EPFL

Thèse n° 8985

Effect of wastewater treatment and environmental exposure on an enterovirus population

Présentée le 2 décembre 2022

Faculté de l'environnement naturel, architectural et construit
Laboratoire de chimie environnementale
Programme doctoral en génie civil et environnement

pour l'obtention du grade de Docteur ès Sciences

par

Odile Marie Clotilde HERVÁS DE NALDA-LARIVÉ

Acceptée sur proposition du jury

Prof. R. Bernier-Latmani, présidente du jury
Prof. T. Kohn, directrice de thèse
Dr J. Hewitt, rapporteuse
Prof. C. Tapparel, rapporteuse
Dr T. Julian, rapporteur

École polytechnique fédérale de Lausanne

2022

I hope that this small stone will someday contribute to building something beautiful.

Acknowledgements

This thesis would have not been possible without the contribution of many people, whom I would like to thank.

First, thank you to Tamar, who trusted me with this thesis, thank you for your patience and your guidance over the years. Thank you for your availability and for having such high standards for science, that pushed me to give my best. Thank you for your adaptability, for always seeking improvement and for caring about the people in your lab. I have learned many things around you over the past five years both scientifically and personally.

Thank you to all my labmates over the years, who made this adventure enjoyable and from whom I've learned so much! Thank you to Matt, who so kindly introduced me to Rotavirus, to molecular biology, to cell culture, and to statistics when I first arrived. Thank you to Virginie, who also trained me in the laboratory and helped me with my experiments. Thank you to Simon for sharing so much of his knowledge and for the nice non-science- related conversations, and to Camille for the nice moments and for the life you brought to the lab, organizing our beer outings, I enjoyed our Arizona trip together. Thank you to Jason and Suzanne for the really nice laughs and talks, to Margot for sharing all these years of work, thanks for your determination and your authenticity. Thanks to Manu, I really enjoyed our time working together and your enthusiasm, and to Chaojie for our always interesting conversations. Thanks Xavi for our work and non-work related conversation and for sharing your knowledge with me. Thank you to Aline and Shannon for being so nice office mates and for the nice times inside and outside the lab, and to Marie for our never-ending conversations and for your attention towards your colleagues. Thank you Anna for your friendship and for always being so available for your colleagues, whether for science or for a nice coffee and chat. A big thanks to Htet Kyi for all the work we've done together, you've made the lake experiments so enjoyable, thanks for all your work for the lab and your constant enthusiasm. Thank you to all the labmates with whom I have shared realy nice moments of laugther and science, to Jade for our nice time working together, to Qingxia, Loïc, Shotaro, Federica, Alex, Andrii and Margaux, our adopted colleague, to all the students who made the LCE life so lively.

Thank you to all the people who have supported me technically over the years. Special thanks to Pierre and Manu for their help with the qPCR and extraction machines, for allways being available, for our nice chats and for always helping to improve things. A big thanks to Dominique, who was always here to help with so many things around the lab central lab, and to Sylvain, Karine and Florian for their availability and kindness. Thank you to Maxime and Michel from the mechanical workshop for the design of these nice diffusion chambers, I enjoyed working with you. A big thans to the LéXPLORE team for their availability, and specially to

Guillaume and Sébastien for their constant enthusiasm and for making my experiments possible despite the technical issues. Thank you to Bastien and Elisa for teaching me how to use the Fluidigm machine, to Denis Hostettler and François Murdter for their help at the WWTP of Morges and VIdy, and to Reto from the ETH statistics deprtment for his help. A special thanks to the people who have helped me when nothing compelled them to: to Carita Savolainen-Kopra and Soile Blomqvist, who provided the viruses for my experiements, and to Arslan who helped me solve issues with my environmental chamber. Thanks also to Florence for her insight regarding my environmentl chamber.

Thank you to the people who loved science so much that they shared and transmitted this love: Nico, Dyllon, Jason, Manu, Marie. Thank you to all these authors whom I will never meet but who inspired me with their work and their nice articles.

Thanks to the people who have particularly helped me in the last few months, allowing me to hand this thesis in time: Tamar, Aline, Anna, Shannon, Htet Kyi, Tono, Marie, Noelia, Flaubert and Léopoldine.

Finally, thank you to the friends who have brought joy and support to these five years. Merci à ma famille pour leur présence, leur soutien et leurs blagues. Merci à mes parents de m'avoir permis de faire des études et de m'avoir encouragée à entreprendre ce doctorat qui me faisait envie. Gracias a mi familia española por vuestro apoyo y vuestra acogida cuando vamos a buscar un poco de descanso.

Gracias Tono, por tu apoyo incondicional, especialmente estos últimos meses. Gracias por nuestra vida juntos y por caminar conmigo hacia nuestras metas. Merci Esther et au nouveau petit bout' de toujours me ramener à l'essentiel et à la joie.

Odile

Abstract

Enteroviruses are ubiquitous surface water contaminants, where they can persist for long periods of time and can pose a threat to human health. Enterovirus genotypes display variability in their sensitivity to natural stressors, and the persistence of enteroviruses in the environment will thus depend on the genotypes present and the stressors encountered. Enteroviruses are discharged into the environment through treated sewage, and their composition depends on the genotypes circulating as well as their persistence through the sewage treatment. This thesis aims to evaluate how a population of enteroviruses is shaped by sewage treatment prior to environmental discharge, and to evaluate the diversity of responses among genotypes when exposed to Lake Geneva.

The main reason for the lack of knowledge about the diversity of responses to stressors among enterovirus genotypes is the absence of methods allowing for infectivity measurements of several enterovirus types in a mixed sample. Firstly, we developed an integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR) method to simultaneously and specifically quantify the infectious concentrations of eight enterovirus genotypes commonly encountered in sewage: coxsackieviruses A9, B1, B2, B3, B4 and B5, and echoviruses 25 and 30. The outcome of this method was calibration curves for the eight genotypes allowing the infectious concentration of each genotype to be inferred based on the increase in qPCR signal after amplification on cells. The method was able to accurately quantify the infectious concentration of a virus after inactivation by heat, and the concentration of a virus within a wastewater matrix.

The method developed was then used to evaluate how activated sludge and chlorination treatment shaped the population of interest at the genotype level. We found that the extent of inactivation by activated sludge varied greatly among genotypes, but also among sludge samples. Overall, our results suggest that activated sludge effluent will be depleted in CVA9 and CVB1 while E25 will persist. Our data also show that inactivation of enteroviruses in the sludge is predominately due to microbial activity, and to a lesser extent to chemical inactivation. Chlorination also caused a wide range of inactivation rates among genotypes, with CVB5 and CVB3 being the least susceptible and E30 being the most. E25 and CVB5 were found to gain protection against chlorination from exposure to activated sludge-derived EPS.

Finally, the ICC-RTqPCR method developed was used to evaluate the diversity in decay in Lake Geneva among our population of eight enteroviruses. An environmental chamber was used to expose the enteroviruses to Lake Geneva for five days during winter and spring. A wide range of inactivation among genotypes was found

during both seasons, but the relative sensitivity of the genotypes differed between seasons. Inactivation was globally greater at higher temperatures, though the inactivation and its variation at different temperatures was not very large, with a maximal inactivation of 2.3 $\log_{10}$ and most genotypes being inactivated by 1 $\log_{10}$ or less over five days. Furthermore, inactivation was found to be microbially mediated both in spring and in winter.

Overall, this thesis contributes to a better understanding of the variability of responses to sewage treatment and environmental exposure that exists among a population of enteroviruses. It highlights particularly persistent genotypes, and shows the importance of considering the diversity that exists among enterovirus genotypes when predicting the effect of an inactivating treatment or environmentally-associated stressor.

Keywords

Waterborne virus, infectivity, wastewater, coxsackievirus, echovirus, environment exposure, dialysis chamber, disinfection, wastewater.

Résumé

Les entérovirus sont des contaminants omniprésents dans les eaux de surface, où ils peuvent persister pendant de longues périodes et constituer une menace pour la santé humaine. Les génotypes d'entérovirus présentent une variabilité dans leur sensibilité aux facteurs de stress naturels, et la persistance des entérovirus dans l'environnement dépendra donc des génotypes présents et des facteurs de stress rencontrés. Les entérovirus sont rejetés dans l'environnement via les eaux usées traitées, et leur composition dépendra des génotypes en circulation ainsi que de leur persistance à travers le traitement des eaux usées. Cette thèse vise à évaluer comment une population d'entérovirus est façonnée par le traitement des eaux usées avant son rejet dans l'environnement, ainsi que la diversité des réponses parmi les génotypes lorsqu'ils sont exposés à l'environnement du lac Léman.

La principale raison du manque de connaissances sur la diversité des réponses aux facteurs de stress parmi les génotypes d'entérovirus est l'absence de méthode permettant de mesurer l'infectiosité de plusieurs types d'entérovirus dans un échantillon mixte. Dans un premier temps, nous avons développé une méthode de transcriptase inverse-PCR quantitative combinée à une culture cellulaire (ICC-RTqPCR) pour quantifier simultanément et spécifiquement les concentrations infectieuses de huit génotypes d'entérovirus couramment rencontrés dans les eaux usées : les coxsackievirus A9, B1, B2, B3, B4 et B5, et les échovirus 25 et 30. Le résultat de cette méthode s'est materialisé par des courbes d'étalonnage pour les huit génotypes permettant de déduire la concentration infectieuse de chaque génotype en fonction de l'augmentation du signal qPCR, après amplification sur les cellules. La méthode a permis de quantifier avec précision la concentration infectieuse résiduelle d'un virus après inactivation par la chaleur, et la concentration d'un virus dans une matrice d'eaux usées.

La méthode développée a ensuite été utilisée pour évaluer comment les boues activées et le traitement par chloration façonnaient la population d'intérêt au niveau du génotype. Nous avons constaté que l'étendue de l'inactivation par les boues activées variait considérablement entre les génotypes, mais aussi entre les échantillons de boues. Globalement, nos résultats suggèrent que les effluents de boues activées seront appauvris en CVA9 et CVB1 alors que E25 persistera. Ils montrent également que l'inactivation des entérovirus dans les boues est due à l'activité microbienne, et dans une moindre mesure à l'inactivation chimique. La chloration a également provoqué un large éventail d'inactivations parmi les génotypes, CVB5 et CVB3 étant les moins

sensibles et E30 le plus affecté. Il a été constaté que E25 et CVB5 obtenaient une protection contre la chloration due à l'exposition à des substances polymériques extracellulaires dérivées de boues activées.

Enfin, la méthode ICC-RTqPCR développée a été utilisée pour évaluer la diversité d'inactivations dans le lac Léman parmi notre population de huit entérovirus. Une chambre environnementale a été utilisée pour exposer les entérovirus au lac Léman pendant cinq jours en hiver et en été. Une large gamme d'inactivations parmi les génotypes a été observée au cours des deux saisons, mais la sensibilité relative des génotypes différait d'une saison à l'autre. L'inactivation était globalement plus élevée à des températures plus hautes, bien que l'inactivation et sa variation à différentes températures ne soient pas très importantes, avec une inactivation maximale de 2,3 $\log_{10}$, la plupart des génotypes étant inactivés de 1 $\log_{10}$ ou moins sur cinq jours. De plus, l'inactivation s'est avérée être due à l'activité microbienne à la fois en été et en hiver.

Globalement, cette thèse contribue à une meilleure compréhension de la variabilité des réponses au traitement des eaux usées et à l'exposition environnementale qui existe au sein d'une population d'entérovirus. Elle met en évidence certains des génotypes qui ont le mieux ou le moins bien résisté aux différents facteurs de stress évalués et montre l'importance de considérer la diversité existant parmi les génotypes d'entérovirus pour prédire l'effet qu'un traitement ou une exposition à l'environnement aura sur les entérovirus.

Mots-clés

Virus d'origine hydrique, infectiosité, eaux usées, coxsackievirus, échovirus, exposition à l'environnement, chambre de dialyse, désinfection, eaux usées.

Contents

List of Figures

List of Tables

List of Equations

Chapter 1 Introduction

1.1 Context

1.1.1 Water sanitation, human health and viruses

Water is essential to life, and access to water has always been the main factor influencing human settlement. Although wastewater has always been a nuisance, and the Greeks and Romans already evacuated it, it took several discoveries in the second half of the 19th century to show that soiled water could be a vector of disease. For instance, Dr. John Snow demonstrated that an epidemic of cholera in London was due to water contamination, William Budd demonstrated that typhoid fever was also transmitted through contaminated drinking water, and Koch and Pasteur developed germ theory. Following these and other empirical observations, the benefit of water treatment became clear. The soiled water was physically separated from human activities, though the water bodies receiving the soiled water were oftentimes the drinking water source of the same or other communities. With the increasing population density and the increase of water use, the quality of the water sources has deteriorated and made it necessary to treat water before consumption. In the late 19th century early 20th century, wastewater started to be collected and treated systematically, and most developed countries implemented conventional drinking water treatment and chlorine disinfection by 1940. The number of deaths by typhoid fever in the United States decreased with the increased chlorination of water in the early 20th century.

Although the first microorganisms that where discovered to be responsible for water contamination were bacteria (Salmonella typhi, Vibrio cholera), amoebae were soon found to also be of concern (amoebic dysentery), and in the mid-20th century, viruses were also found to be responsible for fecal-oral infection routes. Unlike bacteria, viruses are obligatory parasites and cannot replicate outside their host, so human viruses do not multiply in water. However, their small size makes them difficult to detect and eliminate with treatments that commonly target bacteria, and their low infectious dose make them a threat even at low concentrations.

1.1.2 Enteroviruses

Waterborne viruses are viruses that can be transmited through water, for example while bathing, drinking, consuming food exposed to contaminated water. One important class of waterborne virus that is responsible for human disease are Enteroviruses. Enterovirus-based diseases can present with a broad range of clinical

manifestations such as a mild rash, foot and mouth disease, upper and lower respiratory diseases, meningitis and paralysis; overall, individual genotypes are associated with a wide range of illnesses[1,2]. Although most infections remain asymptomatic, they regularly cause severe outbreaks[3–5], making them a target of interest for water-treatment facilities.

Enterovirus is a genus of positive-sense single-stranded RNA viruses that belong to the *Picornaviridae* family. They are small non-enveloped viruses of about 30 nm in diameter with a genome size of 7.2–8.5 kb, and their capsid is an arrangement of four proteins VP1, VP2, VP3 and VP4[6]. There are 15 species of the genus *Enterovirus*, seven of which affect humans: *Enterovirus A, Enterovirus B, Enterovirus C* (which encompasses polioviruses), *Enterovirus D, Rhinovirus A, Rhinovirus B* and *Rhinovirus C*. There are 103 non-polio enterovirus genotypes[7], 56 of which were originally distinguished in serotypes by serologic studies and classified into three groups based on pathogenicity[8]: coxsackie A viruses (CVA), coxsackie B viruses (CVB) or echoviruses (E). Due to the difficulty in classification based on pathogenicity, new serotypes were numbered based on their order of identification[8], for example enterovirus 68 (EV-D68). Currently, typing is done through sequencing of the VP1[9], and species classification is based on protein amino acid similarity[10] and the different types are called genotypes. Enteroviruses have high mutation and recombination rates, which can lead to the apparition of new strains and viral tropism.

1.1.3 The enterovirus life cycle

The different members of the *Enterovirus* genus have different tropisms. Rhinoviruses species infect cells in the respiratory tract, while enterovirus species are thought to be swallowed and resist acidic condition and body temperature[2]. Enteroviruses then reproduce in the gastrointestinal tract and/or therespiratory tract, depending on type. This causes the virus to be shed in the stool and through respiratory secretions, and reinfection can occur through fecal-oral and/or respiratory transmission. The first step of enterovirus infection is viral binding to one or more cell receptors, where the location of the available receptor in the body defines the tropism as well as the entry route. The variety of cell receptors that can be bound by enteroviruses likely explains the wide spectrum of associated diseases[11]. The cell receptors can promote attachment but also uncoating of enteroviruses. After binding, the virus enters the cell by endocytosis and will undergo uncoating and release its RNA into the cytoplasm. Attachment to receptors and/or change in pH in the endosome trigger the uncoating process. Once the genome enters the cytoplasm, it is fully translated by ribosomes into a single polyprotein, which is then cleaved into ten proteins including the capsid proteins and gene-replication proteins. The newly expressed viral polymerase replicates the genome by first creating a negative strand that serves as a template for the creation of new positive strands. The synthesized positive strands can be used for the translation of more proteins, replication or encapisdation into new virus particles. New virus particles form by capsid protein assembly and are released through host cell lysis or non-lytically

in vesicles that can contain several virions. Enteroviruses disseminated from the primary replication site can infect other tissues, such as the central nervous system.

1.1.4 The burden of enteroviruses

Enteroviruses are widely circulating viruses, and although most infections are asymptomatic, a number of genotypes have emerged as public health concerns. The most well-known enterovirus is poliovirus (PV), which will most often cause minor disease such as fever, sore throat or malaise, but in 1-2% of the infections will enter the central nervous system and causes paralysis[12]. At the beginning of the 20th century, large outbreaks of poliomyelitis[12] led to the development of two effective vaccines, and poliovirus is close to being eradicated. Non-poliovirus enterovirus genotypes are also public health concerns, such as EV-A71, which causes outbreaks of hand-foot-mouth disease, leading to potentially serious neurological symptoms[13–15]. Additionally, EV-D68 caused outbreaks of severe respiratory diseases in the United States[16,17], CVA24 caused large outbreaks of acute haemorrhagic conjunctivitis[1], and E30 caused meningitis-related upsurges in Europe in 2018[3].

Infected individuals shed high amounts of enteroviruses in their stool, at up to 10^6 infective particles per gram of stool[18]. Once shed, enteroviruses are released to the sewage, where they are partially removed by wastewater treatment before being discharged into the environment. Enteroviruses have been found to retain their infectivity for weeks in groundwater or seawater[19], and have been detected in river or bathing water[20,21], tap water[22] and chlorinated water[23]. Because the water source is often no longer available when symptoms arise, it can be difficult to establish direct causality between water use and disease. Regardless, the detection of infectious enteroviruses in recreational and drinking water as well as their long persistence strongly suggest the possibility of contamination through water consumption or bathing. A number of studies have established a link between bathing and enterovirus infection, especially in young children[4,5,24].

To support the effort of poliovirus eradication, sewage surveillance of enteroviruses is performed in many countries, providing information on circulating types[25–29]. A comparison between enteroviruses detected in sewage and in clinics highlights that the same genotypes can be found, though their relative abundances differ[28,30]. This could be due to a difference in persistence in the environment, a different excretion rate or a bias in the detection methods.

1.2 Fate of enteroviruses in the natural environment: state of knowledge

1.2.1 Occurrence and diversity of enteroviruses in natural water

For clinical samples, non-PV enteroviruses occurrence peaks in spring[31]. Consistently with this infection peak, enteroviruses are also detected in natural waters during those months[32]. Infectious enteroviruses have been

detected in freshwater in concentrations ranging from 0.5–56 infectious units per liter, in seawater in concentrations ranging from 0.05–16 infectious unit per liter, and in marine sediments in up to hundreds of infectious units per kg[33]. A variety of genotypes have been detected, and the prevalence of some genotypes varies from one study to another, reflecting prevalence in the community[34], survival of the different genotypes, and sometimes a bias in the concentration procedures[35]. Rao et al.[34] detected E7, E29 and PV2 in seawater from seawater receiving treated sewage and E7, CVB4, E29, CVB3, PV2, PV1 and PV3 in sediments from the same site. Lucena et al.[35] measured enteroviruses in two rivers and in coastal seawater close to beaches. In rivers, they mainly found the three types of PV (89% of all enterovirus typed) as well as CVB2, CVB3, CVB5 and E1. In coastal seawater, they detected three types of PVs as well as E11. Tani et al.[20] measured enteroviruses monthly in one river in Japan for five years and detected coxsackieviruses B1, B2, B3, B4 and B5; echoviruses 3, 5, 6, 7, 11, 14, 16, 18, 19 and 21; and PV 1, 2 and 3. The detection of certain genotypes and their prevalence was highly dependent on the year and month of sampling. For example, PV2 and PV3 were eradicated in 2015 and 2019 and are therefore unlikely to be found in current monitoring efforts.

1.2.2 Factors affecting virus persistence

Many studies have evaluated how long enteroviruses retain their infectivity in natural environments. For instance, Matossian and Garabedian[36] studied the inactivation of PV1 in seawater, and Lo et al.[37] studied the stability of CVB5, E6 and PV1 in ocean and estuarine water. Nasser et al.[38] studied the inactivation of CVA9 in stream water and seawater, and Yates and al.[39] studied the inactivation of PV1 and E1 in groundwater. As shown in the review by Boehm et al.[40], the persistence of enteroviruses in the environment will vary a lot. It depends on local conditions and the genotypes. Under the same conditions, the persistence of different genotypes has been shown to differ. However, not many genotypes have been studied, with researchers usually focusing on poliovirus and a few other genotypes.

A number of factors affect enterovirus persistence in the environment, including temperature, biological activity, metabolites, light, and adsorption to solids. Temperature has an important impact on enterovirus in natural waters, with higher temperatures resulting in increased inactivation, even in sterile seawater or lake water[37,41]. Biologically mediated inactivation plays an important role in environmental enterovirus inactivation, and several studies have shown the importance of microbial activity. Lycke et al.[42] and Shuval et al.[43] showed that filtering or heating natural water reduces its inactivation capacity, likely due to removal or inactivation of the microbial community. Olive et al.[41] showed that both the bacterial and eukaryotic fraction of natural water contributed to the inactivation of E11. Metabolites also seem to play a role: Cliver and Hermann[44] showed that human enteroviruses were susceptible to certain proteolytic enzymes. This could explain the results of some studies showing that filtering natural sea water did not affect the inactivation capacity[36,45]. Viruses can also be inactivated through light, either through the adsorption of UV light or through

photo-oxidation via the excitation of sensitizer molecules. However, the former is limited in water beds due to the attenuation coefficient of water[46], and Silverman et al.[47] showed that reactive intermediates played a limited role in the inactivation of PV3. Finally, adsorption onto solids can protect enteroviruses from inactivation. Several studies in natural waters have shown that enteroviruses adsorbed onto solids remained infectious for longer[48,49] and can gain protection from thermal inactivation[50]. Other parameters, such as salinity, have been cited to impact enterovirus inactivation, but as results have been contradictory[37,45,51], salinity is likely not a major environmental inactivator of enterovirus. Figure 1.1 summarizes the factors that can impact enterovirus persistence in the environment.

The magnitude of inactivation or shielding achieved by these various processes varies for different genotypes. Cliver and Hermann[44] showed that CVA7, CVA9 and CVB2 were more susceptible to pronase than CVB1, CVB3, PV1, PV2 and PV3. Lo et al.[37] showed that CVB5 was more stable than E6 and poliovirus 1 (PV1) at 25°C in sterile water. In a study by Smith and al.[48], the increase in survival provided by the presence of sediment was not the same for all genotypes tested (PV1, E1, CVB3, CVA9), which was confirmed by LaBelle and Gerba[49] (PV1 and E1). Thus the extent of overall enterovirus inactivation in the environment depends on the genotypes discharged, such that it is necessary to understand how more genotypes will be affected by exposure to the environment.

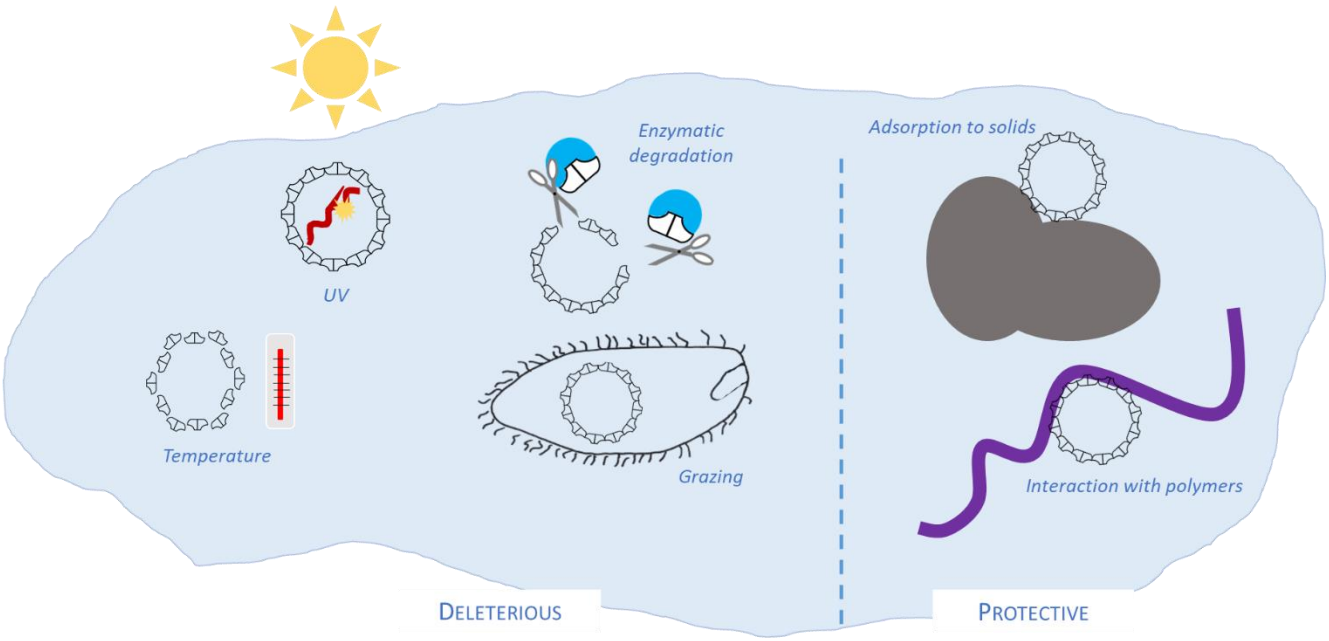


Figure 1.1: Natural processes that can impact persistence of enteroviruses in the environment in a protective or deleterious way.

1.3 Fate of enteroviruses during wastewater treatment: state of knowledge

1.3.1 Virus removal in water and wastewater treatment

Water and wastewater treatment are intimately linked since the sources of drinking water often receive treated wastewater. The goal of water treatment is to render the water safe for human consumption by removing harmful components, such as organic matter, heavy metals, nitrates and especially pathogens. The goal of wastewater treatment is to render wastewater safe to discharge into the environment, such that it causes no harm to the receiving waters, ecosystems, and humans in contact with the receiving water. This is mainly done through the removal of organic matter and nutrients that can cause odours, oxygen depletion and eutrophication of the receiving waters, but also through pathogen control and occasionally micropollutant removal. The quality of treated wastewater, notably regarding pathogens, becomes crucial when little dilution occurs between steps or for the direct potable reuse of treated wastewater.

