistance against chemical disinfectants and temperature. This notion was confirmed by the results of this study. We found that populations adapted to warmer temperatures were significantly more resistant to disinfection by chlorine. Viruses adapted to warmer environments such as those encountered in tropical climates or those expected as a consequence of global warming, are thus harder to control by conventional water and wastewater treatment practices. This finding remains to be validated in disinfection studies using viruses isolated from different climate zones. If confirmed, however, this implies that disinfection practices may have to be adapted with global warming.

Besides becoming more resistant to virus control during water treatment, virus adaptation to higher temperatures may also influence the microbial quality of natural systems. The QMRA model developed in this study shows that the probability of illness due to the contamination of surface water with viruses of the adapted population 30D exceeded the risk threshold for two more days as compared with viruses belonging to the 10D population. This indicates that the temperature adaptation observed in this study may have adverse consequences for public health in case of a contamination event.

Finally, our findings have implications in the context of globalization, which has enabled viruses and other human pathogens to be rapidly transported across large geographical scales. While the populations adapted to warmer environments became more resistant to thermal inactivation both at

10 and 30°C, the populations adapted to 10°C were rapidly inactivated when transferred to lake water at 30°C. This suggests that only viral strains adapted to warmer habitats may be capable of persisting in temperate and cold habitats, whereas viruses originating from cold climates are unlikely to persist in warmer ones. Our results are thus consistent with a sink-source model of viral ecology in which new strains originate in tropical locations and sink in temperate regions, as described for the human influenza A virus.³⁹

In summary, we have shown that human echoviruses exposed to a combination of environmental factors (temperature, sunlight and microbial grazing) adapted to temperature and that this increased tolerance to heat may have implications for the ecology of human viral pathogens, their disinfection by chlorine and their risks to public health. These findings are of particular relevance in a context of global warming, which is projected to cause a doubling in the number, duration and spatial extent of heat waves ⁴⁰, and hence to higher water temperatures, by 2050. We conclude that disinfection practices may need to be adapted according the climate of the region in which disinfection is applied and in future years if global warming projections are confirmed.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at <http://pubs.acs.org>.

- Replicative fitness of all virus populations
- Inactivation data of E11 adapted populations in filtered lakewater at 20°C
- Inactivation data from transplant experiments conducted at 10°C
- Sensitivity Analysis of QMRA model using Spearman's correlation coefficients

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506 **FIGURES**

507 **Figure 1.** Design of the experimental adaptation of human echovirus 11 to different regimes (10°C in
508 the dark, 10°C under simulated sunlight, 30°C in the dark and 30°C under simulated sunlight). The
509 regrowth between exposure to the stressors (sunlight and high temperature) or dilution (for the
510 population adapted to 10 °C in the dark) was done in BGMK cells.

Figure 2. Effect of environmental stressors on the survival of the adapted populations. A). Inactivation of the adapted E11 populations in natural lake water (unfiltered; containing autochthonous microbiota) at 20°C in the dark over a period of 3 days. No statistically significant differences were obtained when comparing the inactivation kinetics between the different populations ($p=0.99$). B). Thermal shift of the adapted E11 populations. All populations were exposed to a range of temperatures (40-50°C) for 1 minute. The differences between the datasets were found to be statistically significant ($p<0.0001$). C). Adaptation to sunlight radiation. Inactivation of the adapted human echovirus populations in lake water filtered through 0.22 μm (without autochthonous microbiota) under sunlight exposure over a period of 3 days at 20°C. No statistically significant differences were obtained when comparing the inactivation kinetics between the different populations ($p=0.71$)

Figure 3. Time required to achieve a virus decay of 2-log (T99). These inactivation times were calculated based on incubation experiments conducted in lake water filtered through 0.22 μm (without autochthonous microbiota) at 10 or 30 $^{\circ}\text{C}$ in the dark over a total period of 56 and 7 days, respectively.

Figure 4. Chlorine inactivation kinetics of the adapted populations of E11. Independent experiments were conducted in duplicate at room temperature using PBS and an initial chlorine concentration of 1 mg/ml. The virus initial concentration was set to 1×10^7 MPN/ml in all the experiments.

Figure 5. Probability of illness from swimming in natural surface waters contaminated with enterovirus at concentrations similar to those reported for wastewater over a period of ten days, based on QMRA model using Monte Carlo methods (n = 10'000 simulations). Median probability of illness for swimmers from exposure to the 30D population remains elevated above an acceptable risk threshold (8:1000 based on U.S. EPA) for almost three days, compared to one day for the 10D.

544 **TABLES**545 **Table 1.** Parameter values used in the QMRA of this study.

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Parameter		Distribution	Value	Units	Reference
Simulation Parameters					
Length		point	10	days	Assumption
Increment		point	1	day	Assumption
Exposure Assessment					
Enterovirus Concentration in Wastewater Effluent	C	gamma	shape = 0.566, scale = 5.097	PFU/ml	Maozeni et al. 2017
Volume Ingested	V	uniform	min = 0.24, max = 0.57	ml	Ashbolt et al. (1997)
Enterovirus Inactivation Rate (10D)	K_{10D}	PERT	min = 0.03, mode = 0.03448, max = 0.04	1/days	This study
Enterovirus Inactivation Rate (30D)	K_{30D}	PERT	min = 0.06, mode = 0.6034, max = 0.07	1/days	This study
Dose-Response and Risk Characterization					
Dose-Response Model (Infection endpoint)	r	PERT	min = 0.00183, mode = 0.00374, max = 0.00562		QMRA Wiki, Cliver (1981)
Illness Probability		uniform	min = 0.24, max = 0.57		Maozeni et al. 2017
Disease Burden Threshold		point	8:1000		U.S. EPA

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Table 2. List of mutations found in the studied populations, as compared to the reference strain of echovirus 11 X80059.1. We sequenced nearly the entire genome for the 10D and 30D populations and only the VP1 region for the 10L and 30L populations. The given position of the mutations is based on the X80059.1 sequence. Nt and AA stand for nucleotide and amino acid, respectively. Syn stands for synonymous mutation.

Region	Position (nt)	X80059.1	10D	30D	10L	30L	AA substitution
VP1	3113	A	A	G	A	A	NS (Ile-219-Val)
VP1	3162	C	C	T	C	C	NS (Thr-235-Ile)
VP1	3164	G	G	G	A	A	NS (Val-236-Ile)
VP1	3190	T	C	T	T	T	Syn (His-244-His)
2A	3347	T	T	C	-	-	NS (Tyr-5-His)
2C	4771	A	A	G	-	-	Syn (Pro-230-Pro)
3C	5691	C	C	T	-	-	NS (Ala-97-Val)
3C	5818	C	C	T	-	-	Syn (Asn-139-Asn)