The required water treatment train will depend on the source of water and its composition and will typically encompass different combinations of a sedimentation step, a filtration step (such as with activated carbon, membranes, or combined with coagulation) and a disinfection step. Wastewater treatment train typically consists of mechanical steps to remove particles that is followed by a biological treatment to remove organic matter and sometimes nutrients and that ends with a disinfection step in some plants.

Some water treatments were found to remove viruses to a certain extent, like coagulation-flocculation-sedimentation[52,53], slow sand filtration[54–56] or filtration through microfiltration, ultrafiltration or reverse osmosis membranes[57–59]. However, the removal is highly dependent on the operating conditions and setup. Thus, the control of viruses mainly relies on disinfectants, such as chlorine, chlorine dioxide, monchloramine, UV or ozone. In wastewater reuse trains, ozonation, high dose UV, free chlorine treatments are respectively credited with 6-, 6-, and 4 log viral reductions[60]. In wastewater treatment trains, no virus removal is expected in the primary treatment[61], though biological treatment reduces the viral load by 1–2.5 log[62–64]. In wastewater reuse trains, conventional activated slude is credited of 1.9 log removal of viruses. The reduction in viral titer caused by disinfection can vary depending on the treatment[64] and on the quality of the effluent in terms of organic content and nutrients[65].

The disinfectant-based inactivation of enteroviruses has been studied in the laboratory using laboratory strains[66–72], though this has rarely been studied in environmental isolates[23,73–75] .This can cause complications, as some isolates have been observed to have higher disinfection resistance than their representative laboratory strains[23,73,74] (Payement et al. 1985, Meister et al. 2018, Torii et al. 2020), though this is not always the case[23,75] (Payment et al. 1985, Torii et al. 2022). Regardless, most of these studies include only some of the same four enteroviruses genotypes (E1, E11, CVB3, CVB4) or CVB5, which has been repeatedly shown to be

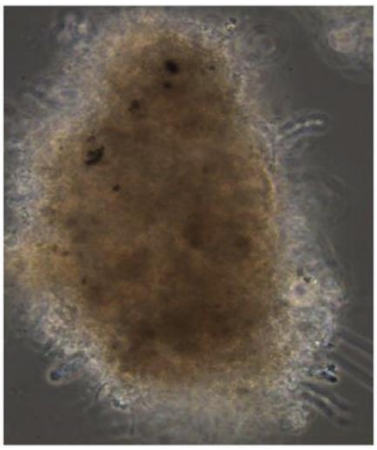
more chlorine resistant than the other genotypes tested. Moreover, current guidelines for water treatment are usually based on data for one virus. For example, the USEPA guidelines for chlorine doses for viral disinfection are based on the disinfection of hepatitis A described by Sobsey et al.[66] with a 3-fold security factor[76]. The WHO guidelines[77] are based on the inactivation data of CVA2 extrapolated by White[78] and the assumption that CVA2 is chlorine resistant. It remains to be seen whether these guidelines and inactivation kinetics are valid for a wider variety of enteroviruses and other viruses.

The reduction of enterovirus load in activated sludge is generally estimated by measuring the enterovirus concentration in the influent and in the effluent by qPCR or infectivity assays, although these two methods give different information. However, there are difficulties to the quantification, particularly due to low viral concentrations, the presence of inhibitors in the matrix, and the estimation of residence time in the treatment. Abatment of enteroviruses through activated sludge is always measured for the global population of enteroviruses[62,64,79–81] also evaluated which genotypes were present and how often they were detected, their study was not quantitative, as it is also difficult to individually quantify enteroviruses via these techniques. More data is therefore required on the variability of the responses of different genotypes to activated sludge treatment as this might affect the prediction of overall removal by activated sludge treatment.

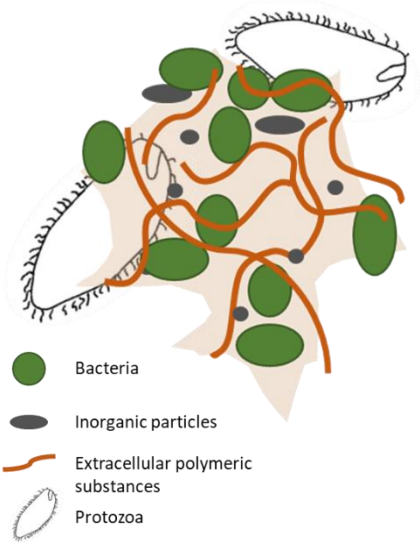
1.3.2 Activated sludge: a black box

1.3.2.1 *What is activated sludge?*

One of the most widely used biological treatments for wastewater, activated sludge is a complex matrix comprising multiple microorganisms and metabolites, and wherein a number of processes take place. Mainly, activated sludge consists of a suspension of aerobic bacterial culture that is aerated and then fed wastewater. The bacteria consume the organic matter to obtain energy and material for the synthesis of new cells, removing it from the water and increasing the bacterial biomass. The bacteria aggregate in flocs, that also contain inorganic particles and natural polymers exuded by the bacteria called extracellular polymeric substances (EPS), which give their structure to the flocs. Other microorganisms are also present in the sludge, such as protozoa, rotifers or even fungi. Protozoa and rotifers contribute to a good floc structure by consuming bacteria that do not flocculate or small floc particles that do not settle. The age of the sludge greatly impacts on the type of bacteria and other microorganisms that are present in the flocs and the processes that will take place.



Tansel, 2017



A B C

Figure 1.2: Activated sludge basin at a wastewater treatment plant (A), picture of a floc of activated sludge (B) and schematic with details of activated sludge composition (C).

The main objective of activated sludge is organic carbon removal, though ammonia oxidation (nitrification) can also occur under certain conditions. Heterotrophs play an important role in this carbon removal, and autotrophs such as Nitrosomonas and Nitrobacter play an important role in the nitrification process. These bacteria develop at a longer sludge age (sludge retention time, SRT).

1.3.2.2 *Fate of viruses in activated sludge*

Although the main objective of activated sludge is not pathogen removal, pathogen concentrations can be impacted by the sludge. For example, pathogenic bacteria can be removed by competition with the bacteria present in the activated sludge[82]. Viruses have also been shown to be removed in activated sludge. Table 1:1 presents studies of enterovirus removal by activated sludge or a combination of activated sludge and other treatments, and Figure 1.3 summarizes the main processes that can impact the fate of enteroviruses in activated sludge. Some studies measured the removal of infectious enteroviruses, reporting between 0.7–2 log reduction[62,63,83]. Other studies examined removal in terms of genome copies, finding between <0.2–3 log reductions, though this gives no information about the infectivity of the viruses. Reductions in terms of genome copies were globally greater than reductions in infectivity. In wastewater reuse trains, conventional activated sludge is credited with a 1.9-log removal of viruses[60].

Table 1:1: Reported removal of enteroviruses in activated sludge treatment trains. Red shading indicates studies that include infectivity data measured with cell culture.

Study	Treatments considered	Measurement type	Enterovirus reduction reported
Lodder and Roda Husman, 2006[83]	Activated sludge and phosphorus removal	Cell culture	0.7–1.8 log removal, average of 1.3 log removal
Costán-Longares et al. 2008[63]	Activated sludge	Cell culture, Identification of isolates by sequencing	1–2 log reduction
Hewitt et al. 2011[62]	Several plants with treatments including: -Moving bed biofilm reactor -Tricking filter -Activated sludge -Waste stabilization pond -a combination of these	Cell culture, and RT-qPCR	1–1.3 log reduction in culturable virus (for the group of plants that include some activated sludge) More than 2 log removal in qPCR
Katayama et al. 2008[84]	Several plants with treatments including: -Conventional activated sludge (4/6) -Nitrifying-denitrifying sludge (1/6) -Anaerobic-anoxic-oxic sludge (1/6)	RT-PCR	2–3 log removal (average of the 6 plants)
LaRosa et al. 2010[80]	Activated sludge + chlorination	RT-qPCR and ICC-RTqCPR	34% removal by qPCR ICC-RTqPCR only informs how many influent and effluent samples contained infectious enteroviruses
Kitajima et al. 2014[79]	Activated sludge + chlorination	RT-qPCR	2 log removal
Qiu et al. 2015[64]	Activated sludge	RT-qPCR and ICC-RTqPCR	2.6 log removal by qPCR ICC-RTqPCR only informs how many influent and effluent samples contained infectious enteroviruses

1.3.2.3 *State of research: processes of removal and inactivation of enteroviruses by activated sludge*

Adsorption onto solids has been described as one of the main sources of virus removal in activated sludge. Gerba et al.[85] found that 67–99.8% of the enteroviruses tested were adsorbed onto the sludge after two minutes of stirring. Haun et al.[86] modelled virus elimination in activated sludge, estimated a two-phase elimination of adsorption followed by inactivation, though other studies have found that adsorption was not the main removal mechanism. For instance, Kelly et al.[87] found no accumulation of viruses in the sludge, and suggested that inactivation was occurring. This was corroborated by Chaudry et al.[88], who found that inactivation was a bigger contributor to virus removal than attachment to solids in a membrane bioreactor with mixed liquor suspended solids. Kelly and al.[87], Malina et al.[89], Knowlton and Ward[90], Kim and Unno[91], Chaudry et al.[88] and Haun et al.[86] all showed or suggested that viruses were inactivated in activated sludge or in bacterial cultures isolated from activated sludge. Kelly and al.[87], Knowlton and Ward[90] and Kim and Unno[91] showed no inactivation in the presence of the supernatant after the removal of solids. They concluded that inactivation was due to microbial activity, and that inactivating compounds are either short-lived or active only when associated with microorganisms[90]. Knowlton and Ward[90] found that untreated mixed liquor suspended solids (MLSS) inactivated PV1 and released its RNA. Protists isolated from activated sludge have been shown to graze on poliovirus[91]. Sludge has also been found to be protective towards viruses. For instance, Nakajima et al. found that poliovirus adsorbed to activated sludge maintained their infectivity for longer period of time, and displayed higher thermal resistance than free viurses[92].

Regarding the structure of the flocs, Kim and Unno[91] found that flocculating bacteria isolated from activated sludge caused adsorption and inactivation of poliovirus, while a mixture of non-flocculating and flocculating bacteria caused only adsorption, and that virus could be fully recovered. In contrast, Kelly et al.[87] showed that the mechanical stability of the activated sludge floc was not vital to virus removal—sludge dispersed by agitation in a blender maintained the same removal capacity. This hints that the nature of the bacteria is crucial to viral inactivation. Floc-forming ability is linked to the secretion of EPS, which contains a high concentration of hydrolytic enzymes[93–95] that might enhance viral inactivation, and this EPS might be conserved despite the dispersion. Another hypothesis is that the type of bacteria has an impact on viral inactivation through the biological reactions that take place, such as nitrification.

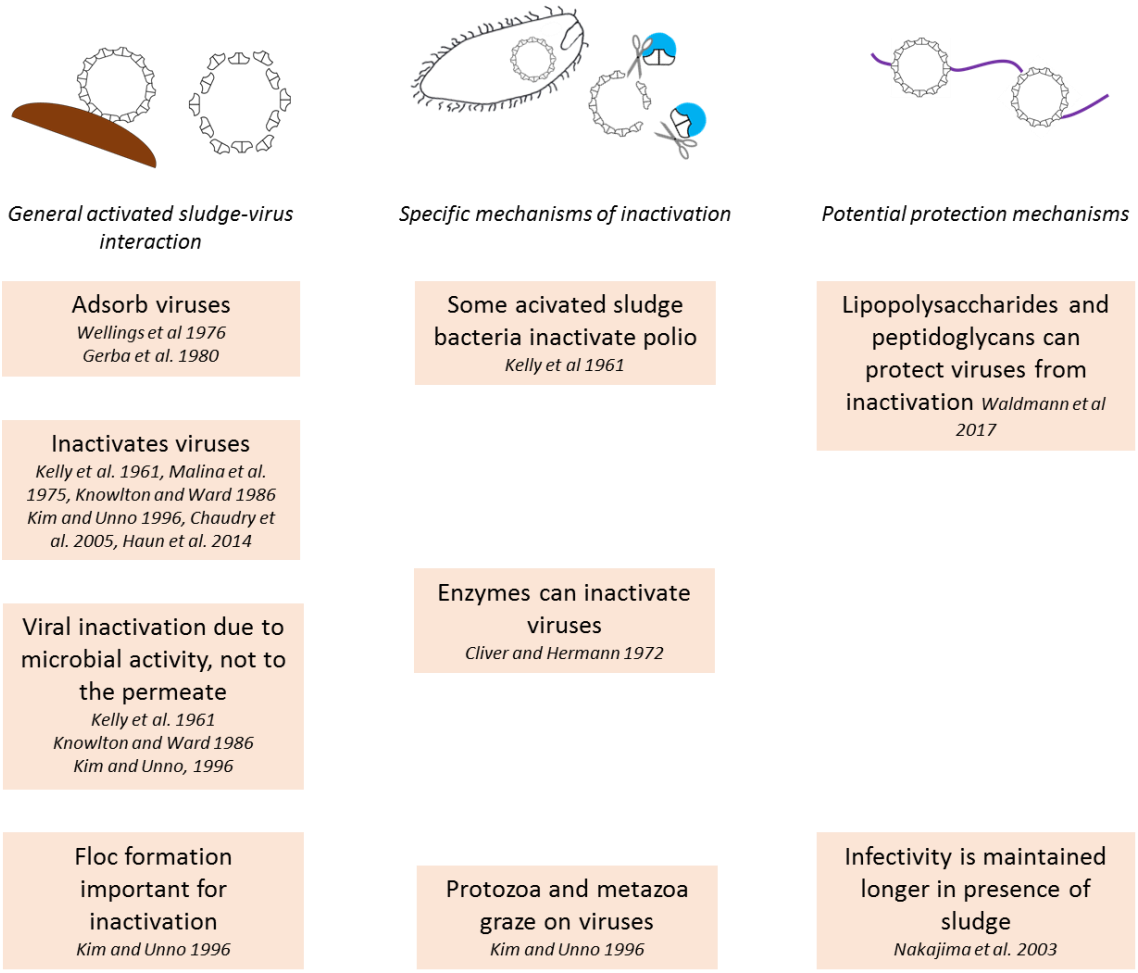


Figure 1.3: Processes reported to impact the fate of enteroviruses in activated sludge.

1.3.2.4 *Potential for shaping the enterovirus population*

The degree of removal and inactivation caused by activated sludge is expected to vary among the genotypes of an enterovirus population. For instance, as adsorption of enteroviruses has been described as one of the main mechanisms of virus removal[85,91], the genotype-specific adsorption shown by Gerba et al.[85] and Tao et al.[96] would affect the degree of removal of each genotype differently. The presence of microorganisms and proteolytic enzymes can also induce biologically mediated inactivation that is genotype specific[44,97]. Activated sludge reactors operate at temperatures between 4–32°C[98], and this range of temperatures can also cause differentiated inactivation for different genotypes[37]. Finally, polysaccharides and peptidoglycans, two types of molecules present in the EPS of the sludge, have been shown to interact with the enterovirus capsid and influence its thermal and environmental stability in a genotype-specific fashion[99,100].

These genotype-specific interactions that can occur in activated sludge treatment indicate that not all genotypes will be removed or inactivated to the same extent. Thus treating a population of enteroviruses will

shape the ratios of genotypes and shift the prevalence of specific genotypes. However, this has not been studied, as the fate of enteroviruses in activated sludge is measured as a bulk parameter.

1.3.3 Chlorination

1.3.3.1 *Chlorination process*

Chlorine is a strong oxidizing agent, and free chlorine is one of the most commonly used disinfectants for water and wastewater. It is used in wastewater treatment as the final disinfection before discharge, and can be used as the primary disinfectant in water treatment or as a secondary (or residual) disinfection step for preventing pathogen regrowth in the distribution system. Free chlorine in water is created by the addition of chlorine gas ($Cl_{2[g]}$), sodium hypochlorite (NaOCl) or calcium hypochlorite ($Ca[OCl]_2$). When dissolved in water, chlorine will form a mixture of free available chlorine in the form of hypochlorous acid (HOCl) and hypochlorite (OCl-), as shown in Eqs. 1 and 2, with HOCl being the stronger oxidant. The relative proportion of each is pH dependant, with HOCl prevalent at neutral and low pH values.

$$Cl_{2(aq)} = \mathrm{HOCl} + H^+ + Cl^- \qquad \textit{Equation 1:1 Chlorine dissolution in water}$$

$$HOCl = OCl^- + H^+ \qquad \textit{Equation 1:2 Hypochlorous acid dissociation}$$

Free chlorine efficiently inactivates pathogens, but it will also react rapidly with ammonia and organic compounds to form combined chlorine compounds, such as chloramines. Chloramines are weaker oxidants often used as secondary disinfection in water treatment trains because they cause fewer disinfection by-products (such as trihalomethanes) than free chlorine, though they have been found to form other disinfection by-products[101,102]. Chlorine treatment can also be performed with chlorine dioxide (ClO_2).

1.3.3.2 *State of research: mechanisms of chlorine-induced enterovirus inactivation*

The mechanisms through which chlorinated compounds inactivate viruses are not well understood. Free chlorine has been shown to affect both viral proteins and the genome. For instance, Wigginton et al.[103] showed that free chlorine impacted replication and injection functions of MS2, commonly used as an enteric virus surrogate. Their results show that free chlorine causes high levels of genome and protein damage, and suggest that not all chlorine-induced genome damage reduced the infectivity of MS2, and that the extensive effect on proteins did not necessarily have biological consequences. They linked a site specific cleavage in the capsid protein to an inhibition of the genome injection function, and they did not observe any reduction in binding ability of MS2 upon chlorine treatment. Torrey et al.[104] determined that free chlorine caused little to no significant loss of genome functionality in E11 compared to the induced loss of infectivity, inferring that the loss of infectivity must be linked to protein damage. Based on their results and studies highlighting different mechanisms for different ranges of FC concentrations, they further hypothesized that the mechanism

of inactivation by free chlorine is dependent on the concentration of the free chlorine and the species[105,106]. Chlorine dioxide has been shown to affect viruses differently than free chlorine[103,107,108], though that is beyond the scope of this thesis.

1.3.3.3 *Genotype-dependent differences in inactivation*

Enteroviruses have been reported to be more resistant to chlorine treatment than other waterborne viruses, such as hepatitis A virus[66], and different enterovirus genotypes display differing susceptibilities to treatment with free chlorine[23,73,75], monochloramine[69] or chlorine dioxide (Harakeh et al. 1987). Though there are some studies on the variability in inactivation efficiencies between genotypes and strains[23,72,75] and differences between laboratory strains and environmental isolates[73], most studies focused on only a few genotypes. Table 1:2 presents some of the log reductions reported in the literature for different genotypes at given free chlorine doses.

Table 1:2: Literature reported log10 inactivations of enteroviruses genotypes by different doses of free chlorine. Colored areas represent the range of inactivation reported for several strains of the same genotype, while colored bars indicated inactivation reported for one strain of the genotype.

Torii et al. 2021 & 22[74,75]	Chlorine Dose CT 0.5 mg.min.L^{-1}	Log10 inactivation							
	1	2	3	4	5	6	7	8
CVB3								
CVB4								
CVB5								
E11								to 12.5
Meister et al. 2018[73]	CT 0.5 mg.min.L^{-1}							
	1	2	3	4	5	6	7	8
CVB1								
CVB4								
CVB5								
E11								
Payment et al. 1985[23]	CT 0.4 mg.min.L^{-1}, approximation of CT							
	1	2	3	4	5	6	7	8
CVB4								
CVB5								
Cromeans et al. 2010[69]	CT 0.4 mg.min.L^{-1}							
	1	2	3	4	5	6	7	8
CVB3								
CVB5								
E1								
E11								
Black et al. 2009[68]	CT 2.4, 2.5 and 2.9 mg.min.L^{-1} for E12, CVB5 and E1 resp.							
	1	2	3	4	5	6	7	8
CVB5								
E1								
E12								
Liu et al 1971[72]	CT 2.5 mg.min.L^{-1} in Potomac river water (in green results in demand free water							
	1	2	3	4	5	6	7	8
CVA9								
CVB1								
CVB2								
CVB3								
CVB4								
CVB5								
E11								
E5								
E7					>			
E29								
E9								
E8								
CVA6								
E12								
CVA5								
E1								

1.4 Challenges in measuring enteroviruses: infectivity and qPCR assays

Two types of methods are commonly used to measure viral concentration in water: cell–culture- and quantitative polymerase chain reaction (qPCR) methods, both providing different types of information. In the former, the presence of a virus is detected by observing its cytopathic effect on a susceptible cell line, and its concentration is assessed by plaque assay or by end point dilution methods. There are drawbacks to cell culture, including the absence of available cell lines for replicating certain viruses, the time required for measurement, the possible absence of visible CPE and the presence of components in the viral matrix that might induce false-positive cell death. Moreover, cell culture cannot differentiate between viruses in a sample when the host cells are susceptible to more than one virus, and the cell culture data might be biased by differences in viral fitness.

Enterovirus reduction by wastewater treatment trains and surveillance in sewage are normally performed using cell culture for quantification, followed by serotyping or sequencing of viruses isolated with cells to identify the type. However, qPCR and deep sequencing are being increasingly used in enterovirus surveillance in sewage and in environmental samples for quantification[32,109,110] and evaluation of the genotype diversity respectively[32,111,112]. qPCR allows the amplification of viral RNA using primers that can be specific to a virus or even a genotype, the quantification is generally faster and is not biased by the ability of a virus to amplify in the cell culture, although it has other drawbacks. Sample volume used in qPCR is much smaller than that used in cell culture, such that samples with low concentration may result in false negative. Additionally, water concentration methods can inhibit qPCR reactions. However, the main drawback of qPCR is that it can measure viruses with intact RNA but that are no longer infectious. Similarly, deep-sequencing methods allow to evaluate the diversity of the enterovirus population without the bias of cell culture. However, deep-sequencing is not quantitative and it also induces a bias for some genotypes in the genome amplification step. Like qPCR, this method cannot identify if the viruses detected are infectious or not. It is to be noted that some authors consider that most viruses detected by qPCR are infectious, and that cellular methods underestimate the infectivity by two to three orders of magnitude[113].

The concentration and types of enteroviruses found in a water sample will thus depend on the method used for its determination. For example, Hewitt et al.[62] reported from 0.7–3.52 log10 plaque forming units (PFU) $.L^{-1}$ and 2.84–6.67 log10 genome copies.L^{-1} in sewage, and Tao et al.[112] detected different enterovirus genotypes in sewage with cell culture followed by sequencing than with deep sequencing. Overall, there is no method that allows to assess the infectivity of individual enterovirus genotypes in water samples. For this, we would need a method that allows to evaluate the infectivity of the enteroviruses in a specific manner for each genotype. It would allow to evaluate the fate of individual genotypes through treatments or in the environment and give insight on the diversity in persistence.

1.5 Research objectives and approach

The objective of this thesis is to unravel how wastewater treatment and exposure to a lake environment will shape a typical enterovirus population. To account for the different sensitivities of the genotypes to treatments or natural stressors, this work seeks to move away from treating enteroviruses as a bulk parameter when assessing the effect of treatments. We want to highlight which genotypes among a relevant population will likely persist or be inactivated throughout the engineered treatment or exposure to the natural environment.

Chapter 2: We have highlighted that both cell culture and qPCR have limitations when it comes to estimating the infectious concentration of enterovirus genotypes in a sample. The goal of this chapter is to first establish what is a typical enterovirus population that can be found in sewage in Europe. Second, this chapter discusses the development of an integrated cell culture RT-qPCR (ICC-RTqPCR) method that can specifically quantify the infectious concentration of each genotype of the population in a mixed sample.

Chapter 3: Using the method developed in Chapter 2, the objective of this chapter is to assess how wastewater treatment, specifically activated sludge and chlorination, can shape an enterovirus population. We will question which genotypes persist through each treatment, which genotypes are readily inactivated, and whether the effect of activated sludge on a population is consistent across sludge samples. We also assess the mechanisms of enterovirus removal in activated sludge as well as the potential protection from chlorination that enteroviruses might acquire in the activated sludge.

Chapter 4: Using the method developed in Chapter 2, the objective of this chapter is to assess how exposure of an enterovirus population to a lake environment will shape its composition. We will examine which genotypes will be readily inactivated or persist. We decouple the effect of thermal and chemical versus biological inactivation and assess the effect of seasonality on the persistence of enteroviruses. The results of this chapter provide inactivation kinetics data for the whole population of enteroviruses in Lake Geneva in winter and in spring. To evaluate the fate of enteroviruses from sewage to Lake Geneva, we also compare these results to those obtained in Chapter 3.

Chapter 2 An integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR) method to simultaneously quantify the infectious concentrations of eight environmentally relevant enterovirus genotypes

A modified version was published as:

Larivé, O., Brandani, J., Dubey, M. & Kohn, T. An integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR) method to simultaneously quantify the infectious concentrations of eight environmentally relevant enterovirus serotypes. *J. Virol. Methods* **296**, (2021)

Odile Larivé designed the experimental plan, performed most of the experiments, analyzed the data and wrote the chapter. Jade Brandani designed most of the primers used in this study. Manupriyam participated to the ICC-RTqPCR experiments.

2.1 Introduction

Enteroviruses are a group of common and globally circulating viruses. Of the 15 known enterovirus species 15 species of the genus *Enterovirus*, seven of them affect humans: *Enterovirus A, Enterovirus B, Enterovirus C* (which encompasses polioviruses), *Enterovirus D, Rhinovirus A, Rhinovirus B* and *Rhinovirus C*. Enteroviruses cause a diverse array of clinical outcomes, ranging from asymptomatic infections to mild rashes, encephalitis, meningitis or paralysis[1]. Enteroviruses are frequently encountered in sewage, where several genotypes are typically present in a single sample [26,27,32,112]. Once discharged into the environment, enteroviruses can retain infectivity for up to four weeks in groundwater, wetland, and seawater [19]. Infectious enteroviruses have also been detected in lake water [114], seawater[21], and river water [20].

Knowledge of infectious enterovirus concentrations in environmental samples is critical for monitoring water and wastewater treatment performance, or for assessing health risks arising from exposure to contaminated waters. Because different enterovirus genotypes are affected by natural and engineered stressors to a varying extent [73,115], individual genotypes should ideally be monitored individually. Traditional cell culture-based assays used to assess infectious virus concentration, however, cannot differentiate between different genotypes present in a single sample (e.g. wastewater). Integrated cell culture – reverse transcriptase quantitative PCR (ICC-RTqPCR) overcomes this shortcoming by combining cell culture with viral genome detection by qPCR. The virus is briefly propagated on cells, and its amplification is measured by qPCR with primers specific to the virus of interest. The amplification measured is proportional to the initial concentration of infectious virus, and the measurement can be genotype-specific, even in a mixed sample. ICC-RTqPCR can thus determine infectious virus concentrations in a timely and specific manner. Furthermore, because cells are exposed to virus-containing samples for shorter duration compared to traditional cell culture assays, the risk of cell death arising from cytotoxic matrix components (e.g. wastewater constituents) is reduced.

ICC-PCR was originally described by Reynolds et al.[116] to detect infectious enterovirus in environmental samples. Since its introduction, several studies have used ICC-PCR for its more rapid and sensitive detection of different waterborne viruses compared to cell culture[22,114,117–121]. A quantitative assay using specific primers was developed by Mayer et al.[122] to simultaneously measure the infectious concentration of three enterovirus genotypes (echovirus 12, coxsackievirus B6 and poliovirus type I) in disinfection studies. Ryu et al.[123] then expanded on this work to include four genotypes representing the four enterovirus species relevant to human health. While only including a small number of genotypes, these two studies demonstrated that ICC-RTqPCR is a promising technique to quantify enterovirus infectivity in a sample containing multiple species and genotypes.

The objective of this study was to develop an ICC-RTqPCR that specifically targets the enterovirus genotypes most frequently encountered in environmental samples in Europe. We first identified the genotypes of interest, and then developed and calibrated an ICC-RTqPCR assay to measure their infectious concentrations. Finally, we confirmed the ability of the assay to quantify residual infectious concentrations after inactivation, and in challenging environmental matrices (wastewater). Ultimately, this assay will enable a genotype-specific monitoring of enterovirus fate during water and wastewater treatment or

2.2 Materials and methods

2.2.1 Genotype selection

A literature review was conducted to identify the enterovirus genotypes commonly found in sewage. As part of the effort to eradicate poliovirus, enteroviruses in sewage are monitored worldwide. Our focus was on surveillance papers reporting the genotypes found in European sewage[25–28,112,124–126]*. Genotypes detected were assigned a rank from 1 to 13 based on their prevalence in each study (percentage of total enteroviruses detected), a low rank corresponding to high prevalence. Only enteroviruses detected at a percentage superior to 0.5% and in more than one study were considered. If a genotype was not detected in one study, it was assigned the maximal rank of 14 for that study. The mean rank of each genotype over all studies was calculated. Prevalence data for each study and the final ranking of each genotype are given in Table 2:1.*

2.2.2 Viral stock preparation

For each selected genotype, we obtained one environmental isolate. Coxsackieviruses B4 (CVB4) and B5 (CVB5) were isolated from Lausanne sewage as described elsewhere [73]. Sewage isolates of the remaining genotypes were kindly provided by Soile Blomqvist and Carita Savolainen-Kopra (Finnish National Institute for Health and Welfare) and included coxsackieviruses B1, B2, B3, A9 (CVB1, CVB2, CVB3, CVA9) and echoviruses 3, 6, 7, 11, 13, 25 and 30 (E3, E6, E7, E11, E13, E25, E30).

Stocks of all the enterovirus genotypes were produced by propagating them once in their corresponding cell line, except for CVA9, which was propagated on Buffalo Green Monkey Kidney (BGMK) cells. Based on literature[127–134] BGMK were selected for their ability to propagate coxsackievirus B. Rhabdomyosarcoma (RD) cells were selected because they are efficient at propagating echovirus and are the most efficient cell line to propagate coxsackievirus A. BGMK (provided by Spiez Laboratory, Switzerland) and RD (ATCC CCL-136) cells were grown at 37°C in 5% CO2, on Minimum Essential Medium (MEM, Life Technologies) and Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) respectively, supplemented with 10% fetal bovine serum

(FBS, Life Technologies) and 1% penicillin/streptomycin (Life Technologies). The maintenance media was prepared by supplementing 2% FBS instead of 10%.

Confluent flasks of BGMK or RD were infected with the virus in maintenance media and incubated at 37°C until full cytopathic effect (CPE) was observed. After three cycles of freeze-thawing, the cell lysate was centrifuged for 10 min at 300x*g* and the supernatant was filtered through a 0.22 µm polyethersulfone (PES) membrane (Durapore, Millex) or 0.2 µm PES membrane (Sarstedt). The viral stock was then aliquoted and stored at -20°C. Viral stocks were enumerated on their respective cells by endpoint dilution in 96 well plates, and their infectious concentration was assessed by the most probable number (MPN) method [135] with 5 replicates per dilution. Two different virus generations were used for this study: the first was propagated once from the original isolates, while the second was propagated once from the first generation stock.

2.2.3 RNA Extractions

All RNA extractions were performed on 200 uL of sample using the Maxwell® 16 Viral Total Nucleic Acid Purification kit (Promega) according to the manufacturer's instructions with the Maxwell® 16 Instrument, and the RNA extracts eluted in 50 uL of molecular grade water. The RNA extracts obtained from the ICC-RTqPCR of virus spiked in a wastewater matrix were additionally treated using the OneStep™ PCR Inhibitor Removal Kit (Zymo). RNA extracts were stored at -20°C for a maximum of 7 days prior to RTqPCR.

2.2.4 Primer design

For each genotype, a consensus sequence of the VP1 region was created with approximately twenty sequences from the NIAID Virus Pathogen Database and Analysis Resource (ViPR) [136]. The VP1 region is the variable region of Enterovirus which allows typing [9]. The following criteria were used for the selection: (i) originated from a European country (excluding Russia for potential geographic distance), (ii) as recent as possible (most were after 2000). The accession numbers of the sequences are listed in Table A.1. Based on the consensus sequence, primers were designed in VP1 regions that were relatively conserved within a genotype using the Geneious software version 9.1.8 [137] (Table A.2). Since the main design criterion was genotype specificity, the region was the main criteria used for design, disregarding other common design guidelines (GC content, high melting temperature, secondary structure). The primers were tested with their target genotype and discarded if they did not amplify it.

In addition to the specific primers, we also employed general enterovirus primers described elsewhere [138] that capture all genotypes simultaneously.

2.2.5 Reverse transcription (RT)-qPCR analysis

RT-qPCR was performed on a Mic qPCR Cycler (Bio Molecular Systems) using the One Step SYBR® PrimeScript™ RT-PCR Kit (Takara). Each reaction (20 µL) contained 3 µL of template, 10 µL of 2x One Step SYBR RT-PCR Buffer III, 0.4 µL of TaKaRa Ex Taq HS (5 U/ul), 0.4 µL of PrimeScript RT enzyme mix II, 250 nM of each primer and 5.2 µL of water. The following thermocycling conditions were used for all specific primes: RT at 42°C for 5 min, 10 sec at 95°C (RT inactivation/initial denaturation), followed by 40 cycles of 95°C for 5 sec, 52°C for 20 sec and 60°C for 30 sec. The same thermocycling conditions were also used for the general enterovirus RT-qPCR assay, except for an annealing temperature of 57°C instead of 52°C. Each sample was run once, and the Cq values were determined using the micPCR software (v2.10.0; Bio Molecular Systems).

DNA standards for each genotype were purchased (gblock, Integrated DNA Technologies) and were serially diluted to produce standards ranging from 3.3 to 3.3E+06 to genome copies per µL. An overall calibration curve was created for each genotype by pooling individual curves from multiple qPCR runs. The limits of quantification (LOQ) of each assay were determined in R using a curve-fitting method developed by Klymus et al.[139] with a coefficient of variation (CV) threshold of 35%. For samples with a genome copy (gc) concentration below the LOQ, the concentration was set to the LOQ and labeled accordingly. QPCR data were excluded if they exhibited interfering peaks in the melt analysis.

No template controls containing deionized water were included in all qPCR runs and did not result in an amplicon peak. Selected samples were analyzed at 1:10 and 1:100 dilutions to check for PCR inhibition. Inhibition was only detected in samples containing wastewater, and therefore these samples were subjected to an inhibitor removal step (see “RNA extractions”).

2.2.6 Microfluidic quantitative PCR to determine primer specificity

In order to verify that the primers were genotype-specific, each primer was evaluated against all genotypes included in the study using a Microfluidic quantitative PCR (MFqPCR) platform (Biomark HD, Fluidigm). MFqPCR allows the simultaneous run of multiple singleplex qPCR reactions that take place in nanoliter chambers (9.1 nL) situated at high density on a single chip.

RNA extracted from each genotype stock was quantified using the general enterovirus RT-qPCR assay and adjusted to equal concentrations. The RNA was reverse transcribed using the SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific) and was subsequently diluted 5-fold to avoid inhibition in the following steps. Since the MFqPCR uses very small reaction chambers, the cDNA was pre-amplified to ensure that at least one DNA molecule is found in each chamber. This pre-amplification was performed using 1 µL of Preamp Master Mix (Fluidigm), 0.5 µL of a mix of all primers at a concentration of 500 nM each, 2.25 µL of DNase-free water, and 1.25 µL of cDNA. The following thermocycling program was used: 2 min at 95°C, followed by 14 cycles of

15 sec at 95°C and 4 min at 60°C. Finally, 5 µL of preamplified DNA was incubated with 2 µL of an exonuclease I solution (1.4 µL of DNase-free water, 0.2 µL of Exonuclease I reaction Buffer (New England BioLabs), 0.4 Exonuclease I 20 $U.\mu L^{-1}$) for 30 min at 37°C, followed by 15 min at 80°C.

Two MFqPCR runs were undertaken with different cDNA concentrations. After pre-amplification, samples were diluted 5-fold for the first run and 20-fold for the second run before the MFqPCR run. MFqPCR was performed using a 48x48 Dynamic Array™ integrated fluidic circuit. The sample mix contained 2.5 µL 2xSso-Fast EvaGreen Supermix with low ROX (BioRad), 0.25 µL 20x DNA Binding Dye (Fluidigm), and 2.25 µL of the pre-amplified sample (cleaned with exonuclease and diluted). Each primer pair mix contained 2.5 µL 2xAssay Loading Reagent (Fluidigm), 2 µL 1x DNA Tris 0.1mM EDTA, 0.25 µL of forward primer at 100 µM, and 0.25 µL of reverse primer at 100 µM. The sample and primer mixtures were loaded onto the chip and were mixed using an IFC controller MX (Fluidigm) following the manufacturer's instructions. The qPCR was run with the following thermocycling conditions: 1 min at 95°C, followed by 30 cycles of 5 sec at 96°C, 20 sec at 52°C, and 20 sec at 60°C. Data analysis was performed using the Fluidigm Real-Time PCR Analysis software.

Several control samples were included in the analysis. Serial dilutions of the E7 stock solution were used to confirm that the RT and pre-amplification did not affect the relative quantification. Negative controls (water) of the RT, the pre-amplification, and the MFqPCR analysis were also included. Extracts from cell scraping also underwent the whole process from RT on, to check whether the primers amplified the cell extracts.

2.2.7 PCR efficiency

The PCR efficiency of each primer pair was determined based on serial dilutions of DNA standards, according to Green and Sambrook (2012). Preliminary experiments revealed that similar efficiencies were obtained when using viral RNA instead of DNA standards (Table A.3). Due to design constraints, we aimed for an amplification efficiency of at least 0.8 for the specific primers. For primers with an amplification efficiency of 1, a 10-fold dilution of samples results in a 3.3 Cq difference. With a 0.8 efficiency, a 10-fold dilution factor results in a 3.9 Cq difference.

2.2.8 ICC-RTqPCR protocol

A detailed protocol of the ICC-RTqPCR can be found in Figure A.1.. Briefly, 6 well plates of BGMK or RD were prepared. When cells reached full confluency, the growth media was discarded, and replicate wells were inoculated with 1 mL of the sample. The first replicate well was scraped immediately and the entire content, sample and scraped cells, was collected and frozen at -20°C until processing (**t0 sample)**. The plate with the second replicate was placed in the incubator at 37°C for 24 hours before the well was scraped and the content collected and frozen (**t24 sample)**. The samples were then thawed and centrifuged for 10 min at 1000x*g* to remove cell debris and the supernatant was transferred to a new tube. RNA was extracted from 200 µL of

the sample as described above, and the viral genome copy concentrations in each sample were determined by RT-qPCR. Finally, the increase in genome copies over 24 hours (Δgc (24h)) was determined as the difference in genome concentration between the t0 and t24h samples.

To calibrate the ICC-RTqPCR assay, Δgc(24h) was measured for standards with known concentrations of infectious virus. A stock solution containing all thirteen genotypes at equal infectious concentration was made and was serially diluted to produce standards ranging from 50 to 5000 $MPN.L^{-1}$ for each virus. The dilutions were made in 2% MEM or DMEM for infection on BGMK or RD, respectively. Calibration standards were subjected to the ICC-RTqCPR protocol described above, in duplicates for each dilution and on both cell lines. Preliminary data showed that Δgc(24h) reached a plateau for standard concentrations of 1E+04 $MPN.L^{-1}$ or higher, therefore 5000 $MPN.L^{-1}$ was the maximum concentration considered. Three repeats of the calibration curve were made with different cell batches. The first two repeats of the ICC-RTqPCR calibration curves were done with the first generation viral stocks, while the third repeat was done using the second generation.

2.2.9 Competition among genotypes during cell culture

To evaluate if the presence of other genotypes in solution inhibits the replication of any given virus during cell culture, Δgc(24h) was compared between solutions containing multiple genotypes and single genotypes. To this end, we compared the two highest standards of the first ICC-RTqPCR calibration curve (which used mixed samples) to samples containing only one genotype at the same concentration.

The single genotype experiments were performed on the same day and using the same individual viral stock solutions and cell batches as the first ICC-RTqPCR calibration curve.

2.2.10 Inactivation experiment

Aliquots (100 µL) of CVB5 at a concentration of 7E+06 $MPN.L^{-1}$ were exposed to 55°C in the thermocycler for 0, 2, 5, or 10 seconds. Each exposure time was tested in duplicate and the duplicate samples were combined and diluted 70- to 7000-fold in 2% MEM, to fall within the linear range of ICC-RTqPCR. The diluted samples were then enumerated by endpoint dilution once and by ICC-RTqPCR in triplicate.

2.2.11 Measurement of virus in wastewater matrix

A 24-hour composite influent sample was collected from the Vidy wastewater (WW) treatment plant (Lausanne, Switzerland). It was filtered through a 2.7 µm glass filter (Whatman®) and a 0.22 µm PVDF filter (Durapore, Millex). Part of the filtered WW was sterilized by autoclaving (SWW). The CVB4 stock was diluted in filtered WW and in SWW to obtain a concentration of 200 $MPN.L^{-1}$. Virus in WW and in SWW were then measured in duplicate by ICC-RTqPCR. The WW and SWW without added CVB4 were also measured by ICC-

RTqPCR, to check for the possible interference by indigenous CVB4. All ICC-RTqPCR inocula were 500 µL instead of 1 mL, to decrease cell toxicity. The infectious concentration of the spiked virus was measured once by endpoint dilution and the Δgc(24h) was measured in duplicates for each sample.

2.2.12 Data analysis

Statistical analyses were performed in R using the packages *stats* [140], *car*[141], and the EPA MPN calculator for MPN calculations [142]. The two-tailed Student t-test for equal variances was done in R using the t.test function, and the bartlett.test function to test for the equivalence of variance. ANCOVA analysis was done using the Anova function with Type III sum of squares. All statistical tests were performed with a statistical significance threshold of α=0.05. The prediction intervals for the inactivation experiment and the wastewater spiking experiment were calculated using Equation 2:1, with x being the infectious concentration and y the Δgc(24h), n being the number of standards used for the calibration curve, m the number of replicate to calculate x_0, $s_{y/x}$ an estimation of the random error in the y-direction, and b the slope of the calibration curve [143].

$$x_0 \pm t_{n-2} * \frac{s_{y/x}}{b} * \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}}$$

Equation 2:1: prediction intervals for inactivation measured with ICC-RTqPCR

2.3 Results

2.3.1 Selection of enteroviruses

2.3.1.1 *Identification of predominant enterovirus genotypes.*

Based on the occurrence found in the literature, the following list of genotypes in order of prevalence was produced: CVB5, CVB4, E11, E6, CVB3, E3, E7, CVB2, CVB1, E25, E30, CVA9, E13 (Table 2:1). The five most common genotypes were present in all studies considered, whether in clinical[26,124,125] or environmental samples[25–28,112,124–126]. A mixture of these 13 genotypes at equal infectious concentration constituted our stock solution for the ICC-RTqPCR calibration curve, representing the diversity that may be found in a sewage sample.

Table 2:1: Prevalence of the enterovirus genotypes in European sewage and global rank. The global rank corresponds to the sum of all individual ranks, divided by the number of studies.

	Prevalence in the study (% of total enteroviruses detected)			**Global rank**
Study	Delogu et al. 2018	Hovi et al. 1996	Benschop et al. 2017	
Cells used / Genotype	(RD, L20B)	(GMK, Vero, HA, HES)	(L20B, RD, Ht29, HEp2)	
CVB5	24.4	17.3	8.3	2.3
CVB4	13.8	18.7	6.7	2.7
E11	13.6	16.6	16.8	2.7
E6	10.4	15.2	17.1	3.0
CVB3	7.9	7.8	6.5	6.0
E3	2.7	2.6	9.2	6.3
E7	8.4	0.8	5	8.0
CVB2	6.7	11.1	1.6	8.3
CVB1	1.5	3.5	2.7	8.7
E25	1.1	0.9	6.5	9.0
E30	1.4	1.3	3.2	9.3
CVA9		0.6	2.3	12.3
E13	0.5		1.8	12.7

2.3.1.2 *Primer specificity, efficiency and LOQ*

The ability of a primer pair to amplify its target genotype (specific amplification) was compared to its ability to amplify the other genotypes (non-specific amplification). A difference in Cq (ΔCq) between specific and non-specific amplification of 3.9 was considered acceptable, a ΔCq of 7.9 good and a ΔCq of 11.8 very good, representing at least 10-, 100- and 1000-fold greater amplification of the specific target compared to the non-specific one, respectively, for primers of efficiency ≥ 0.8. Figure 1 presents the ΔCq values for each combination of primer pair-genotype, for the primers used in this study. Most primers exhibited good or very good specificity, but three primer pairs were rated not acceptable: E13 did not amplify its own genotype, and E6 amplified E11. E6 and E13 were therefore removed from further consideration. In addition, CVB2 amplified E6. In this case, however, the lack of specificity was not prohibitive, because E6 does not amplify on BGMK cells, which are used for the ICC-RTqPCR analysis of CVB2. The full specificity results for both repeats of Fluidigm runs with qPCR replicates are presented in Figure 2.1. For the remaining primers, standard curves were produced and PCR efficiency was determined (Table 2:2). Most primers achieved efficiencies > 0.8, except E7, which was removed from further consideration. However, primers for E3 and E11 exhibited interfering melt peaks (data not shown), and were therefore also discarded.

Genotype \ Primer Pair	CVA9	CVB1	CVB2	CVB3	CVB4	CVB5	E3	E6	E7	E11	E13	E25	E30
CVA9		>	12.0	>	>	14.4	>	>	>	>	7.8	>	>
CVB1	23.5		12.7	>	22.2	7.2	>	>	>	23.2	9.8	19.9	>
CVB2	>	>		>	>	>	>	>	>	>	>	>	>
CVB3	>	>	12.4		>	>	22.5	>	>	>	>	>	>
CVB4	16.7	>	10.9	>		17.7	19.0	>	>	>	>	>	13.1
CVB5	18.4	11.6	>	5.4	25.2		>	>	>	>	>	18.3	>
E3	>	20.9	12.9	12.4	>	>		>	>	>	>	17.5	>
E6	>	>	**1.0**	15.5	>	>	12.2		9.9	>	>	>	15.1
E7	>	>	>	>	>	>	17.0	>		>	>	>	16.6
E11	17.8	>	>	12.7	>	11.1	14.8	**3.2**	10.7		>	>	>
E13	>	>	>	>	10.1	>	>	>	5.6	>		>	17.8
E25	>	18.1	>	>	>	17.3	>	>	>	>	>		>
E30	22.5	>	>	>	24.0	>	>	>	>	>	>	>	

Figure 2.1: ΔCq (amplification of specific genotype – amplification of non-specific genotype) for each combination of primer pair and genotype. The diagonal corresponds to specific amplification, and the > sign to the absence of non-specific amplification. ΔCq under 3.9 are in pink and bold, indicating non-specificity of the primers.

The final eight genotypes maintained for ICC-RTqPCR analysis were thus CVB5, CVB4, CVB3, CVB2, CVB1, E25, E30, and CVA9. For these genotypes, LOQs and calibration curve parameters were determined and are shown in Table 2:2.

Table 2:2: PCR efficiencies for all specific primer pairs and LOQ and calibration curve parameters for the final primer sets maintained for ICC-RTqPCR development. All values are determined based on multiple pooled qPCR runs.

Primers	Efficiency	LOQ (gc/run)	Slope of standard curve	Intercept of standard curve	R^2 of the standard curve
CVA9	0.96	10	-3.41	29.86	1.00
CVB1	1.09	1041	-3.11	36.29	0.95
CVB2	1.04	10	-3.23	28.33	0.93
CVB3	0.88	10	-3.63	32.68	0.99
CVB4	0.91	16	-3.56	33.65	0.93
CVB5	0.97	10	-3.39	30.21	0.94
E3	0.99				
E7	0.54				
E11	0.85				
E25	0.96	16	-3.43	32.28	1.00
E30	0.86	26	-3.71	33.90	0.98

2.3.2 Competition between genotypes in mixed samples

Typical environmental samples contain several genotypes, which may compete with one another during amplification on cells, and may hence influence ICC-RTqPCR results. We therefore compared the ICC-RTqPCR signal of samples containing all thirteen genotypes included in this study to samples containing only a single genotype at a time (Figure 2.2).The comparison was performed for the eight genotypes for which the ICC-RTqPCR analysis is possible, namely CVB5, CVB4, CVB3, CVB2, CVB1, E25, E30, and CVA9. For the CVB genotypes, the Δgc(24h) values for single and mixed samples matched well. For the two echoviruses (E25 and E30) and CVA9, in contrast, there was a statistically significant divergence between single and mixed sample (two-sided t-test for CVA9 (5000 MPN.L^{-1}): p=0.016; E30 (1000 MPN.L^{-1}): p=0.043; E30 (5000 MPN.L^{-1}): p=0.032). The confidence intervals for the difference in means between the two groups are presented in Table A.4. We consider a five-fold difference in Δgc(24h) (0.7 $\log_{10}$) as not biologically relevant since the error is comparable to the variability observed for replicate samples. Thus, the competition in samples CVA9 (5000 MPN.L^{-1}) and E30 (1000 MPN.L^{-1}) can be ignored, while E30 (5000 MPN.L^{-1}) and perhaps E25 (5000 MPN.L^{-1}) are affected

by competition by other genotypes. Consequently, only samples up to 1000 MPN.L^{-1} were included in the further analysis of E25 and E30 in mixed samples.

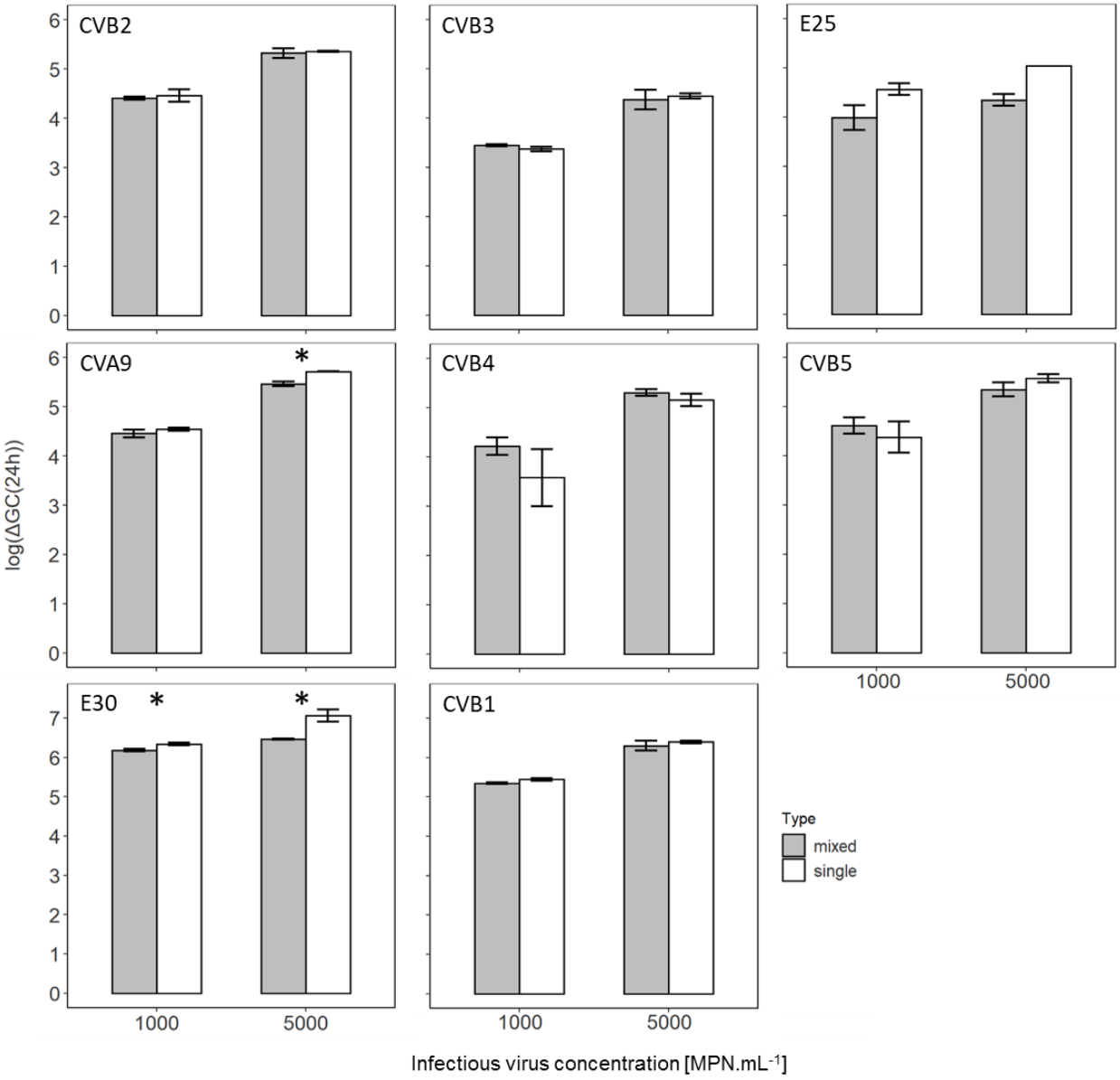


Figure 2.2: Comparison of the Δgc(24h) produced by each genotype in a mixed sample versus alone (single). The value on the x-axis denotes the concentration of each individual virus in solution measured by endpoint dilution. The error bars represent the standard deviation for the two replicates and the stars indicate a statistically significant difference (t-test). For sample E25 5000 MPN.L^{-1} only one replicate of the single sample yielded results.

2.3.3 ICC-RTqPCR standard curves

Three replicates of the ICC-RTqPCR calibration curves were obtained to assess the influence of the difference between cell batches on the relation between MPN and Δgc(24h). As shown in Figure 2.3, variation was observed between the three repeats of the ICC-RTqPCR calibration curves. A log-log linear regression was fitted to explain the relation between $\log_{10}$(MPN) and $\log_{10}$ (Δgc(24h)) for each calibration curve repeat, as the most parsimonious model allowing to make predictions about their dependency (plots of the regression residuals and their linearity are available in Figure A.3). An ANCOVA analysis of the three repeats shows that the slopes between the three calibration curves were not significantly different for any of the genotypes, while there was a significant difference in the intercepts of the three calibration curves for all the genotypes (see statistical parameters in Table A.5). The pooled standard curves are plotted in Figure 2.3 and their slopes and intercepts are given in Table A.5.

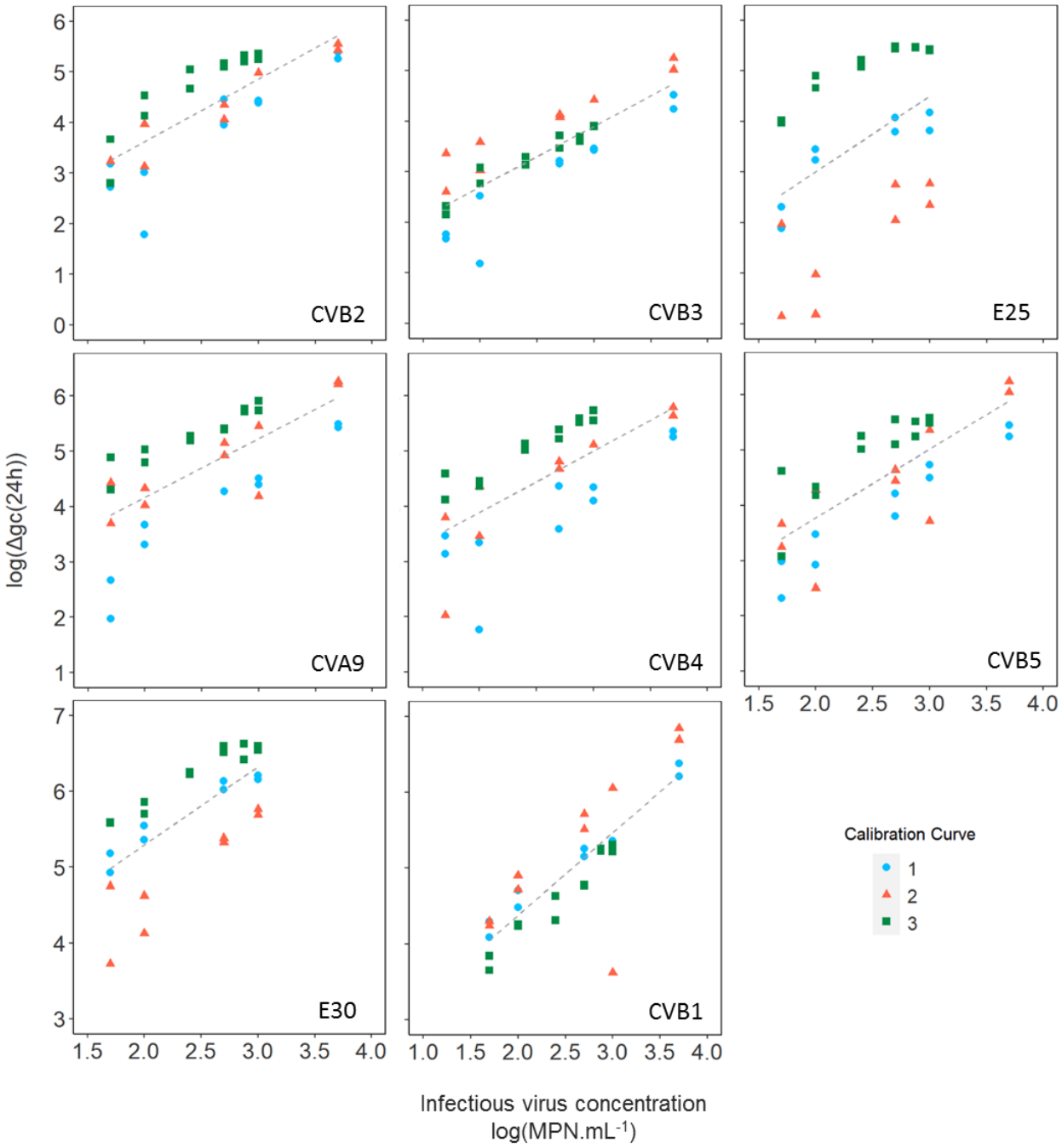


Figure 2.3: ICC-RTqPCR calibration curves: three repeats for each genotype. The dotted grey line corresponds to the pooled calibration curve regression equation. The following t0 samples were replaced by the LOQ in the Δgc(24h) calculation, with no impact on the calculated Δgc(24h): CVB1 3d Calibration Curve, log(MPN.L^{-1})=1.7 316 rep. 2; CVB5 3d Calibration Curve, log(MPN.L^{-1})=1.7 rep. 1 and 2.

2.3.4 Measuring inactivation curves by ICC-RTqPCR

The ICC-RTqPCR method relies on an increase in genome copies caused by viral replication on cells. In samples with a high background of viral RNA from inactivated, non-replicating viruses, a small increase in genome

copies caused by few infectious viruses may be difficult to detect. In order to test that the method allows the accurate estimation of infectious virus among inactivated ones, a sample of CVB5 was inactivated by heat (Figure 2.4A), and the infectious concentration of the samples measured by both endpoint dilution and by ICC-RTqPCR were compared (Figure 2.4B). ICC-RTqPCR-measured concentrations corresponded well to the concentration measured by endpoint dilution. All time points fall close to the 1:1 line, thus the infectious concentration of a sample containing inactivated virus can be determined by ICC-RTqPCR with reasonable accuracy. The accuracy was lowest for the sample inactivated during 10 seconds, possibly due to the lowest ratio of infectious to total virus present in this sample.

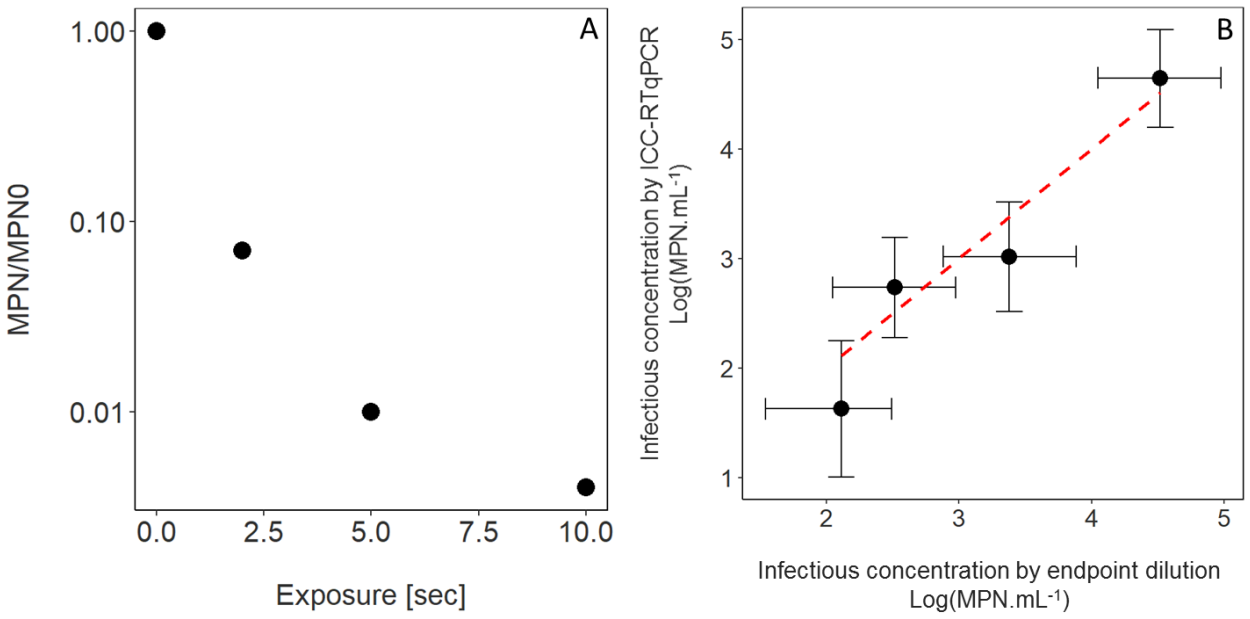


Figure 2.4: A) Inactivation curve of CVB5 upon exposure to heat, expressed as the residual fraction of infectious virus (MPN/MPN 0) measured by endpoint dilution. B) Infectious concentration of the samples shown in panel A) as measured by ICC-RTqPCR method (mean of triplicates) vs measured by endpoint dilution. The third replicate ICC-RTqPCR calibration curve (Figure A.4b) was used to determine log10(MPN) in the ICC-RTqPCR measurement. The dotted red line corresponds to a 1:1 relation. The y error bars correspond to 95% prediction intervals on the log10(MPN) (Equation 2:1) and encompass the error associated with the measurement of Δgc(24h), as well as to the regression estimation. The x error bars correspond to the 95% confidence interval of the MPN calculation with the Cornish & Fisher method determined by the EPA MPN calculator.

2.3.5 ICC-RTqPCR in wastewater

To test the ability of the method to correctly measure the infectious concentration of a virus in a complex matrix, we spiked CVB4 virus into filtered WW and SWW and compared the infectious concentration measured by endpoint dilution to the determined by ICC-RTqPCR method (Figure 2.5). The red line corresponds to

a 1:1 relation between endpoint dilution measurement and ICC-RTqPCR measurements. The ICC-RTqPCR measured concentrations in both SWW and WW were not significantly different from the concentration measured by endpoint dilution of $\log_{10}$(MPN)=2.3 (single sample t-test; SWW: p=0.69, 95%CI=[0.9,3.8] and WW: p=0.10, 95%CI [1.3; 2.6]). In addition, there was also no statistically significant difference between the CVB4 concentrations measured in SWW and WW (two-sample t-test, p=0.08, 95%CI=[-0.1;0.9]). CVB4 can thus be measured accurately by ICC-RTqPCR in wastewater matrices.

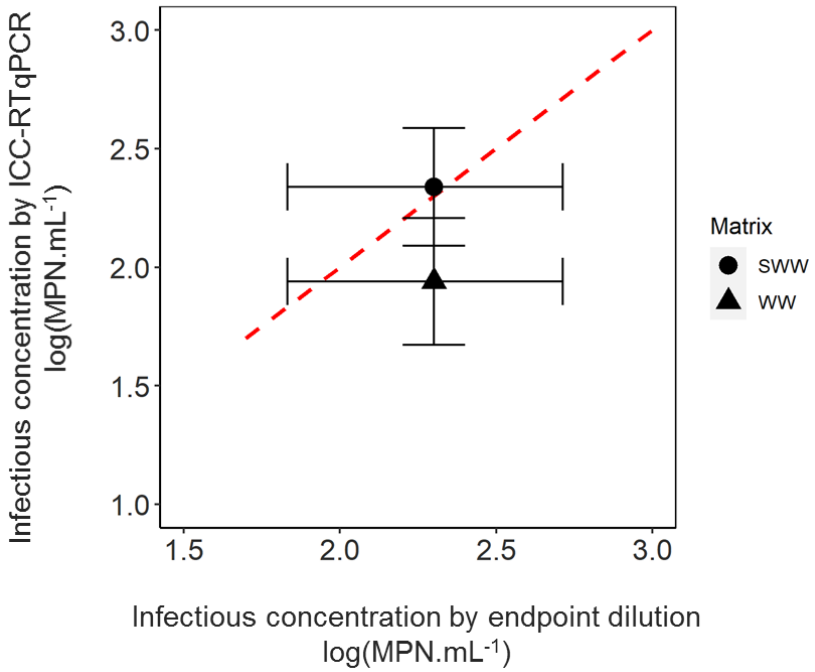


Figure 2.5: Comparison of infectious CVB4 concentrations measured in filtered and sterilized wastewater by ICC-RTqPCR and by endpoint dilution. The third calibration curve replicate (Figure A.4a) was used to determine the mean infectious concentration by ICC-RTqPCR. The dotted red line corresponds to a 1:1 relation. The y error bars correspond to 95% prediction intervals on the log10(MPN) (Equation 2:1) and encompass the error associated with the measurement of Δgc(24h), as well as to the regression estimation. The x error bars correspond to the 95% confidence interval of the MPN calculation with the Cornish & Fisher method determined by the EPA MPN calculator.

2.4 Discussion

The ICC-RTqPCR method developed in this work was designed to target eight of the most commonly encountered enteroviruses in environmental samples and to capture a broad range of circulating strains of a given genotype. The method performed comparably in simple laboratory solutions and s, and was thus not influenced by cytotoxic solution components or competing viruses present in the sewage. It has a sensitivity that

is similar to endpoint dilution [144]. Calibration curves produced in this work ranged from infectious concentrations of 50 MPN.L^{-1} to 1000 or 5000 MPN.L^{-1}, depending on the genotype considered. Over this concentration range, the calibration was mostly independent of the presence of other genotypes. The upper range can be expanded by diluting the sample prior to analysis, as was done in the inactivation experiment. The method is thus well-suited for studies that cover a wide range of infectious virus concentrations.

We successfully applied the ICC-RTqPCR to measure the inactivation curve of CVB5 during heat treatment, reaching an inactivation of 2.4 $\log_{10}$. This range of inactivation is comparable to that reported for echoviruses in UV experiments using the ICC-RTqPCR method developed by Ryu et al.[123], but narrower than the dynamic range of traditional endpoint dilution assays [73]. In disinfection studies, the extent of inactivation measurable by ICC-RTqPCR is dependent on the maximal increase in genome copy numbers during cell culturing. Specifically, if inactivation is extensive (greater than the increase in genome copy numbers during culturing), the ICC-qPCR signal produced by the residual infectious viruses may not surpass that of the inactivated viruses present in the sample. As a result, Δgc(24h) is no longer quantifiable, and the sample is not measurable by ICC-RTqPCR. Furthermore, the measurable inactivation range is genotype-specific. For example, CVA9 was found to replicate more extensively than CVB3 over 24 hours. It is therefore expected that ICC-RTqPCR can quantify lower residual concentrations of CVA9 compared to CVB3.

A critical aspect of any ICC-RTqPCR method targeting more than one virus, or single viruses in mixed samples, is the specificity of the PCR primers. Here we aimed at simultaneously measuring many closely related genotypes, and this complicates the design of primers that are both efficient and specific. We identified eight primer pairs that were acceptable with respect to both efficiency and specificity, whereby specificity was aided by the use of two different cell lines. Most of the primers included in the final method displayed good specificity, exhibiting at least >100-fold more efficient amplification of the target genotypes compared to the other genotypes considered. Nevertheless, a low level of non-specific amplification was found for many of the primer pairs used, and several genotypes included in our initial list of relevant strains had to be excluded from the final ICC-RTqPCR method for lack of suitable primers. Very high primer specificity was achieved by Hu et al.[145], who designed primers targeting 9 different enterovirus genotypes. Their method included a GeXP multiplex amplification by two sets of primers: universal and gene-specific chimeric primers. They tested each set of primers against a mixture of 28 enterovirus genotypes and checked the size of the sequence produced by each combination, finding that all their primers were specific to their target. Several other studies designed specific primers for two to four genotypes, which they evaluated against a number of other genotypes using methods such as multiplex qPCR or PCR [123,146–148]. None of these studies reported non-specific amplification. Compared to these studies, however, our approach faced additional challenges: first, our primers were designed based on a consensus sequence of multiple strains to ultimately allow for the detection of

circulating environmental viruses. Such breadth was not a criterion in the work of Hu et al.[145], and notably their primers for CVB3 were unable to amplify the environmental CVB3 strain used herein (data not shown). Second, we designed primers with a low annealing temperature to enable a single thermocycling protocol for all genotypes. For future assay design, allowing for different thermocycling conditions would allow for higher annealing temperature and higher specificity. The imperfect specificity of some of the primer sets designed herein requires that we be prudent with the interpretation of future ICC-RTqPCR results. In environmental samples, a non-target genotype may be present at a higher concentration than the target genotype, which may result in the production of a significant but non-specific ICC-RTqPCR signal. Nevertheless, to study the composition and dynamics of a known population of enteroviruses with comparable concentrations, the method is well-suited.

ICC-RTqPCR measurements are associated with considerable variability between experiments. Specifically, we found that the relation between infectious concentration and increase in genome copies after 24 hours replication on cells was not constant from one experiment and cell batch to the other. This is consistent with the finding from Ryu et al.[123], who found a different linear regression equation for PV1 than Mayer et al.[149], despite using the same experimental setup. This variability may stem from the cell culturing step of the assay. Both the age of the cells, as well as differences in the cell densities between experiments may alter the virus replication efficacy. Alternatively, replication may also be influenced by the adaptation of the viruses to the cell line: in this work, viruses that had undergone two passages on cell culture (used in the third standard curve) exhibited a greater increase in genome copies during cell culturing compared to viruses that had only been passaged once. Finally, variability may be associated with the relative starting concentration of each genotype, though this aspect was not assessed in this study. Overall, we conclude that for maximum accuracy, a calibration curve should be obtained for each experiment, in particular when absolute quantification is required. However, despite the differences in absolute genome copies produced in each experiment, the slopes of the different calibration curves were not found to be significantly different for any of the genotypes. A pooled standard curve with a global slope for each genotype can thus be used in the context of experiments, where relative, rather than absolute changes in infectious virus concentrations are desired, such as in disinfection studies. Finally, because the slopes of the ICC-RTqPCR calibration curves do not differ much between genotypes, a global curve could even be used to study the inactivation of an unknown enterovirus, in the absence of a specific calibration curve.

Despite some shortcomings, the ICC-RTqPCR method developed herein promises to be a unique and useful tool to study enterovirus population dynamics. Other studies have demonstrated that different enterovirus genotypes differ in their susceptibility to various disinfectants and environmental stressors [73,115]. These find-

ings were established using endpoint dilution methods and samples containing individual genotypes. In contrast, this ICC-RTqPCR can capture several genotypes in a single sample, thereby removing experimental variations. It also greatly reduces the amount number of cell culture needed as well as the time from experiment to result. We expect that this method will allow us to move beyond studying enterovirus as a bulk parameter, and instead focus on the fate of eight of the most relevant individual genotypes. In future work, this method should be tested on real environmental samples.

2.5 Conclusion

We have designed a method that can simultaneously determine the infectious concentration of eight enterovirus genotypes commonly found in sewage, based on the increase in qPCR signal after amplification on cells. This ICC-RTqPCR method is able to specifically quantify the infectious concentrations of eight enterovirus genotypes among 13 other genotypes. It furthermore was able to accurately measure residual infectious concentration in a background of inactivated virus, and could determine with good accuracy the concentration of a virus within a wastewater matrix. This method will be valuable to study changes in the composition of an enterovirus population over time in the environment or during water and wastewater treatment processes. It will also allow to readily assess discrepancies in the fate of different enterovirus genotypes when exposed to specific natural or engineered control processes, such as disinfection or grazing by microorganisms.

2.6 Acknowledgments

This work was supported by the Swiss National Science Foundation (grant no. 31003A_182468). The authors thank Soile Blomqvist and Carita Savolainen-Kopra (Finnish National Institute for Health and Welfare) for providing environmental enterovirus isolates, Virginie Bachmann for laboratory assistance and the EPFL Gene Expression Core Facility for help with the Fluidigm experiments.

Chapter 3 Sewage treatments shapes the composition of an enterovirus population

Odile Larivé designed the experimental plan, performed most of the experiments, analyzed the data and wrote the chapter. Shotaro Torii designed the chlorination method. Nicolas Derlon provided useful advice, analysis and expertise regarding the batch experiments, enzyme and EPS in sludge.

3.1 Introduction

Enteroviruses are commonly circulating human viruses that comprise 106 genotypes. Enteric enteroviruses reproduce in the gastrointestinal and are therefore shed into the sewage, where they are subsequently subjected to various sewage treatment processes. Sewage treatment lowers the viral load, but it is well known that treatment is typically incomplete, such that infectious viruses are discharged into the environment[62,63]. This study investigates how different treatment processes alter the load and composition of the enterovirus population during sewage treatment. Specifically, we focus on the effects of the of activated sludge treatment and chlorination.

While the primary purpose of activated sludge treatment is the removal of nutrients and biological oxygen demand, activated sludge has also been found to reduce pathogen concentrations. Typical reductions in the viral load during activated sludge treatment range from 1 to 3 $\log_{10}$[62–64,84]. The reduction in viral load attributable to chlorination in full scale treatment plants is more difficult to measure, because virus concentrations in chlorinated effluent are often very low[64]. Costán-Longares et al.[63] reported a reduction in the enterovirus load of 2.3 to 4.2 $\log_{10}$ for tertiary treatment trains that include chlorination. In water reuse treatment trains which recycle sewage for potable or non-potable purposes, a 4 $\log_{10}$ virus removal credit is typically attributed to high dose chlorination[60].

Even though many different enterovirus genotypes can be found in sewage[25,27,28], the reduction of the enterovirus load during sewage treatment is typically estimated for the global population of enteroviruses[62,64,79,80,84]. This is due to the difficulty in measuring infectious enterovirus genotypes individually. However, it is known that activated sludge and chlorination remove or inactivate different enterovirus genotypes to differing extents. During activated sludge treatment, enteroviruses are removed by both adsorption to the sludge[85,91] as well as by microbially mediated inactivation[87,90,91]. Gerba et al.[85] and Tao et al.[96] found that the adsorption of enteroviruses onto sludge was genotype-specific. Similarly, inactivation in activated sludge can be expected to be genotype-specific, for several reasons. Frist, not all genotypes are equally susceptible to antiviral activity by microorganisms or their metabolites[44,97,150]. Second, activated sludge operation temperatures between 4°C and 32°C[98] can cause differentiated inactivation for different enterovirus genotypes[37]. And third, polysaccharides and peptidoglycans, two types of molecules present in extracellular polymeric substances (EPS) produced in activated sludge, have been shown to interact with the enterovirus capsid and influence its thermal and environmental stability in a genotype-specific fashion[99,100]. Similar to activated sludge, the susceptibility of enteroviruses to disinfection treatment has also been shown to vary among genotypes when in the form of free chlorine[23,73,75], monochloramine[69] or chlorine dioxide[151]. activated sludge and chlorination thus both have the capacity to shape the enterovirus population prior to discharge into the environment.

The goal of this study was to evaluate how activated sludge and chlorination treatment shape an enterovirus population at the genotype level. To this end, a population of eight genotypes commonly found in sewage was exposed to activated sludge and free chlorine, and removal kinetics were monitored for each genotype. Additionally, experiments were conducted to identify the mechanisms of removal in activated sludge.

3.2 Materials and Methods

3.2.1 Preparation of viral stock solutions

Our starting enterovirus population was comprised of eight genotypes typically found in sewage[152]. One environmental isolate was obtained for each genotype: coxsackieviruses B4 and B5 (CVB4 and CVB5) were previously isolated from Lausanne sewage[73], and coxsackieviruses A9, B1, B2 and B3 (CVA9, CVB1, CVB2, CVB3) and echoviruses 25 and 30 (E25 and E30) isolated from sewage were kindly provided by Soile Blomqvist and Carita Savolainen-Kopra (Finnish National Institute for Health and Welfare).

Buffalo Green Monkey Kidney (BGMK; kindly provided by Spiez Laboratory, Switzerland) cells were used for the propagation of coxsackieviruses while Rhabdomyosarcoma (RD; ATCC CCL-136) cells were used to propagate echoviruses. BGMK and RD cells were grown at 37°C in 5% CO_2, on Minimum Essential Medium (MEM, Life Technologies) and Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) respectively, supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin (Life Technologies). The maintenance media was prepared analogously to the growth media except that the FBS content was lowered to 2%.

To propagate viral stock solutions, confluent T-150 flasks (TPP™, 90150) were inoculated with one genotype in maintenance media and were incubated at 37°C in 5% CO_2 until full cytopathic effect (CPE) was observed. The flask underwent three cycles of freeze-thawing, the supernatant was collected, centrifuged 10 min at 300 x*g* and filtered at 0.2 µm (Sarstedt, 83.1826.001). The stocks were aliquoted and stored at -20°C. Concentrated stocks were prepared by concentrating the filtered supernatant 40- to 270-fold with Amicon centrifugal filters with a cutoff of 100 kDa (Millipore, UFC9100). The concentrated stocks were aliquoted and stored at -80°C.

Titers of the stocks and concentrated stocks were determined by end-point dilution on their respective cell line. Specifically, the stocks were serially diluted in ten-fold series and 100 µL of each dilution was inoculated in five replicates on confluent 96 well plates (CELLSTAR® Greiner Bio-One, 7.655 180). The cytopathic effect

in the wells was observed after five or six days of incubation at 37°C and 5% CO_2, and the Most Probable Number of infectious virus was calculated using the EPA MPN calculator [142].

3.2.2 Virus enumeration

Two different methods were applied to enumerate viruses in experimental samples containing multiple enteroviurs genotypes: reverse transcription (RT)-qPCR was used to quantify the total (infectious and inactivated) concentration of each genotype in a sample, and integrated cell culture (ICC)-RT-qPCR was used to enumerate the infectious concentration of each genotype.

3.2.2.1 *RNA extractions*

All RNA extractions were performed using the Maxwell® 16 Viral Total Nucleic Acid Purification kit (Promega) according to the manufacturer's instructions with the Maxwell® 16 Instrument, extracting 200 µL of sample and eluting in 50 µL of RNase-free water. All RNA extracts obtained from experiments using activated sludge were additionally treated using the OneStep™ PCR Inhibitor Removal Kit (Zymo). RNA extracts were stored at -20°C for a maximum of 30 days prior to RT-qPCR.

3.2.2.2 *RT-qPCR*

One-step RT-qPCR was performed using genotype-specific primers designed previously (Larive et al. 2021). RT-qPCR was performed on a Mic qPCR Cycler (Bio Molecular Systems) using the One Step SYBR® PrimeScript™ RT-PCR Kit (Takara). Each reaction (20 µL) contained 3 µL of template, 10 µL of 2x One Step SYBR RT-PCR Buffer III, 0.4 µL of TaKaRa Ex Taq HS (5 $U.ul^{-1}$), 0.4 µL of PrimeScript RT enzyme mix II, 250 nM of each primer and 5.2 µL of water. The same thermocycling conditions were used for all primers (RT at 42°C for 5 min, 10 sec at 95°C, followed by 40 cycles of 95°C for 5 sec, 52°C for 20 sec and 60°C for 30 sec). Each sample was run once, and the Cq values were determined using the micPCR software (v2.10.0; Bio Molecular Systems).

DNA standards were purchased for each genotype (GeneBlocks, Integrated DNA Technologies) and were diluted to create calibration curves ranging from 3.34 to $3.34x10^6$ genome copies (gc) $.\mu L^{-1}$. A calibration curve for each genotype of interest was included in each RT-qPCR run. The limit of quantification (LOQ) was determined for each primer pair in R using a curve-fitting method developed by Klymus et al.[139] with a coefficient of variation (CV) threshold of 35%, applied to the measurement of ten replicates of the three lowest concentrations of the calibration curve. Concentrations of samples below the LOQ were set to the LOQ. RT-qPCR data were excluded if they exhibited interfering peaks in the melt analysis. Extraction blanks and no-template controls (molecular grade water) were analyzed in each run and yielded negative results.

3.2.2.3 *ICC-RT-qPCR*

ICC-RT-qPCR was performed according to a method described previously (Larive et al. 2021). Briefly, 1 mL of sample in maintenance media was inoculated onto the confluent well of a 6-well plate (CELLSTAR® Greiner Bio-One, 657160)) in duplicate plates. One plate was immediately processed (t0 sample), while the other plate was placed at 37°C and 5% CO_2 for 24 hours prior to processing (t24 sample). Each well was scraped and the entire content of the well was recovered and frozen at -20°C. The frozen samples were stored for a maximum of 2.5 months and were then thawed, centrifuged for 10 min at 1000x*g* and the supernatant was collected. RNA was extracted from 200 µL of the t0 and t24 supernatants, the genome copy number in each supernatant was quantified by RT-qPCR, and the increase in genome copies over 24 hours (deltagc(24h)) was calculated. Deltagc(24h) is proportional to the infectious concentration of virus present in the sample, and calibration curves relating the deltagc(24h) to the infectious concentration have been previously established for each genotype used in this study (Larive et al. 2021). The slope of these calibration curves was found to be reproducible, while the intercept varied depending on the age of the cells used. Here, we therefore used the calibration curve slopes determined previously, but determined the intercept for each ICC-RT-qPCR run by analyzing a standard of known infectious concentration in parallel to the samples.

3.2.3 Activated sludge experiments

3.2.3.1 *Reactor setup*

Activated sludge was collected on three different days (sludge 1 on the 26th of June 2021, sludge 2 on the 30th of September 2021, and sludge 3 on the 9th of February 2022) from the sewage treatment plant of Morges, Switzerland. The sludge was stored at 4°C until use, for a maximum of 5 hours. Depending on the experiment, 200 mL to 900 mL of activated sludge were placed in a beaker stirred continuously with a magnetic stirrer and aerated to reach a dissolved oxygen concentration of at least 2 mg.L^{-1}. Temperature, pH and oxygen in the sludge were monitored with a MeterMulti 3630 IDS (WTW, Xylem Analytics Germany GmbH) and results reported in Figure B.1. Viruses were spiked into the reactor as a mixture of all eight enteroviruses genotypes at approximately equal concentrations. Samples were taken at different time points to determine the physical and chemical parameters of the sludge (see section 3.2.3.2), as well as the virus concentrations in sludge and supernatant fractions (see section 3.2.3.3). At the end of each experiment, the residual sludge volume in the beaker was measured to determine the volume of evaporated liquid, and concentrations were adjusted correspondingly.

Over the course of the experiments, temperature was around 24°C, between 20 and 22°C and around 24°C in sludges 1, 2 and 3 respectively. pH was around 7.7, 8 and between 8.2-8.6 in sludges 1, 2 and 3 respectively and DO always superior to 4 mg.L^{-1} (Figure B.1).

3.2.3.2 *Measurement of chemical and physical sludge parameters*

Total suspended solids (TSS) were measured as recommended by standard methods (APHA et al., 2005). Briefly, 5 to 10 mL of activated sludge were filtered through a 0.7 µm glass fiber filter (Whatman, WHA1825047) and the weight of the solids on the filter was determined after drying for one hour at 105°C in the oven and equilibrating for 15 minutes in a desiccator. TSS results are reported in Table B.1.

For chemical characterization, 5 to 30 mL of activated sludge were sampled, centrifuged for 10 min at 4000x*g*, and the supernatant was filtered at 0.2 µm and exposed to UV for 20 min for sterilization. Samples were stored at 4°C for a maximum of 30 days before being analyzed. Dissolved organic carbon (DOC), dissolved inorganic carbon (DIC) and total nitrogen (TN) were measured with a DOC/TOC Analyzer (Elementar Vario TOC Cube). Ammonium (NH4+), nitrate ($NO3^-$), nitrite ($NO2^-$), phosphate (PO_4^{3-}), and chloride (Cl^-) ions were measured by ion chromatography (Thermo Scientific Intergrion HPIC). DOC and DIC are reported in Figure B.2, while ions and TN are reported in Figure B.3.

Over the course of the experiments, TSS was in the range between 1.4-0.9, 1.4-0.9, 2.4-1.1 g.L-1, in sludges 1, 2 and 3 respectively. DOC was in the range between 10-40, 15-10, 5-25 mgC.L-1, in sludges 1, 2 and 3 respectively. TN was in the range between 20-45, 30-45, 60-30 mgN.L-1 and ammonium in the range between 5-0, 14-0, 60-30 mgN.L-1, in sludges 1, 2 and 3 (Figure B.2 and Figure B.3).

3.2.3.3 *Inactivation experiments*

Experiments were conducted to determine the inactivation kinetics of each enterovirus genotype in activated sludge reactors. In a first step, inactivation curves were established. To this end 7.5 mL of a stock solution containing all enterovirus genotypes at an average concentration of ~$2x10^7$ MPN.mL^{-1} each (ranging from $7.4x10^5$ to $3.8x10^7$) were spiked into a reactor of 900 mL of activated sludge. Samples were taken after 30 min, 4 h, 21 h, 45 h and 68 h, and TSS, and chemical sludge parameters were determined. In addition, the infectious and total concentrations of each genotype were determined in both the supernatant and the sludge fraction. To this end, triplicates of 5 mL of activated sludge were sampled and centrifuged for 20 min at 4000x*g* at 4°C. The supernatant was filtered through a 0.2 µm PES (Sarstedt, 83.1826.001) filter and the filtrate was collected and termed the supernatant fraction. The sludge pellet was amended with 5 mL beef extract (100 g.L^{-1} beef extract (Merck Millipore, B4888-50G, pH 7.2) and was resuspended by briefly vortexing followed by shaking for 30 min at RT at 400 rpm. The resuspended pellet was then centrifuged for 20 min at 4000x*g* at 4°C and the supernatant was filtered through a 0.2 µm PES filter (Sarstedt, 83.1826.001). The filtrate was retained and termed the sludge fraction. 5 mL of 2% MEM was added to both the sludge and supernatant fraction samples before storage at 4°C for a maximum of one week (with the exception of one instance where it was kept for 15 days) prior to enumeration by ICC-RTqPCR, and RT-qPCR. Depending on the sludge sample, this protocol allowed for the recovery of 10 to 61% of total virus. Recovery was measured

after 30 min exposure to the activated sludge, to ensure sufficient time for partition between sludge and supernatant while also limiting the extent of inactivation.

Next, the experiment was repeated two more times using two different new samples of sludge (sludge 2 and sludge 3), to assess the variability in genotype inactivation among sludge samples. For this purpose, samples were only taken at times 30 min and 45 h. The two repeats simultaneously served as the control samples of the two experiments described below (section 2.3.4).

3.2.3.4 *Experiments to determine the role of microbial activity in virus inactivation*

To determine the role microbial activity versus adsorption in the removal inactivation and degradation of enteroviruses, a batch of activated sludge (sludge 2) was split into three portions, as presented in Figure 3.1. One portion was maintained as described above (section 3.2.3.3), used as is and termed control sludge. The second portion was sterilized in a water bath at 65°C for more than 40 min and was then cooled down on ice before the start of the experiment. This portion was termed sterilized sludge. The third portion was decanted and only the supernatant was kept while the solids were discarded. This portion was termed decanted sludge. 900 mL of each portion were placed in three beakers, and were spiked with 2.3 mL of a solution containing all enterovirus genotypes at an average concentration of ~$5x10^7$MPN.mL^{-1} each (ranging from $8.7x10^6$ to $5x10^7$ MPN.mL^{-1}). Triplicates samples were taken after 30 min and were enumerated for total concentrations of each genotype. Triplicate samples were also taken after 44 h and were enumerated for both infectious and total concentrations of each genotype as described above. TSS and chemical parameters were measured as described above. An additional sample taken after 5.5 h was only analyzed for TSS and chemical parameters.

A second experiment aimed to evaluate if proteases produced by the activated sludge bacteria could account for the observed antiviral effect. The activated sludge (sludge 3) was split into two portions of 210 mL. To one of the reactors, 2 mL of a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail, Thermo Scientific) was added. This cocktail inhibits aspartic acid, cysteine and serine proteases. The reactors were stirred for 15 minutes and were then amended with 2 mL of a solution containing all enterovirus genotypes at an average concentration of ~$4x10^7$ MPN.mL^{-1}each (ranging from $2.34x10^6$ to $4x10^7$ MPN.mL^{-1}). Triplicates samples were taken after 30 min and were enumerated for total concentrations of each genotype. Triplicate samples were also taken after 45 h and were enumerated for both infectious and total concentrations of each genotype as described above. Samples for chemical analyses were taken before spiking the protease inhibitor cocktail, and then after 19.5h, 27h and 45h. Samples for TSS measurements were taken before spiking the protease inhibitor cocktail and after 45 h.

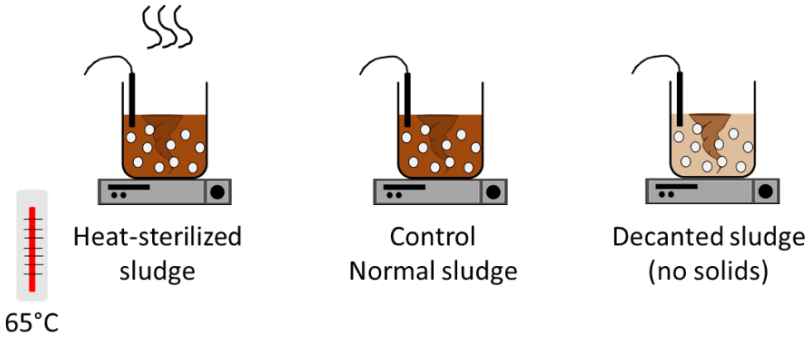


Figure 3.1: schematic of the three activated sludge conditions used to study the mechanisms of enterovirus decay.

3.2.3.5 *EPS Extraction and quantification*

EPS were extracted from one activated sludge to assess their effect on chlorination following activated sludge treatment (see section 2.4.). The extraction was performed following the protocol by Felz et al. (2016). 50 mL falcon tubes were filled with activated sludge and centrifuged at 4000x*g* at 4°C for 20 minutes. The supernatant was discarded and the wet weight of the pellets was determined. Pellets were combined to obtain a mass of 3 g of wet sludge. The wet sludge was then placed in a 250 mL baffled flask filled up to 50 mL with deionized water, and 0.25 g Na2CO3 anhydrous (Fluka) were added to the flask to obtain a 0.5% (w/v) Na_2CO_3 concentration. The baffled flask containing the mixture was placed in a 1L beaker containing 150 mL of tap water heated to 80°C. Both flask and beaker were covered separately with aluminum foil, and the baffled flask was stirred for 35 min at 400 rpm. The mixture was then transferred to a 50 mL tube and centrifuged at 4000x*g* at 4°C for 20 min. The supernatant collected comprised the EPS extract. The extract was then concentrated approximately 12-fold using Amicon (Millipore, UFC900324 and UFC901024) ultrafiltration filters with a cut-off of 3 kDa and 10 kDa to achieve an EPS concentrate of about 1250 $mg.L^{-1}$ equivalent glucose.

The amount of polysaccharide in the EPS concentrate was determined using the phenol- sulfuric acid protocol based on Felz et al. (2019) and Dubois et al. (1956). Briefly, 400 µL of a sample were pipetted into a cuvette (Brand™, 759115), 10 µL of 80% phenol (Sigma Aldrich, 33517-100G) were added, followed by 1 mL of concentrated sulfuric acid (95-98%, Sigma-Aldrich, 258105). The cuvette was left 10 min at room temperature, then 10 minutes in the water bath at 25°C. The absorbance was read at 490 nm on a UV-Vis spectrophotometer (UV-2550, Shimadzu) instrument. Glucose (Acros Organics, 410955000) diluted in MilliQ water to concentration between0.1 and 100 $mg.L^{-1}$were used as a calibration curve to allow an approximate quantification.

3.2.4 Chlorination experiments

20 mL beakers were soaked overnight in a 200 mg.L^{-1}sodium hypochlorite solution (Reactolab SA, Vaud, Switzerland) to remove chlorine demand. A working solution of sodium hypochlorite at a concentration between 2 and 4 mg.L^{-1} free chlorine was prepared in 1mM phosphate buffer. Before the experiment, the beakers were rinsed twice with MilliQ water and once with the working solution. They were then filled with 12.5 mL of the working solution and spiked with an enterovirus solution containing all eight genotypes to a concentration of approximately 1.1x10^8 MPN.mL^{-1} per genotype. Two different enterovirus solutions were used for spiking: one solution consisted only of viruses in phosphate-buffered saline (PBS; pH 7.4, Gibco™, 18912014, 10 mM Na3PO4, 2.68 mM KCl, 140 mM NaCl) and the second solution contained viruses in 1:1 PBS:EPS concentrate (pH adjusted to 7.4). This latter matrix served to simulate the effect of activated sludge-derived EPS on the downstream chlorination process. Both PBS-free and PBS-containing enterovirus solutions were prepared in in triplicate, were incubated for 2 hours at room temperature, and were then spiked into separate reactors containing the chlorine working solution. Aliquots of 500 µL were taken after 10, 30, 45, 70 and 90 seconds and collected in a tube containing 5 µL of 5000 mg.L^{-1} sodium thiosulfate (Sigma-Aldrich, Germany) to quench residual chlorine. The free chlorine in the reactor was measured prior and at the end of the experiment to estimate the exposure, using DPD Free Chlorine Reagent, Swiftest™ and DR300 pocket colorimeter (Hach Company, Loveland, CO). The chlorine dose (CT; mg·min·L^{-1}) was calculated as the integral of free chlorine concentration over time of exposure, assuming a first-order decay of free chlorine concentration between the start and the end of the run.

3.2.5 Data analysis

All data analysis was performed with R version 4.0.0[140]. To determine the enterovirus decay rate constants in activated sludge, a segmented linear regression was fitted to ln-transformed decay curves of both solids and sludge using the *segmented* function from the segmented package in R, in order to determine the bending point of the curve. A linear regression was then fitted to the first segment, and the negative of the slope corresponded to the decay rate constant *k(solids)* or *k(supernatant) (h^{-1})* (Equation 3:1 and Equation 3:2).

$$\frac{C_t}{C_0} = e^{-k(supernatant).T}$$

Equation 3:1: virus decay kinetics in supernatant

$$\frac{C_t}{C_0} = e^{-k(solids).T}$$

Equation 3:2: virus decay kintetics in solids

A one-way ANCOVA (α=0.05) was run for supernatant and solids separately using the *aov* function, to determine if the values of *k* for the eight genotypes were significantly different. If a significant effect of genotype

was found, a post-hoc analysis (α=0.05) was then performed using the *TuckeyHSD* (α=0.05) function to determine which pairs of genotypes had significantly different values of *k*.

To determine chlorine inactivation rate constants *k(chlorine)*, we fitted a Chick-Watson model (Equation 3:3) to the ln-transformed decay curves of each genotype in EPS and PBS, assuming a pseudo first-order reaction of virus inactivation with respect to the disinfection dose.

$$\frac{C_t}{C_0} = e^{-k(chlorine)[HOCl].T}$$

Equation 3:3: virus inactivation kinetics for chlorination

Here C is the concentration of infectious virus at time T (min), C_0 is the concentration of infectious virus at T=0, k is the inactivation rate constant ($mg^{-1}{\cdot}min^{-1}{\cdot}L$) and [HOCl] is the free chlorine concentration ($mg.L^{-1}$). The model was fitted when at least two disinfectant doses resulted in quantifiable virus. To determine if the inactivation by free chlorine differed depending on the genotypes, a one-way ANOVA (α=0.05) was run, on each disinfection doses separately, using the *aov* function in R. It was then followed by a pairwise genotypes comparison using the *TukeyHSD* (α=0.05) function. To determine if the addition of EPS had a significant effect on the decay rates, an ANCOVA analysis (α=0.05) was performed using the *Anova* function from the car package in R with Type III sum of squares.

3.3 Results

3.3.1 Inactivation kinetics of different enterovirus genotypes in activated sludge reactors

The infectious concentration of each genotype was monitored in the supernatant and in the solid fractions of activated sludge (sludge 1) over 68 hours (Figure 3.2). In the supernatant, all genotypes decayed below the LOQ within 21 hours. In the solids, infectious viruses were still detected for all genotypes except CVA9 and E30 after 68 hours. Infectivity loss in the solid phase appeared to follow two phase kinetics, with a rapid initial decay, followed by a later, slower one. The initial phase (k values reported in Table 3:1) decay of the different genotypes were compared.

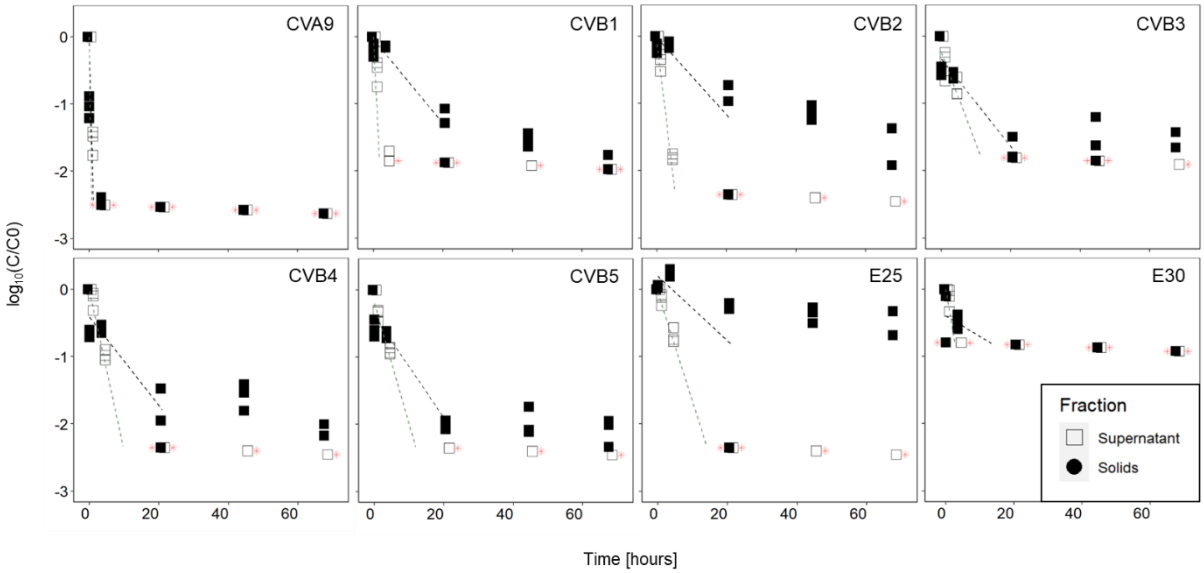


Figure 3.2: Inactivation curves of the different enterovirus genotypes in supernatant and solid fractions of sludge 1. Lines indicate linear regression curve on the first segment of the decay curve

The k values show that the infectivity reduction was faster in the supernatant than in the solids. In the supernatant, statistical analysis determined that the decay kinetics of the different genotypes varied ($p<0.001$) and post-hoc analysis determined that the decays of CVA9 and CVB2 were significantly different from all other genotypes (Table B.2). CVA9 was reduced the fastest, with a 1 $\log_{10}$ reduction in 30 min or less and CVB2 was also among the fastest reduced, with a 1 $\log_{10}$ reduction in less than 2.5 hours. Although CVB1 was reduced faster than CVB2, the difference was only found significantly different from CVA9 and E25 decay, probably due to the fact that only four data points were used to fit the linear regression of CVB1. In the solids, statistical analysis determined that the decay kinetics were not significantly different across genotypes ($p=0.38$), despite an important difference in k value between CVA9 and the rest of genotypes. This result is probably due to the fact that only four data points were used to fit the linear regression of CVA9 in solids.

Table 3:1: Decay rate constants (±95% confidence intervals) measured by ICC-RTqPCR in the supernatant and solid fractions of activated sludge (sludge 1), along with the calculated time to reach 1 log_{10} reduction in infectious virus titer.

Genotype	k(supernatant) (h^{-1})	+/- 95%CI	hours to reach 1 log_{10} reduction	k(solids) (h^{-1})	+/- 95%CI	hours to reach 1 log_{10} reduction
CVA9	-7.1	4.2	0.3	-4.8	3.7	0.5
CVB1	-2.4	4.3	0.9	-0.1	0.0	10
CVB2	-1.0	0.1	2.5	-0.1	0.1	10
CVB3	-0.3	0.3	10	-0.1	0.0	10
CVB4	-0.5	0.1	5	-0.2	0.1	10
CVB5	-0.4	0.2	5	-0.2	0.0	10
E25	-0.4	0.1	5	-0.1	0.1	10
E30	-0.7	3.7	3.3	-0.1	0.4	33.3

3.3.2 Variability in virus decay among different sludge samples

To determine if the extent of infectivity loss and the observed differences among genotypes were generalizable across sludge samples, we analyzed virus removal in two additional batches of sludge. Figure 3.3 shows the inactivation each genotype after 45 hours in the supernatant (Figure 3.3A) and solid fractions (Figure 3.3B) of all three activated sludge samples tested (sludge 1, sludge 2, sludge 3). In the supernatant (Figure 3.3A), the three sludges exhibited variability in the magnitude of infectivity loss, with most genotypes in sludges 1 and 2 decaying below the LOQ within 45 h. In contrast, most genotypes retained quantifiable infectious concentrations in the supernatant of sludge 3 and displayed a wide range of infectivity loss. CVA9 was readily removed from the supernatant (>2.8 log_{10}), while E30 was the most stable (<0.8 log_{10}). In contrast, in the supernatant of sludge 2, E25 was the least removed (<1.5 log_{10}) and was the only genotype that remained quantifiable after 45 h.

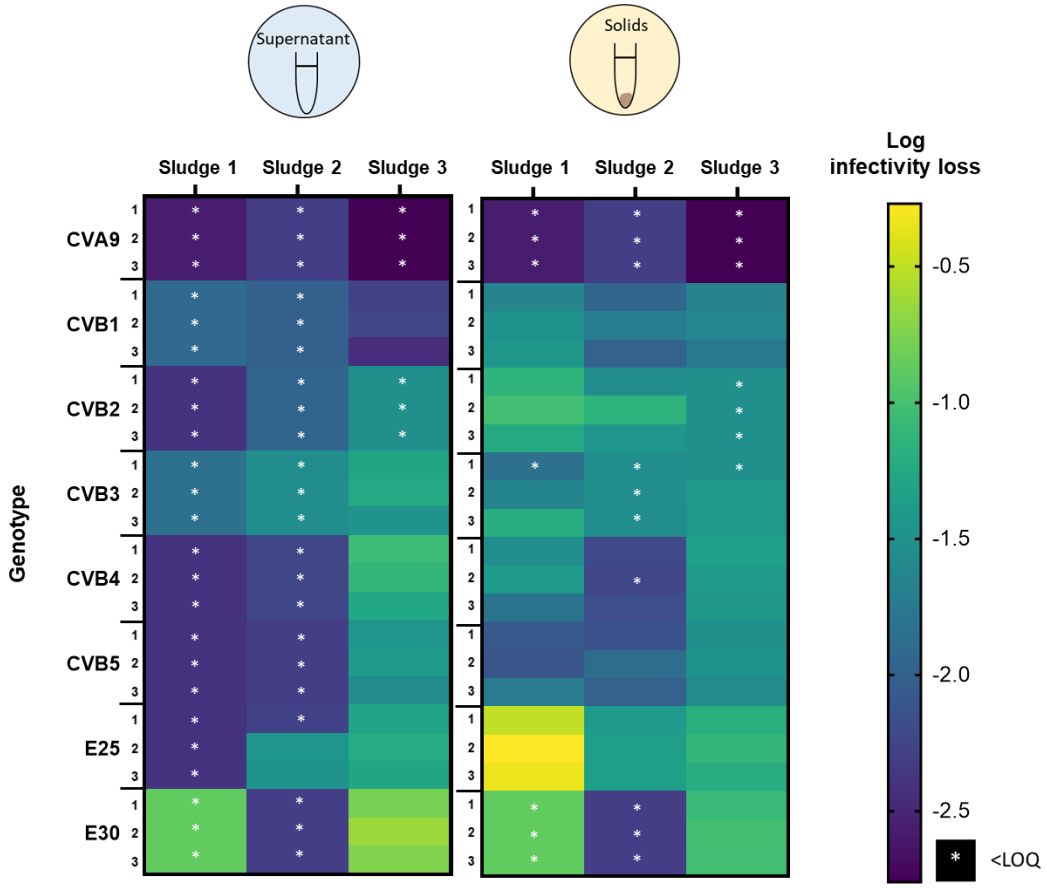


Figure 3.3: heat map of infectivity loss of each genotype in three different activated sludge sample in (A) supernatant and (B) solids fraction after 45 hours. Each combination of genotype and sludge was tested in three replicates. White stars indicate that samples at 45h were below the LOQ; the extent of inactivation was therefore determined based on the LOQ and hence indicates the minimal extent of inactivation in a sample.

In the solid fraction (Figure 3.3B), infectious concentrations of most genotypes were still measurable after 45 hours. However, there was variability in the range of infectivity loss between genotypes, depending on the sludge. Virus decay in the solid fraction ranged from 0.3 to >2.6 $\log_{10}$ in sludge 1, from 1.2 to >2.3 $\log_{10}$ in sludge 2 and from 0.6 $\log_{10}$ to >2.8 $\log_{10}$ in sludge 3. CVA9 was readily removed in the solids of all sludges, while E25 was among the least removed. CVB5 was consistently well removed compared to the other genotypes (except CVA9), whereas the other genotypes exhibited variable relative removal across the three sludge samples. For example, E30 was the least removed in sludge 3, but was among the most removed in sludge 2.

3.3.3 Mechanism of virus removal: adsorption versus biological activity

To determine the mechanism by which activated sludge leads to virus decay, we next investigated the role of adsorption and microbial activity. To this end, virus removal was measured in a regular activated sludge

reactor (control), in the supernatant of heat-sterilized activated sludge (sterilized) and in the liquid phase after decanting of the solids (decanted), as shown in Figure 3.1. In the sterilized reactor, biological activity was likely reduced, but a solid fraction was maintained as a site for virus adsorption. (Note that heat sterilization did not alter the adsorption capacity of the solid fraction compared to the regular activated sludge; see Figure B.5). In the decanted reactor, some microbial activity was likely maintained, while the solid fraction and hence the possibility for adsorption was eliminated.

Figure 3.4 illustrates the infectivity loss and reduction in genome copies of each genotype in the supernatant fraction after 45 hours in the different reactors. In the control reactor, the infectious concentration of most genotypes decayed below the LOQ, corresponding to at least 2 $\log_{10}$ loss in infectious titer (Figure 3.4A). Simultaneously, a loss in genome copies of up to > 4 $\log_{10}$ was observed (Figure 3.4B).

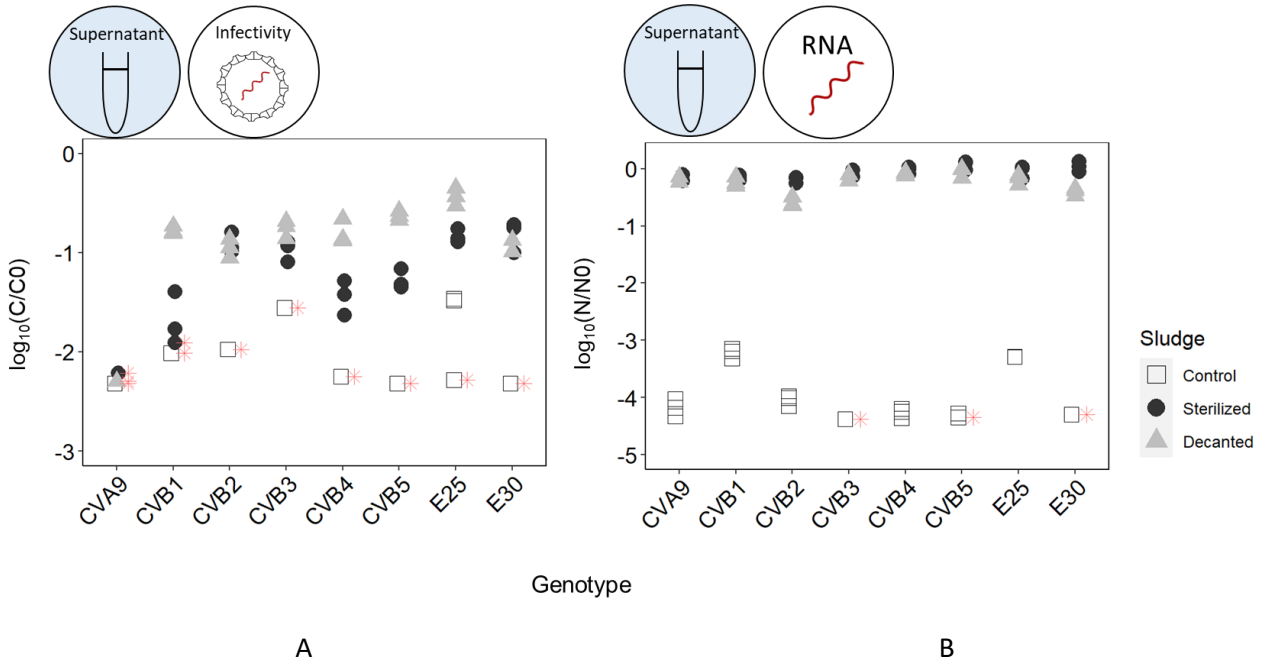


Figure 3.4: (A) infectivity loss (C/C0) and (B) reduction in genome copies (N/N0) of each enterovirus genotype in the supernatant of activated sludge (white squares), heat-sterilized activated sludge (black dots), and decanted sludge without solids (grey triangles) after 45 hours.

When the sludge was sterilized, virus decay for all genotypes except CVA9 was greatly reduced compared to the control (Figure 3.4A). Abatement of microbial activity by sterilization thus led to reduced virus decay. However, a residual decay ranging from 0.7 $\log_{10}$ for E30 to more than 2.2 $\log_{10}$ for CVA9 was nevertheless observed. This residual decay could not be explained by adsorption of viruses to sludge, because qPCR analysis revealed that the genome copies of all genotypes were almost fully recovered after 45 h (Figure 3.4B). Instead, the observed infectivity loss in heat sterilized sludge musts thus result from residual microbial or

enzymatic activity despite heating, or from chemical inactivation by solution components of activated sludge supernatant.

A reduction in virus decay was also observed when solids were removed from the reactor. The infectivity loss observed in the decanted liquid was similar to, or slightly lower than, that observed in the supernatant of the sterilized sludge (Figure 3.4A). This may indicate that insufficient microbial activity was retained in the absence of solids to cause inactivation, and that the observed loss in virus titer mainly stemmed from chemical inactivation. Similar to the heat-sterilized sludge, no genome degradation was observed in the decanted solution (Figure 3.4B).

3.3.4 Effect of protease inhibitors on virus infectivity loss

In order to further evaluate the role of microbial activity in virus inactivation, we compared the fate of our enterovirus population in an activated sludge supplemented with a mixture of protease inhibitors (PI) to a regular activated sludge (control). Proteases have previously been implicated as the antiviral agent in microbial virus inactivation, and their inhibition has been shown to reduce virus inactivation[44,150]. For the two echoviruses studied, the addition of PI reduced slightly the infectious virus loss and to a greater extent the loss in genome copies in the supernatant compared to the control reactor (Figure 3.5). However, the addition of PI resulted in an enhanced infectivity loss for the coxsackieviruses (Figure 3.5A), while reducing the loss in genome copies compared to the control reactor (Figure 3.5B). All coxsackievirus genotypes were inactivated below the LOQ in the PI supplemented sludge, whereas all but CVA9 and CVB2 remained quantifiable by ICC-RTqPCR in the control sludge. A different effect of PI was observed for RNA degradation. For both echoviruses and coxsackieviruses, the loss in viral RNA was reduced in the supernatant of the PI supplemented sludge compared to that observed in the control reactor (Figure 3.5B).

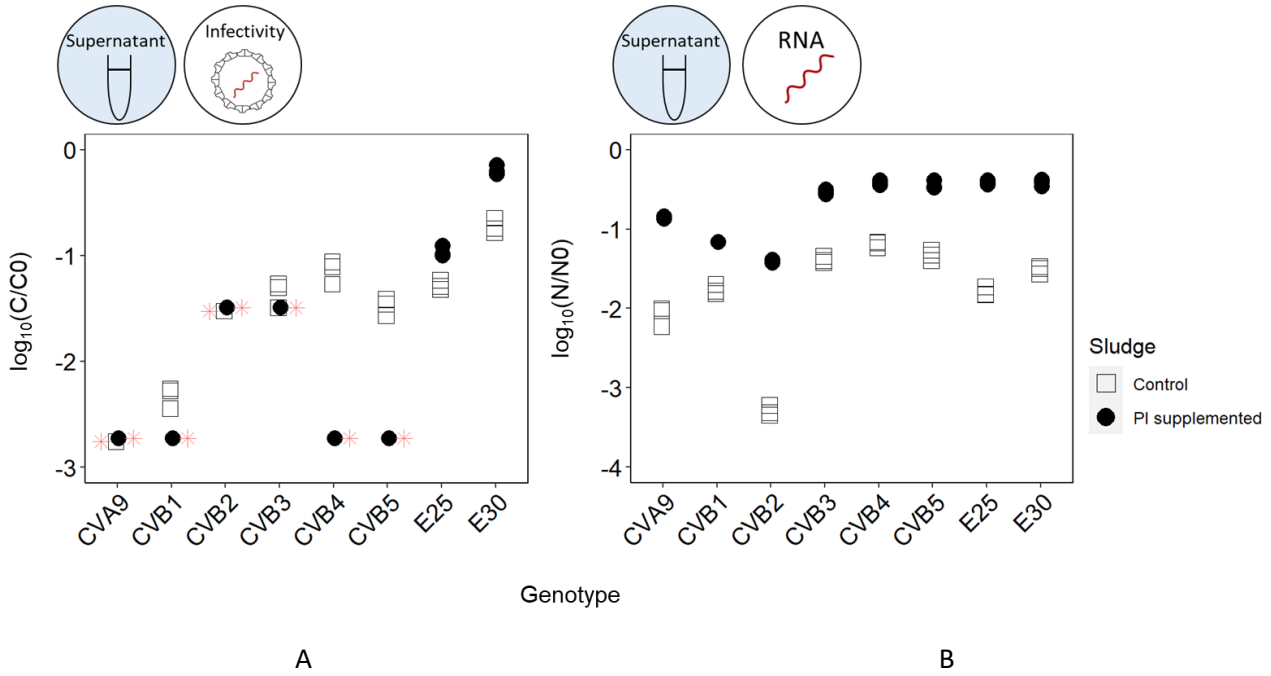


Figure 3.5: (A) infectivity loss and (B) genome copy reduction after 45 hours of each enterovirus genotype in the supernatant of activated sludge (white squares), or activated sludge supplemented with a protease inhibitors (PI) (black dots).

3.3.5 Inactivation of different enterovirus genotypes by chlorination

Finally, we assessed how chlorination affects the fate of different enterovirus genotypes. Exposure of the enterovirus population to chlorine resulted in a broad range of responses from the different genotypes (Figure 3.6, black dots,Table 3:2). At a CT of 0.3 mg.min.L^{-1}, E30 was inactivated below the LOQ with around 2 log10 inactivation, while CVB3 and CVB5 were inactivated by less than 0.5 log10. Statistical analysis determined that there were three groups of genotypes that had significantly different degrees of inactivation across groups but not within the group (Table B.3). CVB3, CVB5 and E25 were reduced the slowest; CVA9, CVB2 and CVB4 exhibited intermediate inactivation; and inactivation of E30 was most significantly faster than all the other genotypes. At a CT of 1 mg.min.L^{-1}, only CVB3 and CVB5 could still be quantified by ICC-RTqPCR with between 1.1 and 1.4 log10 inactivation, while all other genotypes were below the LOQ. CVB3 and CVB5 were both found to have significantly different inactivation than all other genotypes, except each other. It should be noted that the CVB3 primers are not fully selective and also measure CVB5, albeit at a much lower efficiency (Larive et al., 2021). We can therefore not exclude that the inactivation results for CVB3 were influenced by the presence of CVB5, in particular if CVB5 was present at a much higher infectious concentration.

Table 3:2: Log_{10} inactivation of each genotype for different free chlorine exposure.

	Log_{10} inactivation	
Genotype	CT = 0.3 mg.min.L^{-1}	CT = 1 mg.min.L^{-1}
CVA9	1.1	> 2
CVB2	1.3	> 2.2
CVB3	0.3	1.3
CVB4	1.1	> 2
CVB5	0.3	1.2
E25	0.6	> 1.8
E30	> 2	> 2

3.3.5.1 *Effect of EPS addition on chlorination kinetics*

To simulate the effect of activated sludge treatment prior to chlorination, we then evaluated the effect of virus exposure to activated sludge-derived EPS prior to chlorination. Only three genotypes (CVB3, CVB5 and E25) yielded quantifiable data by ICC-RTqPCR. For these three genotypes inactivation by chlorine was reduced in the presence of EPS, though the effect was not statistically significant for CVB3 (p-value = 0.05). Statistical analysis indicated a significant difference between PBS and EPS k values for CVB5 and E25, as shown in Table 3:3.

Table 3:3: Rate constants for the inactivation of enterovirus genotypes by chlorine, in the presence or absence of EPS supplemented matrix. Rate constants were determined based on Equation 3:3, and p values stem from an ANCOVA analysis to determine the effect of EPS addition on virus inactivation. The rate constants and comparison are provided for the genotypes for which at least two disinfectant doses resulted in quantifiable virus.

	PBS		EPS		
Genotype	k	Standard Error	k	Standard Error	P value AN-COVA
CVB3	-3.1	0.1	-2.2	0.3	0.08
CVB5	-2.7	0.1	-2.0	0.2	0.05
E25	-4.1	0.4	-2.3	0.3	<0.01

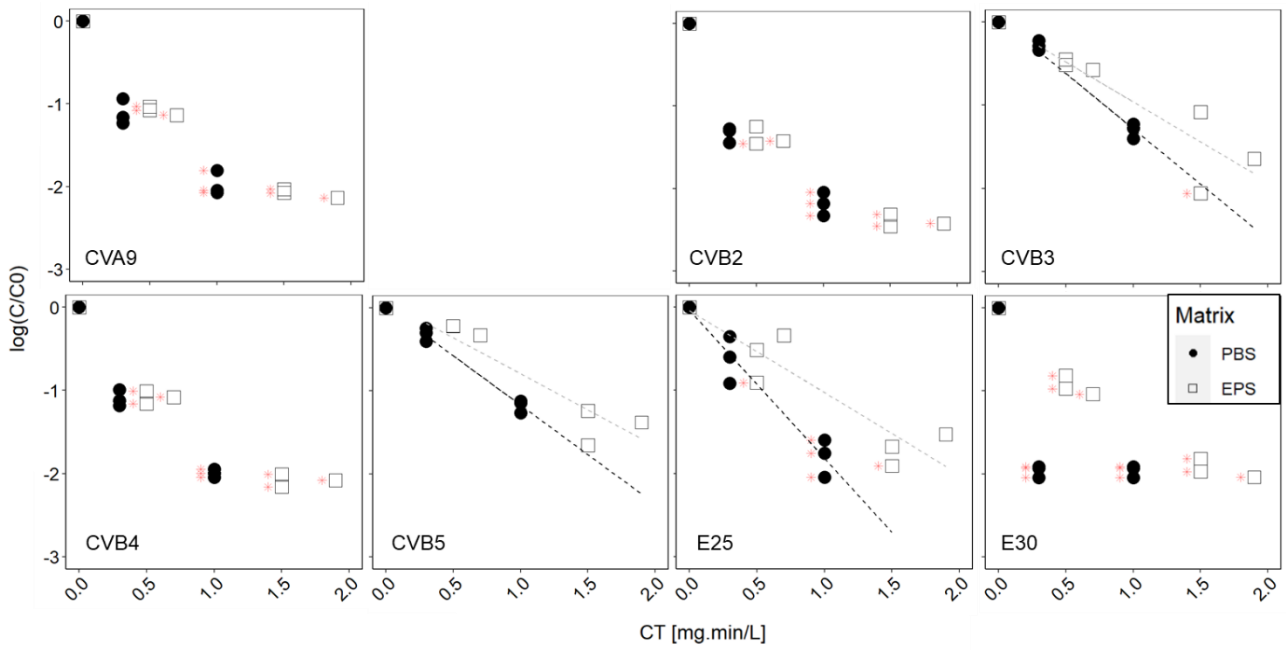


Figure 3.6: Inactivation kinetics of each enterovirus genotype exposed to free chlorine with and without pre-exposure to activated sludge-derived EPS. Grey dotted lines indicate linear regression for the EPS-exposed enteroviruses, while black dotted lines indicate linear regression for the PBS only-exposed enteroviruses. Note that CVB1 did not yield measurable results in this experiment.

3.4 Discussion

In this work, we investigated the effect of activated sludge treatment and chlorination on eight commonly encountered enterovirus genotypes. Inactivation occurred both in the supernatant and in the solid fraction, and the relative degree of inactivation in each fraction was dependent on the activated sludge sample (Figure B.4). In all sludge samples tested, most enteroviruses retained infectivity for 45 hours (Figure 3.3), suggesting that solids may be a source of infectious virus, either during solids treatment or by resuspension into the supernatant. The overall extent of infectivity loss is sludge dependent (Figure 3.3), as is the difference in decay observed among genotypes. Nevertheless, some consistent patterns can be identified. Sludge treatment will likely lead to a depletion of the enterovirus population in CVA9, CVB1 and CVB2 which were removed rapidly in the supernatant of both sludge 1 and 3, and were also removed below the LOQ in sludge 2 (Figure 2 and Table 1). Furthermore, the enterovirus population will be enriched in E25, since it was among the least removed in sludges 1 and 2, and relatively stable in sludge 3. CVB3, CVB4 and CVB5 were also among the most stable in sludge 1 (Table 1) and sludge 3 (Figure 2), though their relative stability could not be assessed in sludge 2.

The variability in virus decay among the three sludge samples is likely linked to differences in sludge characteristics, though it is not clear which sludge parameter drives the observed differences. For all sludge samples

used, the TSS were within the lower range of typical activated sludge reactors (Table B.1). Counter-intuitively, the highest TSS concentration (sludge sample 3) in this study was associated with a low overall virus decay in the supernatant (Figure 3.3). In terms of microbial processes, sludges 1 and 2 appeared to display nitrification (Figure B.3), while sludge sample 3 did not. However, it is not clear whether nitrification activity has an impact on virus stability. Hewitt et al.[62] compared sewage treatment plants with different biological treatment steps including nitrification, and found no significant effect of the treatment process on the extent of enterovirus removal. Furthermore, Bischel et al.[153] found that nitrification of urine did not result in inactivation of virus surrogates. The quality and age of the sludge may nevertheless be of importance, as these parameters influence the population composition of the microorganisms present, which in turn may affect virus decay. For example, Kim and Unno[91] found that the flocculation ability of bacteria influenced the inactivation of poliovirus. This could imply that the nature of the bacteria is crucial to the inactivation of the viruses. Floc-forming ability is linked to secretion of EPS, which has been found to be a place of high concentration of hydrolytic enzymes[93–95], and may thus enhance virus inactivation. Haun et al.[86] found that hydraulic retention time and sludge retention time had an effect on virus removal, but their model estimated that adsorption is the main removal mechanism. Similarly, Naughton and Rousselot[82] also showed that a higher sludge retention time is linked to a greater virus removal. The hydraulic retention time in our experimental setup was higher than in a regular activated sludge reactor, where it is around 4-24 hours[154], with continuous feeding of the sludge. Therefore, the extent of inactivation in a real activated sludge reactor may be expected to be lower than that observed in this study. It is a limitation of this study that we only have three sludges, ideally we would have many more to better generalize the findings. However, this falls beyond the scope of this study.

Our data shows that inactivation mediated by microbial activity in the sludge is an important cause of viral infectivity loss in the supernatant. Removing microbial activity by sterilizing the sludge led to a higher virus stability. Virus stability was also enhanced if microbially active solids were decanted. Efficient virus inactivation by activated sludge thus necessitates the presence of a microbially active solid fraction. However, virus loss was also observed in the absence of biologically active solids, albeit to a lesser extent. Removal of viruses from the liquid by irreversible adsorption to the solids is a negligible source of infectivity loss, since heat sterilization of the sludge resulted in a full recovery of the genome copy number, whereas virus infectivity was only partially retained. Viruses were therefore still inactivated (as opposed to physically removed) in the supernatant when the sludge was heat sterilized. A similar extent of inactivation was also observed in decanted sludge. Assuming that heat sterilization of the sludge was complete and microbial activity in the decanted was minimal, this indicates that inactivation in the absence of microbially active solids is mainly chemically mediated. This assumption is also supported by a shift in inactivation mechanism: in microbially active sludge, virus loss is accompanied by extensive loss in viral RNA (Figure 3.4), indicating that both the viral

capsid and genome were readily degraded. In contrast, in the absence of microbially active solids, the number of genome copies remained constant, consistent with an inactivation mechanism that mainly targets the viral capsid while protecting the RNA from complete degradation. These mechanisms remain to be confirmed with other sludges and at lower concentrations but this would be beyond of the scope of this study.

Adsorption onto the solids has previously been described as one of the main sources of virus removal in activated sludge[85,86]. Here we show that in our system, adsorption is a negligible process in virus infectivity loss. While adsorption onto the solid fraction of the sludge did occur, and while the concentration of enteroviruses on the solid fraction can be important, adsorption did not explain the extent of infectivity loss in the supernatant. Our findings are consistent with other studies which have found that adsorption was not the main virus removal mechanism during activated sludge treatment[87,88]. However, in contrast to our findings, Kelly and al.[87], Knowlton and Ward[90] and Kim and Unno[91] found no inactivation in the supernatant after removal of solids, indicating an absence of chemical inactivation in their experimental system. Nevertheless, these authors reach the same conclusion as this work that virus infectivity loss mainly occurs in the presence of microbially active solids. Specifically, they conclude that inactivation is due to microbial activity, and that antiviral compounds are either short-lived or active only when associated with microorganisms[90] and Ward, 1986). Knowlton and Ward[90] furthermore found that untreated mixed liquor suspended solids (MLSS) caused inactivation of PV1 and release of its RNA, which is consistent with our observations that enterovirus inactivation extended to RNA degradation.

The addition of a protease inhibitor slightly reduced the inactivation of echoviruses, but contrary to our expectations, enhanced that of coxsackieviruses (Figure 3.5). Addition of protease inhibitors was thus likely more complex than simply reducing the overall protease activity in the sludge. For example, the addition of PI contributed to an increase of about 120 mgC.L^{-1} and 20 mgN.L^{-1}in the reactor, which was followed by a TOC of about 150 mg.L^{-1} while TN was reduced byabout 30 mg.L^{-1}. This indicates that the protease inhibitors may have served as a source of carbon and nitrogen, and may have resulted in increased microbial activity and thus viral inactivation. Nevertheless, this does not explain the differences observed between echoviruses and coxsackieviruses, nor does it explain why PIs also protected the viral genome (Figure 3.5). This differential effect on coxsackievirus and echovirus remains to be better understood.

Similar to activated sludge treatment, we found that enterovirus genotypes exhibited a wide range of susceptibilities to free chlorine disinfection. Among the genotypes studied, E30 was the most chlorine susceptible, and CVB5 and CVB3 the most stable. Torii and al.[75], who compared the chlorine sensitivities of CVB3, CVB4 and CVB5 also found CVB5 to be the most chlorine resistant, and Meister et al.[73] found that among environmental strains of CVB, CVB5 and CVB1 were more resistant to free chlorine than CVB4.

We furthermore found that the presence of EPS was protective against chlorine treatment for CVB3, CVB5, and E25, although not significantly so for CVB3. This protection was provided by polysaccharide extracted from wet sludge at a concentration of 625 mg.L^{-1} glucose equivalent. For the other genotypes tested, inactivation in the presence of EPS remained sufficiently rapid that they readily decayed below the LOQ, and we could therefore not determine if there was a protective effect. Waldmann et al.[99] found substantial protection of E30 from chlorination provided by commercial lipopolysaccharides and peptidoglycans at a concentration of 1000 mg.L^{-1}. Our experiment was in the same concentration range, but with no control over the composition of the polysaccharides, and possible uncertainty in the polysaccharide concentration estimation[155]. The protection was less pronounced than that observed by Waldmann et al.[99], though the EPS used herein is likely more representative of the composition and concentration of EPS in sewage. Some protection to chlorination may be provided by attachment of enteroviruses to polysaccharides from the activated sludge, and consequently higher chlorine doses may be required to achieve the targeted extent of inactivation. Finally, the extent of protection will likely differ between, as shown by Waldmann et al.[99], and as we see here with E25 that seems to get more protection than CVB5 and CVB3, though it would require further investigation to confirm it. This might depend on the attachment of the different genotypes to the polysaccharides, as EV-A71 has been shown to attach to heparin sulfate for example[156].

Taken together, our results demonstrate that not only the enterovirus load, but also the population composition will change in a sewage treatment train that includes activated sludge and chlorination. This is illustrated in Figure 3.7, which visualizes how these treatment processes affect the relative genotype abundance in a hypothetical enterovirus population with equal starting concentrations. Hereby, we assume a hydraulic residence time of 6 hours in the sludge with the decay rates observed in sludge 1 supernatant (k(supernatant)), and a chlorine residual of 0.3 mg.min.L^{-1}. It is evident that CVA9 and CVB1 were readily depleted in activated sludge, while E25 is enriched. CVB3, CVB4 and CVB5, which have medium sensitivity to activated sludge treatment, also remain prevalent in the effluent. The prevalence of CVB3, CVB5 and E25 in the effluent was then further enhanced during chemical disinfection, due to their relative resistance to chlorine treatment, in particular in the presence of protective EPS. The range of genotypes is another limitation of this study. When the method was developed, some genotypes prevalent in sewage had to be discarded for technical reasons[152].Furthermore, these genotypes were selected based on cell culture studies and might exclude relevant non-culturable genotypes. Genotypes associated with outbreaks of severe diseases (e. g., EV-D68) might also be interesting to consider in the future, since although they are only sporadicly detected in the population, their fate through the sewage treatment may be relevant if they can cause later infections.

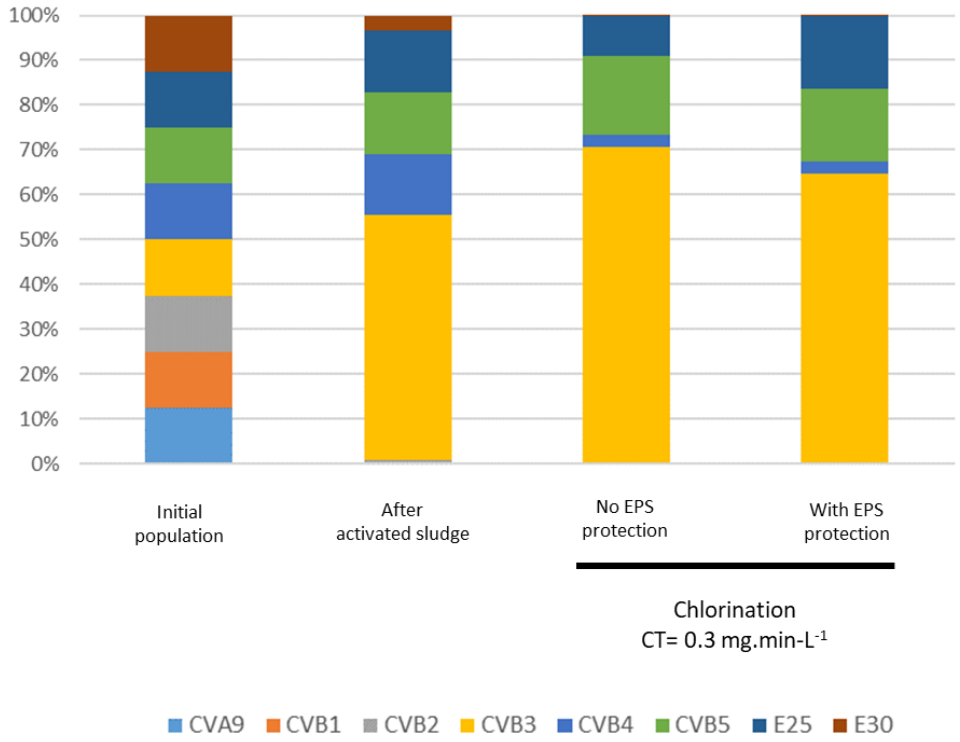


Figure 3.7: Simulation of the change in composition of an enterovirus population subjected to activated sludge treatment followed by chlorination. The initial population is assumed to consist of all eight genotypes studied at equal concentrations. Inactivation rate constants from the supernatant of sludge 1 (Table 3:1) were used to estimate the decay in infectious virus concentrations after 6 hours exposure to the sludge. Inactivation values listed in Table 3:2 were used to estimate titer reductions during chlorination. Since no data was collected for inactivation of CVB1 by free chlorine, a medium value was used. To evaluate the protection provided by EPS, inactivation at 0.3 and 1 mg.min.L^{-1} were estimated for CVB3, CVB5 and E25 based on the k values in EPS matrix (Table 3:3).

3.5 Conclusion

We have investigated the decay of eight ubiquitous enterovirus genotypes during exposure to three different activated sludge samples, and found that microbial inactivation is the main driver of infectivity loss. To a lesser degree, viruses also underwent chemical inactivation, while adsorption to the solids was found to be a negligible contributor to virus removal. Nevertheless, adsorption to sludge does occur and could cause the solids to be a potential source of infectious viruses. The extent of inactivation varied among genotypes and activated sludge samples. Overall, our results suggest that effluent of activated sludge will be depleted in CVA9 and CVB1, while E25 will be prevalent, along with CVB3, CVB4 and CVB5. CVB5 along with CVB3 were less susceptible to chlorination compared to the other genotypes, such that they are further enriched in the final effluent. Finally, E25 and CVB5 were found to gain protection against chlorination from activated sludge-

derived EPS, suggesting that activated sludge treatment prior to chlorination reduces the disinfection outcome for these viruses. When considering the whole sewage treatment process, CVB3 and E25 may be interesting conservative indicators in addition to CVB5 that has been considered as a conservative indicator genotype to monitor treatment success.

Chapter 4 Effect of lake exposure on a population of enteroviruses

Odile Larivé designed the experimental plan, performed most of the experiments, analyzed the data and wrote the chapter. Htet Kyi Wynn designed the mooring setup, participated to the design and testing of the experimental chamber and to the field experiments.

4.1 Introduction

Enteroviruses are commonly circulating human viruses that comprise 81 genotypes. Enteroviruses reproduce in the gastrointestinal and are therefore shed into the sewage. They reach the environment through discharge of treated or untreated sewage, or by direct contamination of the water with feces. Enteroviruses have been found to retain their infectivity for weeks in groundwater or seawater[19], and have been detected in river, sea and bathing water[20,21,34], in tap water[22] or chlorinated water[23]. Detection of enterovirus in recreational water and drinking water, combined with their long persistence, also strongly suggest the possibility of contamination through bathing or water consumption. It can be difficult to establish a direct causality between water use and enterovirus infection, since the water sample is often no longer available when the symptoms arise. Nevertheless, some enterovirus outbreaks have been linked to bathing, especially in young children[4,5,24].

An important determinant modulating the infection risk arising from recreational water use is enterovirus persistence[157]. A number of studies have evaluated the persistence of enteroviruses in natural water samples including seawater, lake, stream and groundwater[36–39,41,49,150]. Persistence of enterovirus in the environment can be influenced by a number of factors including temperature, biological activity and adsorption to solids, though these factors have all been shown to affect different enterovirus genotypes to varying extents. Increasing temperature result in faster inactivation[37,41,51], and Lo et al.[37] showed that thermal inactivation varies with genotypes at 25 °C in sterile water. Several studies have demonstrated a reduction of virus persistence in the presence of microorganisms[42,43], which could be attributed to the action of both protists and bacteria[41] . For both organism types, significant differences were found in their effect on different enterovirus genotypes[97,150]. Furthermore, proteolytic enzymes were identified as antiviral metabolites excreted by microorganisms, and were again found to inactivate different genotypes at different rates[44,150]. Finally, several studies have shown that enteroviruses remained infectious longer in natural waters when adsorbed onto solids[48,49], and that the increase in survival provided by the presence of sediment differed among genotypes tested.

Due to the diversity of environments, weather conditions and genotypes included in these studies, it is difficult to predict the inactivation of an enteroviruses population at any given site. A meta-analysis by Boehm et al.[40] identified a range of inactivation rate constants of enteroviruses in natural waters. However, the inactivation studies included in the analysis typically focused on poliovirus, whereas non-polio enteroviruses were less represented. Furthermore, most of the studies evaluating the persistence of enteroviruses in the environment were performed in the laboratory using batch reactors filled with natural water. This experimental

approach, however, has been shown to underestimate virus inactivation rates compared to those in the environment[37,158].

Understanding how different enterovirus genotypes are affected by exposure to the environment is important to assess the environmental persistence of the enterovirus population as a whole. The goal of this study was therefore to examine the inactivation of different non-polio enterovirus genotypes *in-situ* in lake water. Our study site was Lake Geneva, which is an important source of drinking water and site of recreational activity for close to 800'000 inhabitants, and also serves as the receptacle of several wastewater treatment plant effluents in the region. We selected eight enterovirus genotypes typically found in sewage, and measured their persistence during different seasons and at different depths in the lake.

4.2 Materials and Methods

4.2.1 Environmental chambers

4.2.1.1 *Design*

In order to measure the inactivation of enteroviruses *in-situ*, an environmental chamber was custom-designed on the model of the one used by La Belle and Gerba[49]. This tool allowed to expose viruses safely to the lake environment, while being contained. The chamber was made up of a central PVC cylinder with a sampling port, and was closed on both sides by polycarbonate membranes with a pore size of 15 nm (Whatman Nuclepore WHA111101) sealed with silicone grease (Dow Corning, Z273554-1EA), allowing to retain the viruses in the chamber while smaller molecules can go through, and thus recreating the lake environment in the chamber (Figure 4.1). The membranes were kept in place by a rubber gasket and a side piece the rubber gasket was embedded in. The membranes were protected by a stainless steel mesh, to prevent tearing by debris or animals, and the chamber was held together by screws on each side of the chamber, screwed with equal force using a torque wrench. The dimensions of the chamber were conditioned by the dimensions of the membrane chosen (47 mm) and by the desired volume of the chamber (25 mL). The inner lumen of the central chamber was 3.3 cm and the depth of the chamber is 3 cm. PVC was chosen for its resistance in water with low temperature, and resistance to bleach cleaning and solvents.

1 – PVC central chamber with stainless steel sampling port and cap
2 – polycarbonate membrane
3 – rubber gasket ~2mm thick
4 – PVC side piece
5 – stainless steel mesh
6 – stainless steel screw
7 – stainless steel ring

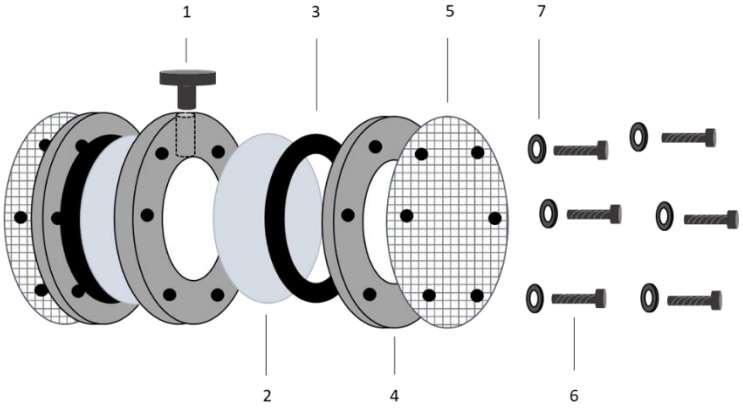


Figure 4.1: Schematic of the environmental chamber constituents and picture of the environmental chamber.

4.2.1.2 *Testing of the environmental chamber*

4.2.1.2.1 Virus containment

To test the water tightness of the chamber, a solution of 20 mL containing $2x10^7$ infectious unit per mL (MPN.mL^{-1}) of coxsackievirus B5 (CVB5) in PBS (Gibco™, 18912014, 10 mM Na3PO4, 2.68 mM KCl, 140 mM NaCl) was placed in the chamber and the chamber was sealed. The same solution was introduced in a sealed falcon tube and placed on the lab bench as a control for natural inactivation of the virus. The chamber was placed in a continuously stirred beaker containing 2 L of PBS. The infectious concentration and the genome copies of CVB5 in the chamber, in the beaker and in the control tube were monitored for 90 hours by end-point-dilution and reverse transcription (RT)-qPCR (see section 2.2.). The test was repeated in a pressure chamber (Drifton, DRIFTON 50-DY) containing 1.4 L of PBS, at a pressure of 2 bar over atmospheric pressure, to mimic the pressure at 20 m under the water.

4.2.1.2.2 Diffusion through the membranes

To estimate the exchange of (bio)chemical substances between the chamber and the environment, the diffusion of methylene blue through the membranes was evaluated. 20 mL of a solution of 20 mg.L^{-1} methylene blue (Sigma-Aldrich, M9140-25G) in tap water was introduced in the chamber, and the chamber was placed in a continuously stirred beaker containing 2 L of tap water. The concentration of methylene blue in the chamber and in the beaker was monitored by measuring the absorbance at 659 nm for a maximum of 22 hours, and the concentration was determined with a calibration curve from 0.1 to 20 mg.L^{-1}. In other experiments, 20 mg.L^{-1} methylene blue in tap water was introduced in a continuously stirred beaker, while the

chamber was filled with tap water and immersed in the beaker and the concentration of methylene blue in the chamber monitored over time.

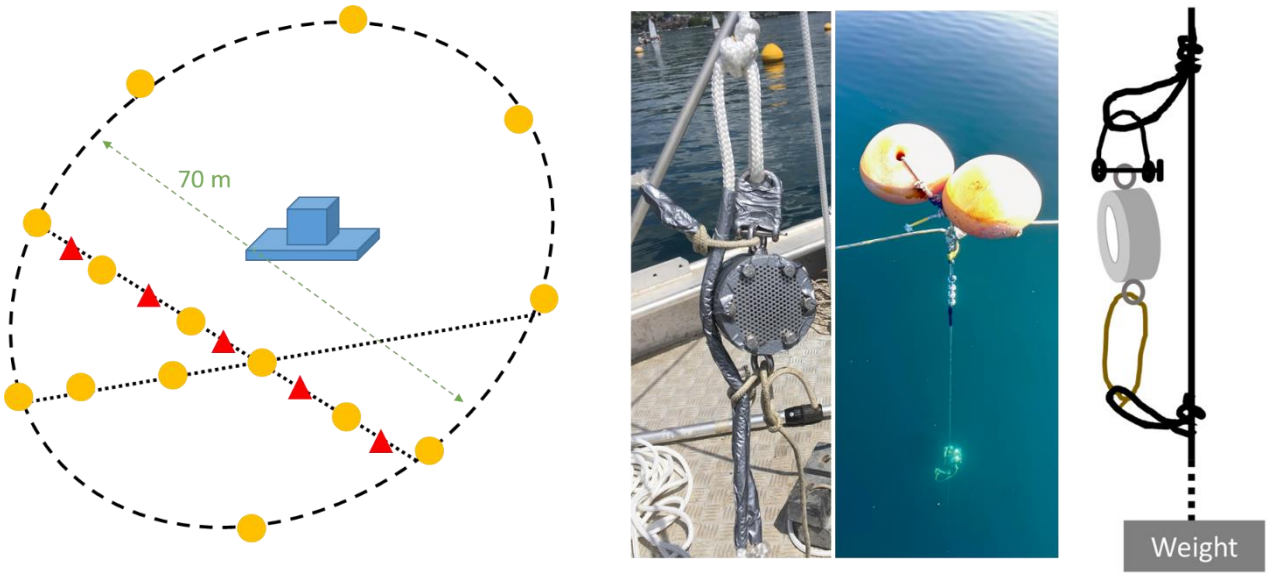
In addition, in the scope of testing the resistance of the chamber in the lake (see below), diffusion tests were also done *in-situ*. The chamber was filled with 25 $mg.L^{-1}$ methylene blue in lake water, and was moored at 20 m depth for 5 days. The methylene blue concentration and the volume in the chamber were monitored at the beginning and at the end of the experiment.

The time to reach 90% diffusion of the methylene blue from inside to outside of the chamber or from outside to inside of the chamber was estimated by calculating the rate of diffusion for the time considered in the experiment, and extrapolating considering a constant rate over the whole experiment.

4.2.1.2.3 Setup and testing of the mooring site

The chambers were moored on the site of the LéXPLORE platform, a floating experimental platform in Lake Geneva (https://lexplore.info/fr/accueil/). The platform is anchored at 570 m from the shore and floats on top a water column of 110 m depth. The platform perimeter is delimited by buoys over a 70 m diameter (Figure 4.2A). Each chamber was moored at a buoy of the perimeter with ropes resisting up to 1600 kg, and weighted with 10 kg of lead or bricks, at least five m distant from the nearest chamber. The chambers were attached to the rope at a depth of 2 m or 15 m with metal shackles in two points to prevent losing the chambers, in a way that the tension of the weight does not apply on the chamber (see Figure 4.2D).

To test if the chambers could withstand being moored five days in the water, a chamber was filled with 25 $mg.L^{-1}$ methylene blue in lake water, and moored at 2 m or 20 m depth for 5 days. After 5 days, the chamber was collected and examined visually for potential damage to the chamber or to the membrane, and the changes in volume of liquid in the chamber and in methylene blue concentration were assessed.



A B C D

E

Figure 4.2: Field setup. (A) schematic of the mooring site of the chambers at the Lexplore platform, the yellow circles are the main buoys of the platform perimeters, and the red triangles are the buoys holding the membranes, attached to the chains of the perimeters between the main buoys. (B) protection of the chamber and of the rope before deployment, to avoid deterioration by friction. (C) Picture of a test chamber deployed at 2 m depth. This depth was selected for test purposes only. In experiments, depths of 6 and 15m were used. (D) attachment of the chamber to the rope to avoid tension due to the weight. (E) Picture of the LéXPLORE platform.

4.2.2 Virus preparation and enumeration

4.2.2.1 *Preparation of viral stock solutions*

Our enterovirus population was composed of eight genotypes, which are among the most frequently detected in sewage[152]. One environmental isolate was obtained for each: coxsackieviruses B4 and B5 (CVB4 and CVB5) were previously isolated from Lausanne sewage[73], and coxsackieviruses A9, B1, B2 and B3 (CVA9,

CVB1, CVB2, CVB3) and echoviruses 25 and 30 (E25 and E30) isolated from sewage were kindly provided by Soile Blomqvist and Carita Savolainen-Kopra (Finnish National Institute for Health and Welfare).

Buffalo Green Monkey Kidney (BGMK; kindly provided by Spiez Laboratory, Switzerland) cells were used for the propagation of coxsackieviruses while Rhabdomyosarcoma (RD; ATCC CCL-136) cells were used to propagate echoviruses. BGMK and RD cells were grown at 37 °C in 5% CO_2, on Minimum Essential Medium (MEM, Life Technologies) and Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) respectively, supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin (Life Technologies). The media for maintenance was prepared in the same way as the growth medium, but lowering the FBS content to 2%.

Viral stock solutions were propagated in confluent T-150 flasks (TPP™, 90150) inoculated with one genotype in maintenance media and incubated at 37 °C in 5% CO_2 until full cytopathic effect was observed. The flask then underwent three cycles of freeze-thawing, and the supernatant was collected, centrifuged for 10 min at 300 x*g* and filtered at 0.2 µm (Sarstedt, 83.1826.001). The stocks were aliquoted and stored at -20 °C. Concentrated stocks were prepared by concentrating the filtered supernatant 40- to 270-fold with Amicon centrifugal filters with a 100 kDa cutoff (Millipore, UFC9100). The concentrated stocks were aliquoted and stored at -80 °C.

The stocks and concentrated stocks were enumerated by end-point dilution on their respective cell line. Specifically, the stocks were serially diluted in ten-fold series and 100 µL of each dilution was inoculated in five replicates on confluent 96 well plates (CELLSTAR® Greiner Bio-One, 7.655 180). The cytopathic effect in the wells was observed after five to six days of incubation at 37 °C and 5% CO_2, and the Most Probable Number of infectious virus was calculated using the EPA MPN calculator [142].

4.2.2.2 *Virus enumeration*

Two different methods were applied to enumerate viruses in experimental samples containing multiple enterovirus genotypes: RT-qPCR was used to detect the total (infectious and inactivated) concentration of each genotype in a sample, and integrated cell culture (ICC)-RT-qPCR was used to enumerate the infectious concentration of each genotype.

4.2.2.2.1 RNA extractions

All RNA extractions were performed using the Maxwell® 16 Viral Total Nucleic Acid Purification kit (Promega) according to the manufacturer's instructions with the Maxwell® 16 Instrument, extracting 200 µL of sample and eluting in 50 µL of RNase-free water. Extracts were stored at -20 °C for a maximum of one month previous to analysis. qPCR inhibition was assessed by quantifying serial-dilution of some samples, and was found non-existent.

4.2.2.2.2 RT-qPCR

One-step RT-qPCR was performed using genotype-specific primers designed previously (Larive et al. 2021). RT-qPCR was performed on a Mic qPCR Cycler (Bio Molecular Systems) using the One Step SYBR® PrimeScript™ RT-PCR Kit (Takara). Each reaction (20 µL) contained 3 µL of template, 10 µL of 2x One Step SYBR RT-PCR Buffer III, 0.4 µL of TaKaRa Ex Taq HS (5 $U.ul^{-1}$), 0.4 µL of PrimeScript RT enzyme mix II, 0.5 µL of each primer at 10 µM, and 5.2 µL of water. Identical thermocycling conditions were used for all primers (RT at 42 °C for 5 min, 10 sec at 95 °C, followed by 40 cycles of 95 °C for 5 sec, 52 °C for 20 sec and 60 °C for 30 sec). Each sample was run once, and the Cq values were determined using the micPCR software (v2.10.0; Bio Molecular Systems).

For each genotype, DNA standards were purchased (GeneBlocks, Integrated DNA Technologies) and were diluted to create calibration curves ranging from 3.34 to $3.34x10^6$ genome copies (gc).μL^{-1}. A calibration curve for each genotype tested was included in each RT-qPCR run. The limit of quantification (LOQ) was determined for each primer pair in R, by applying a curve-fitting method developed by Klymus et al.[139] with a coefficient of variation (CV) threshold of 35% to the measurement of ten replicates of the three lower concentrations of the calibration curve. Samples that exhibited a RT-qPCR signal at a concentration below the LOQ were set at the LOQ, and data were excluded if they exhibited interfering peaks in the melt analysis. Extraction and qPCR controls (molecular grade water) were analyzed in each run and yielded negative results.

4.2.2.2.3 ICC-RTqPCR

ICC-RT-qPCR was performed according to a method described previously[152]. Briefly, 1 mL of sample in maintenance media was inoculated into the confluent well of a 6-well plate (CELLSTAR® Greiner Bio-One, 657160) in duplicate plates. One plate was immediately processed (t0 sample), while the other plate was placed at 37 °C and 5% CO_2 for 24 hours prior to processing (t24 sample). Each well was scraped and the entire content of the well was recovered and frozen at -20 °C. The frozen samples were stored for a maximum of 11 weeks and were then thawed, centrifuged for 10 min at 1000x*g* and the supernatant was collected. RNA was extracted from 200 µL of the t0 and t24 supernatants, the genome copy number in each supernatant was quantified by RT-qPCR, and the increase in genome copies over 24 hours (deltagc(24h)) was calculated. Deltagc(24h) is proportional to the infectious concentration of virus present in the sample, and calibration curves relating the deltagc(24h) to the infectious concentration have been previously established for each genotype used in this study[152]. The slope of these calibration curves was found to be reproducible, while the intercept varied depending on the age of the cells used. Here, we therefore used the calibration curve slopes determined previously, but determined the intercept for each ICC-RT-qPCR run by analyzing a sample of known infectious concentration in parallel to the samples.

4.2.3 Lake Experiments

4.2.3.1 *Biosafety approval*

To be able to perform the *in-situ* testing, a request was made to the Federal Office of Environment to allow experiments in Lake Geneva using human enteroviruses. The request was first submitted on the 12th of July 2021 and was granted on the 21st of December 2021 with the reference number BAFU-217.23-64634/7.

4.2.3.2 *Preparation of the samples and mooring*

Three inactivation experiments in Lake Geneva were performed: "winter" experiments lasted from March 1-3, 2022 (experiment 1) and from March 9-14, 2022 (experiment 2). The "spring" took place from June 15-20, 2022 (experiment 3). All eight enterovirus genotypes were mixed in a solution at equal concentrations 24 to 48 hours prior to the start of the experiment and were stored at 4 °C. One day before the experiment, lake water was collected at the platform, at the depth at which the chambers would be moored at. The lake water was taken back to the laboratory in a refrigerated container. An aliquot was heat sterilized for one hour at 65 °C. Both sterilized and biologically active water samples were stored at 4°C for a maximum of 24 hours before use. The day of the experiment, the enterovirus solution was spiked into the biologically active lake water to a final concentration of $8.2x10^4$, $1.1x10^5$, $2.5x10^5$ MPN.L^{-1} per genotype in experiments 1, 2 and 3 respectively. 20 mL of the virus solution in lake water were immediately filled into each chamber and the chambers were sealed and placed in a biosafety transport box on ice. They were then transported to the platform and were installed within five hours of being filled. Once at the mooring site, the chambers were taken out of their box, attached to a rope and immediately immersed into the lake. One rope held either one chamber at 15 m depth (experiments 1 and 2), or 1 chamber at 6 m depth and one at 15 m depth (experiment 3). The ropes were pulled out of the lake after 48 hours or five days, and the chambers were detached, placed in individual biosafety transport box on ice, and were brought back to the laboratory. The sample volume in each chamber was measured at the end of the experiment to evaluate the dilution due to the exchange with the lake environment through the membranes. Then the samples were frozen at -20 °C within 4 hours after collection. Duplicate or triplicate chambers were deployed for each combination of depth and exposure time.

PBS and sterile lake water (in experiments 2 and 3 only) were spiked at the same virus concentration as the chambers. Sealed reactors shielded against light containing the virus solution in PBS, sterile lake water and lake water were placed in the refrigerator at 4 ° for experiment 1 and 2, and in a temperature-regulated room at 20 °C for experiment 3. These reactors served as controls for thermal inactivation (PBS) and for thermal plus chemical inactivation (sterile lake water).

All lake and control samples were stored for a maximum of four months before being analyzed by ICC-RTqPCR or direct RT-qPRC.

4.2.3.3 *Monitoring of the lake parameters*

During the experiments the following lake parameters were monitored: temperature, conductivity, pH, dissolved oxygen, and turbidity. During experiment 1 and 2, these parameters were measured punctually on the day of the start of the experiment and on each day where a sample was pulled out of the lake, for the entire depth under the platform. In experiment 3, these parameters were measured automatically by a different device over the whole depth under the platform. The temperature in the winter (experiments 1 and 2) was around 7 °C at 15 m depth. In spring (experiment 3), it was 13 °C at 15 m depth and 19 °C at 6 m depth. The pH in experiments 1 and 2 was 7.8, and 8.5 and 8.7 at 15 and 6 m depths, respectively, in experiment 3.

The absorbance spectrum of the surface water was measured on a sample taken on the starting day of the experiment for experiments 1 and 3, and on the starting day and on each day that a sample was pulled out of the water for experiment 2. The absorbance in the UV range (280-320 nm) was always above 0.054 (Figure C.1), indicating that less than $8x10^{-13}$ of solar UVB light reached the mooring depth of the chambers.

4.2.4 Data analysis

Inactivation was measured as

$$inactivation = \log_{10}(\frac{C}{C_0})$$ *Equation 4:1 Calculation of viral inactivation*

where C denotes the virus concentration at a given exposure time to lake water, and C0 is the initial virus concentration in the chamber. Correspondingly to Larivé et al.[152], we consider inactivation equal or under 0.7 $\log_{10}$ as non-biologically relevant, since they are comparable to the variability observed for replicate samples with the ICC-RTqPCR method. Genotypes were considered to show inactivation when at least one of the chambers at a sampling point displayed an inactivation > 0, with a confidence interval including a difference superior to 0.7 $\log_{10}$, as determined by one-sample t-test. To evaluate the difference in inactivation at 15 m between winter and spring, a two tailed t-test was performed. A paired, two-tailed t-test was used to compare the spring inactivation at 6 m and 15 m depth. Differences in inactivation between spring and winter were considered significant if the t-test showed significance, and the 95% confidence interval included a difference superior to 0.7 log10. All statistical tests were performed in R[140] using the t.test function at a significance threshold of α=0.05, and the bartlett.test function to test for the equivalence of variance.

4.3 Results

4.3.1 Virus containment in environmental chambers

A solution containing $2x10^7$ MPN.mL^{-1} of CVB5 in PBS was placed in the chamber and the chamber and the chamber in a beaker or pressure tank filled with PBS. The infectious concentration and total genome copies

of CVB5 were monitored in the chamber, in the beaker or in the pressure tank, and in a control tube for 90 to 96 hours (Figure 4.3). No infectious virus was detected in the beaker or in the pressure tank (Figure 4.3A). At atmospheric pressure, genome copies were detected in the beaker after 43 hours and in one of the triplicates after 90 hours, though all but one measurement were < LOQ (Figure 4.3B). Under a 2 bar pressure, genome copies were detected in the three replicates after 26 hours and in two of the triplicates at 96 hours, though again all but one measurement were < LOQ (Figure 4.3D). What is measured is likely free RNA, since the virus RNA is smaller than the pores of the membranes and no infectious virus was detected in the beaker or in the pressure tank (Figure 4.3C).

The infectious concentration of CVB5 in the chamber remained stable over the course of the experiment. The infectious concentration of CVB5 in the control tube under atmospheric conditions did not vary (Figure 4.3A), while it decreased during the experiment with the pressure tank (Figure 4.3C), likely due to the exposure of the tube to a UV lamp. The genome copy number in both chamber and control tube remained constant during both experiments (Figure 4.3B and Figure 4.3D).

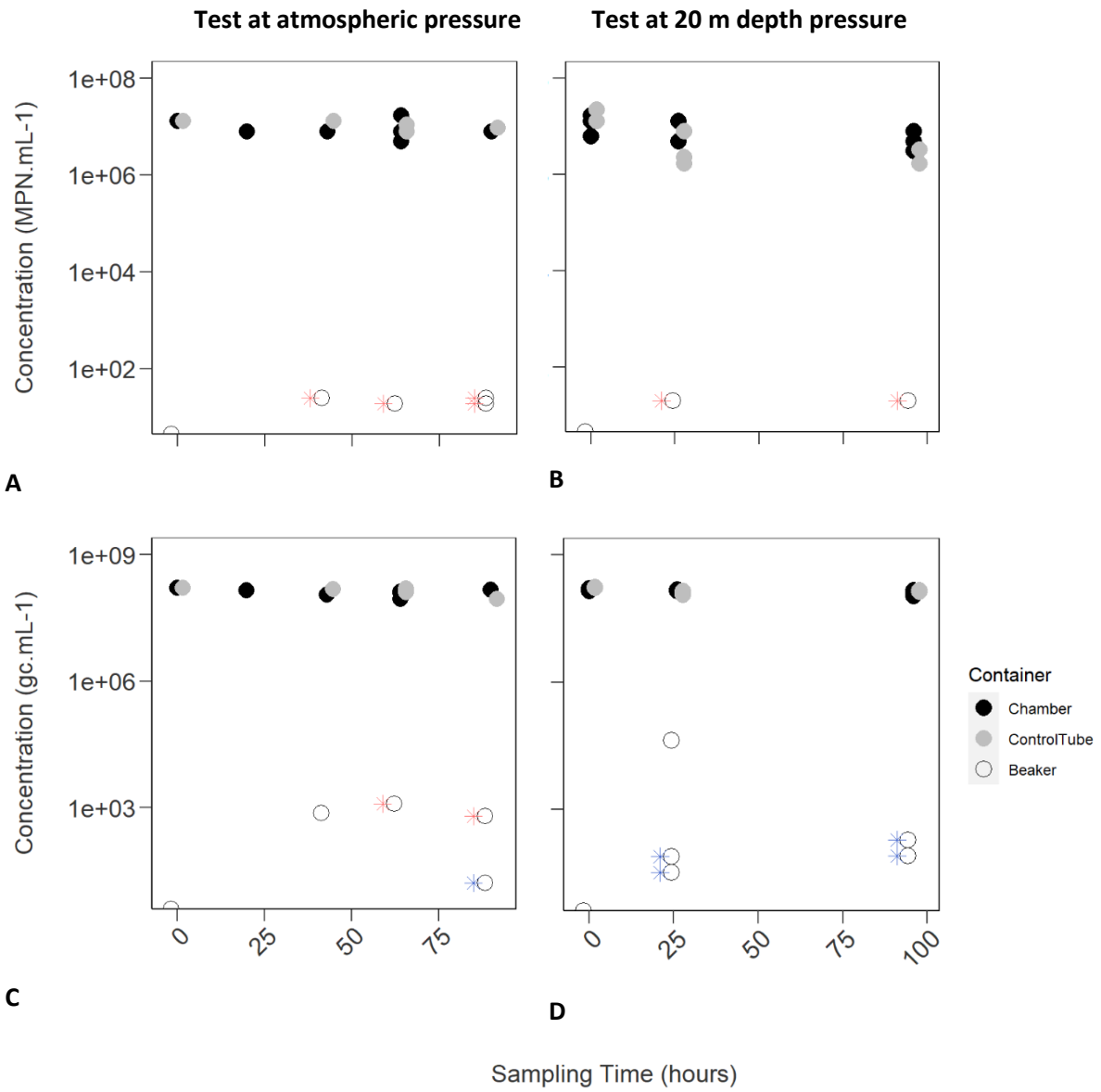


Figure 4.3: Test of CVB5 retention in the chamber at atmospheric pressure (A and C) or at a pressure corresponding to 20 m water depth (B and D). The figure shows the infectious CVB5 concentrations (A and B) and the concentration of genome copies (C and D) in the chamber, the control tube and in the beaker or pressure vessel. Red stars indicate that the virus was not detected, and the data point was arbitrarily set at LOQ. Blue stars indicate that the virus was detected but below the LOQ.

4.3.2 Diffusion through the membranes of the chamber

The diffusion of methylene blue from inside to the outside of the chamber and vice versa was measured, in order to estimate the exchanges between the chamber and the lake environment. The diffusion varied greatly form one experiment to the other, despite similar starting concentrations. In the laboratory, the time estimated for 90% of the methylene blue to diffuse to the outside of the chamber ranged from 27 hours to almost 13 days, while the time for diffusion of 90% of the methylene blue from the outside to the inside of

the chamber ranged from 20 hours to almost 5 days (Table 4:1). In the field, the time estimated for the diffusion of 90% of the methylene blue to the outside of the chamber ranged from 9 to 56 days, with the higher diffusion obtained when the chamber was at 20 m depth. The diffusion to the outside of the chamber was lower in the lake than in the laboratory. The diffusion shows important variations from one experiment to the other, suggesting variability among the membranes. The changes in volume inside the chamber observed after the field experiments show that some exchange of mass occurs with the surrounding environment (Table C.1). This exchange likely depends on the membrane and on the depth of mooring of the chamber.

Table 4:1: Characterization of the diffusion of methylene blue through the chamber membranes. The starting volume in the chamber was 20 mL, save for the first line where it was 25 mL.

Methylene blue concentration ($mg.L^{-1}$)	**Time of testing (hours)**	**Final concentration in chamber ($mg.L^{-1}$)**	**Time for 90% diffusion (hours)**	**Depth (m)**
Diffusion from inside to outside of the chamber, laboratory				
20	17	19	306	beaker
20	17	8.8	27	beaker
20	4	18.3	42	beaker
	7	16.4	35	
Diffusion from inside to outside of the chamber, field testing				
18.6	48	17.6	797	2
18.6	120	17.2	1357	2
27.5	48	23.9	325	2
20.8	120	10.6	219	20
Diffusion from outside to inside of the chamber, laboratory				
20	17.7	2.9	110	beaker
20	4	3.6	20	beaker

4.3.3 Mooring setup

When moored at 20 m depth for five days, visual inspection of the chamber showed no damage to the chamber or to the membranes. When moored at 2 m depth for two or five days, one of the membranes broke on two of the four occasion tested. After close visual inspection, small slits were found on one of the mem-

branes. The breakage of the membranes was concurrent with rough weather over the course of the experiment. 2 m depth was thus dismissed for the testing of the virus persistence. Our experience showed us the important impact that the waves can have on the membrane integrity, and we chose 6 m as second depth, since it maintained the chamber well under the action region of action of the waves, while granting us an interesting temperature gradient in spring.

Due to the effect of waves, we also learned that it is better not to moor the chambers directly under the buoys, but allow a bit of slack so that the movement of the waves affecting the buoy will affect the mooring to a lesser extent. Since the movement can be important under the water, we also avoided the presence of any sharp element and protected the rope with tape against friction. Finally, the weight was placed 5 meters away from the closest chambers to avoid any contact in case of movement, and the chamber was attached to the rope in a way that that weight did not exert any strength on it (Figure 4.2D).

4.3.4 Lake experiment results

4.3.4.1 *Inactivation in sterile controls*

For each experiment, sterile lake water spiked with virus was used as a control for chemical and thermal inactivation of the viruses under similar temperatures as encountered in the lake. The infectious concentration of the genotypes over time in these controls was monitored (Figure 4.4). After 5 days, CVB1 showed about 1 log inactivation in the control of the winter campaign, E25 showed 1 log reduction in the control of the spring campaign with water from 15 m depth and CVB3 showed about 1 log reduction in the controls of the spring campaign in water from both 15 m and 6 m depths. The inactivation observed in the control samples of these three genotypes indicates a small extent of chemical or thermal inactivation. All other controls were stable for the winter campaign and the spring campaign at both depths.

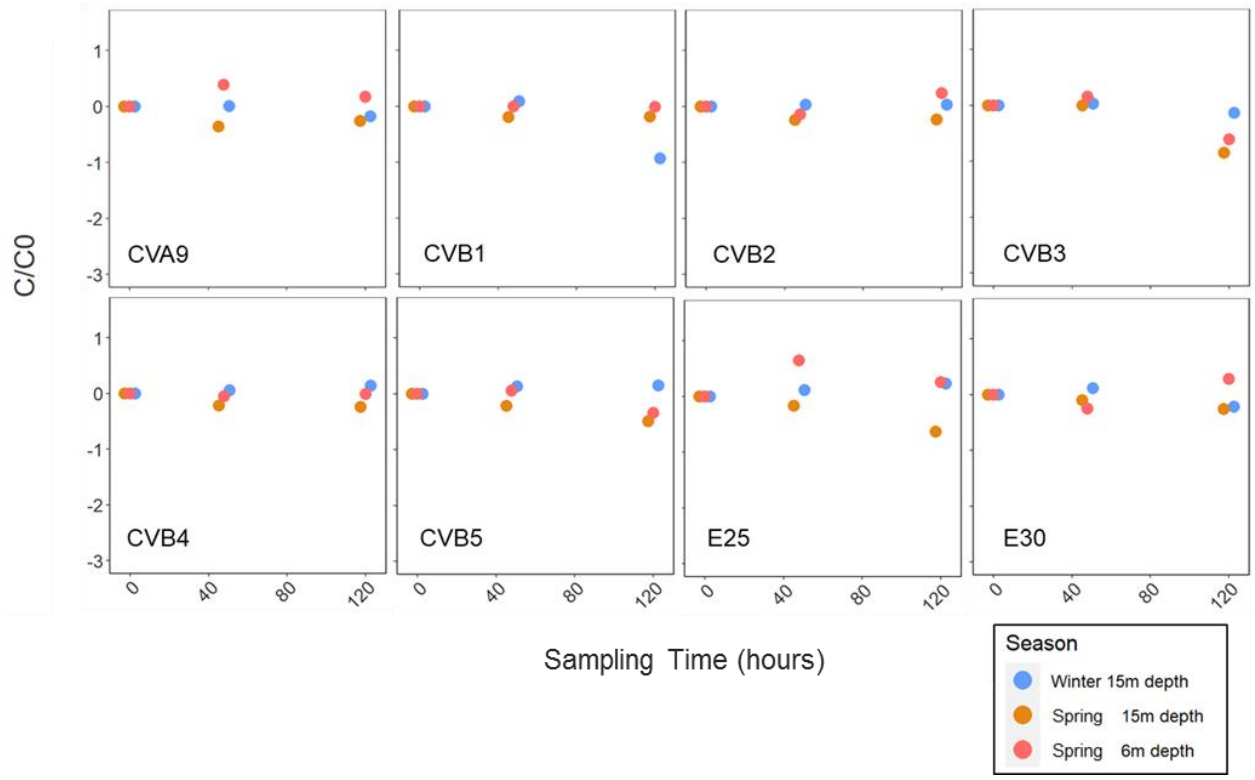


Figure 4.4: Inactivation of the eight genotypes in sterile lake water controls for the lake experiments. In winter, the controls were placed in the refrigerator at 4 °C; in spring, the control was placed in a temperature-regulated room at 20 °C. For winter, the sterile lake control was only done for experiment 2.

4.3.4.2 *Winter vs spring inactivation*

Inactivation of the genotypes at 15 m depth in spring and in winter was monitored and the extent of inactivation was compared between the seasons (Figure 4.5). In winter, biologically relevant inactivation was observed for CVB1, CVB4, E25 and E30 at five days, and for CVB3 at 48h. In spring, biologically relevant inactivation was observed for CVA9 and CVB1 at 5 days, CVB3 and CVB5 at 48 and five days, CVB4 at 48h. Inactivation was significantly greater in spring than in winter for CVA9 and CVB3 at five days, for CVB5 at both 48 hours and five days, and CVB4 at 48 hours. Surprisingly, E25 and E30 were found to be significantly more readily inactivated in winter than in spring.

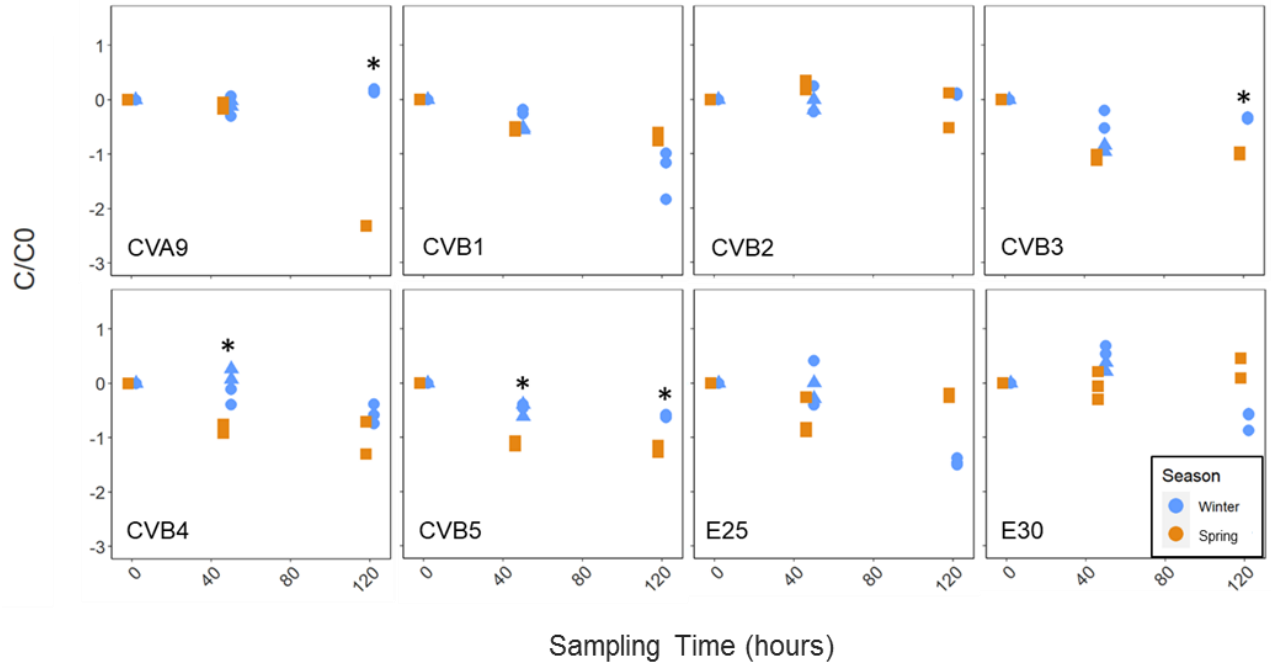


Figure 4.5: Winter vs spring inactivation of each genotype in the chambers at 15 m depth. Squares correspond to experiment 3 (spring campaign), while triangles and dots correspond to experiments 1 and 2 respectively (winter campaign). Black stars indicate a significant (both statistically and biologically) difference between inactivation in spring and winter.

4.3.4.3 *Inactivation at different water depths.*

Inactivation of the genotypes at 15 m depth and at 6 m depth was monitored during the spring campaign (Figure 4.6). At 6 m, inactivation was observed for CVA9, CVB2 and E25 at 5 days, CVB3 and CVB5after 48h and after 5 days, and CVB1 and CVB4 after 48 hours. At 15 m depth, inactivation was observed for CVA9 and CVB1 after 5 days, CVB3 and CVB5 after 48 and five days, and for CVB4 after 48h only. Despite the higher inactivation observed at 6 m compared to 15 m for CVA9, CVB1, CVB2 and E25, the difference in inactivation was only statistically significant and biologically relevant for E25.

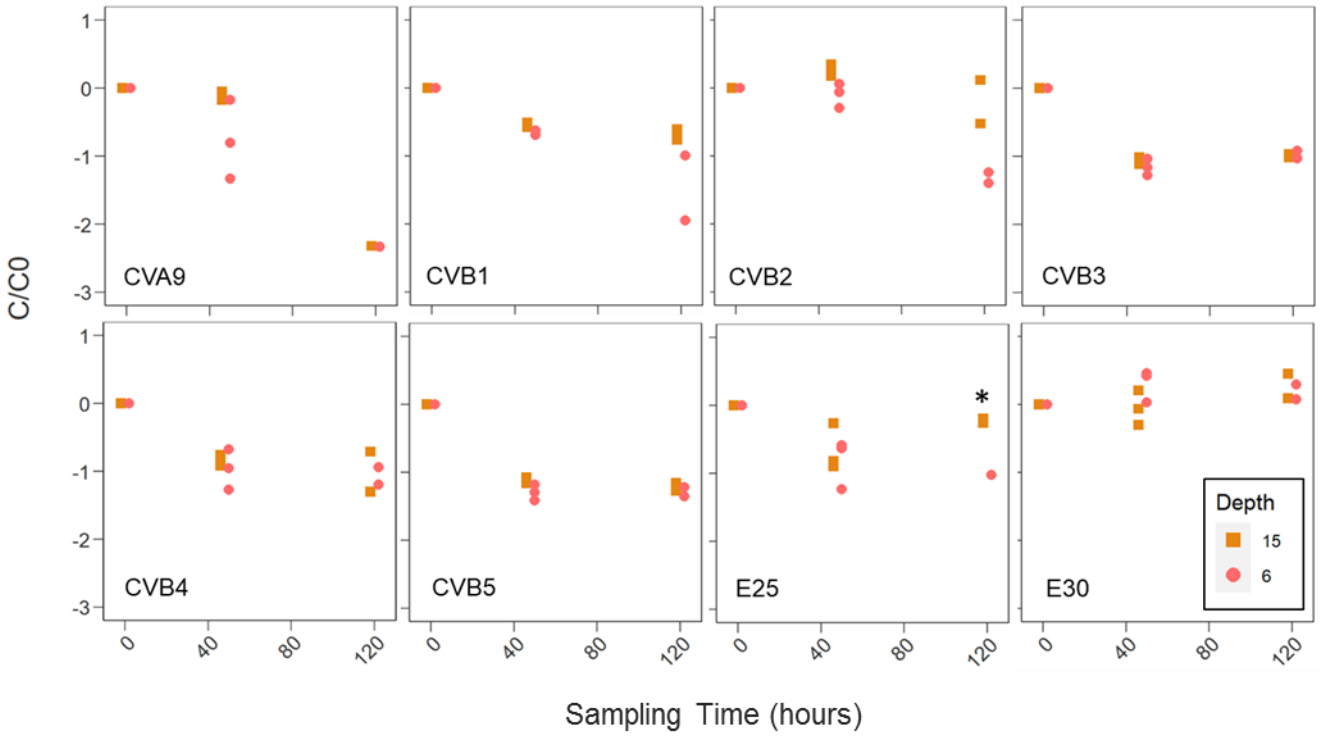


Figure 4.6: Inactivation at 15 m depth (orange squares) vs 6 m depth (red dots) in the spring campaign. The black star indicates a statistically significant difference between inactivation at 6 vs. 15 m.

4.3.4.4 *Variability in genotype response*

The extent and relative inactivation of each genotype in spring and in winter were assessed and compared between seasons, to determine if patterns could be found in the lake water persistence of the different genotypes (Figure 4.7). In spring, the range of inactivation among genotypes was wide: CVA9 was inactivated the most (>2.3 $\log_{10}$), while CVB5, CVB4 and CVB3 exhibited intermediate inactivation. E30 and E25 were the least inactivated (<0.3 $\log_{10}$). In winter, the range of inactivation across genotypes was a bit less, with E25 and CVB1 being the most readily inactivated (1.5 $\log_{10}$ and 1.8 $\log_{10}$, respectively), while E30 and CVB4 exhibited inactivation between 0.4 and 0.9 $\log_{10}$, and CVA9 and CVB2 were the most stable genotypes (no inactivation measured). The relative persistence of the different genotypes thus differed between spring and winter, though they were consistent between replicates within season.

	Spring		Winter		
	chamber 1	chamber 2	chamber 1	chamber 2	chamber 3
CVA9	-2.3	-2.3	0.2	0.2	0.1
CVB1	-0.8	-0.6	-1.0	-1.2	-1.8
CVB2	-0.5	0.1	0.1	0.1	0.1
CVB3	-1.0	-1.0	-0.3	-0.3	-0.4
CVB4	-0.7	-1.3	-0.6	-0.7	-0.4
CVB5	-1.3	-1.2	-0.6	-0.6	-0.6
E25	-0.3	-0.2	-1.4	-1.5	-1.5
E30	0.5	0.1	-0.6	-0.9	-0.6

Inactivation ($log_{10}(C/C_0)$)

-2.3
-1.8
-1.3
-0.8
-0.3
0.2
0.7

Figure 4.7: Extent of inactivation (measured as $log_{10}(C/C0)$ of each genotype after 5 days at 15 m depth, in spring and winter.

4.4 Discussion

This study evaluated the persistence of eight common, sewage-derived enterovirus genotypes in Lake Geneva during two seasons, by means of an environmental chamber. Inactivation was observed for all genotypes in at least one season, and the extent of inactivation measured in winter is within the range of to that reported by Wait and Sobsey[158] for PV1 under similar temperature conditions (with 1 log_{10} reduction in six to seven days at temperatures between 4 and 7.5 °C).

Differences in the inactivation patterns were observed between seasons (Figure 4.7). When immersed in the water column at 15 m depth, CVA9, CVB3, CVB4 and CVB5 were more readily inactivated in the spring, whereas E25 and E30 were more readily inactivated in the winter. The average water temperature at 15 m depth was around 7 °C in winter and 13 °C in spring. At these temperatures, microbial activity is expected to be low, and Olive et al.[41] showed no inactivation of echovirus 11 at 16 °C or lower by lake water bacteriathe eukaryotic fraction. Nevertheless, the sterile lake water controls showed that, aside from few exceptions (CVB1 in winter and CVB3 and E25 in spring; Figure 4.4), little of the observed inactivation could be attributed to thermal or chemical inactivation (Figure 4.4 vs Figure 4.5), suggesting that inactivation is mainly microbial.

Differences in virus persistence were furthermore observed depending on the water depth. In spring, inactivation at 6 m depth was equal to or greater than inactivation at 15 m depth for all genotypes, though the observed differences were mostly subtle and not statistically significant (except for E25). As for the deeper water, inactivation at 6 m could not be explained by chemical or thermal inactivation (Figure 4.4 vs Figure 4.6), and is thus likely mediated by microorganisms in the lake. The greater inactivation at 6 m depth may then be explained either by the expected increase in microbial activity from 13 °C to an average of 18 °C[41], differences in the microbial community composition at different water depths[159], or a combination of both.

Lo et al.[37] as well as Wait and Sobsey[158] have highlighted the role of temperature in the inactivation of enteroviruses in the environment. Our findings are coherent with theirs, since globally a higher temperature - either due to season or to depth of mooring - resulted in increased inactivation for most genotypes.

Consistent with previous reports of differential susceptibility of enterovirus genotypes to microbial predation[44,97,150], we found a wide range of inactivation across genotypes, both in spring and in winter (Figure 4.7). Interestingly, the relative sensitivities of the different genotypes varied between winter and spring. Intuitively, this would hint a change in the main environmental drivers of inactivation, with a shift from a more chemical inactivation in winter to a microbial inactivation in spring. This speculation is supported by the finding that CVA9 was readily inactivated in spring, but not in winter. Corre et al.[150] found that CVA9 was highly sensitive to inactivation by most bacteria isolated from lake water. The high sensitivity of CVA9 in spring but not in winter thus supports that inactivation is driven by microorganisms in the spring but not in the winter. However, Lo et al.[37] also found variability in genotype persistence during *in-situ* experiments including CVB5, E6 and PV1, yet their experiments were done in chambers containing sterile ocean or estuarine water, such that the microbial action would have been limited to the exchange of antiviral metabolites between the chamber and the environment. A shift from microbial to chemical inactivation in winter is also contradicted by our control experiments, which showed that only CVB1 inactivation could be explained by chemical and thermal inactivation in winter (Figure 4.4). Further work is needed to understand the effect of seasons on the different genotypes. For example, the underlying cause may be associated with changes in the microbial population composition between the two seasons, as has been shown in other lakes[159].

An important aspect of this study was the use of the diffusion chamber, which allowed us to perform inactivation experiments in-situ rather than under unrealistic laboratory conditions. While microorganisms could not enter the chamber because of the small pore size of the membranes, smaller molecules, such as nutrients and small biomolecules can pass through and nurish the bacteria contained within the chamber, thus recreating the lake conditions. Ideally, (bio)chemical lake water constituents should diffuse into and out of the chamber at a constant rate, to yield comparable conditions across chambers. Here, the diffusion of methylene blue through the membranes of the chamber varied greatly during the different experiments. As a comparison, LaBelle et Gerba[49] also studied the diffusion time of methylene blue through their chamber, and obtained values of 22 and 27 hours for a 90% diffusion out of the chamber in the field and in laboratory conditions respectively. This is in the same order of magnitude as some of our experiments, but many experiments displayed much longer diffusion time. The difference could be linked to the smaller surface area to volume ratio of our chamber, since the central lumen of our chamber has a diameter of 3.3 cm for a 25 mL volume, against 6 cm for a 20 mL volume for their chamber[160]. The variability could also stem from the quality of the membranes. Finally, the deployment depth may have an effect, since the fastest diffusion in the field was observed for the chamber moored at 20 m instead of 2 m depth. As a comparison, the chambers in

LaBelle and Gerba's study[49] were moored at about 1 to 1.5 m depth. Despite this variability in diffusion, changes in the volume contained in the chambers was observed in almost all experiments (Table C.1), indicating some exchange of matter between the chambers and the surrounding lake water. Correspondingly, replicate experiments exhibited reasonable reproducibility (Figure 4.5 and Figure 4.6), indicating that the diffusion rate is a minor influence on virus inactivation.

4.5 Conclusion

This study shows that temperature is an important factor governing enteroviruses inactivation in the lake. It furthermore shows that persistence of the different genotypes varies greatly, but that the relative persistence of the genotypes is season dependent. As a result, enterovirus populations in the lake are expected to be dominated by different genotypes depending on season (though the final population composition will also be determined by genotype-specific shedding rates and removal efficiencies during sewage treatment). Independent of season, the inactivation observed in the lake was found to be mainly microbial.

Chapter 5 Conclusion

5.1 Achieved results

Enteroviruses are ubiquitous, they persist for long periods of time in aquatic environments and can pose a threat of human infection through use or consumption of contaminated water. The enterovirus genus displays diversity and not all enterovirus genotypes are equally affected by natural stressors. The persistence of enteroviruses in the environment will depend on the genotypes present and the stressors encountered. The enteroviruses mainly reach the environment through discharge of treated sewage and their composition will thus be influenced by the genotypes circulating in the population and their persistence through the sewage treatment. Little is known about the diversity of responses within an enterovirus population when exposed to stressors, and the goal of this study was to evaluate how a typical population found in sewage would be shaped by treatment and by exposure to a lake environment. The main reason for this lack of knowledge is the absence of methods allowing the infectivity of several enterovirus types to be measured in a mixed sample.

Integrated Cell Culture RT-qPCR (ICC – RTqPCR) method allowed the quantification of infectious virus for eight ubiquituous enteroviruses in a mixed sample.

Detection by qPCR following amplification on cells allows for the quantification of specific infectious enterovirus genotypes. In Chapter 2, the study of the prevalence of enteroviruses genotypes in sewage in Europe allowed us to come up with a list of thirteen ubiquitous genotypes. qPCR primers allowing the specific detection of eight of them were designed, and calibration curves were created for the eight genotypes, allowing the infectious concentration of each genotype to be inferred based on the increase in qPCR signal after amplification on cells. The method was able to accurately measure residual infectious concentration in a background of inactivated virus, and could accurately quantify the concentration of a virus in a wastewater matrix.

Virus decay in activated sludge is genotype-dependent and sludge-dependent

Using the ICC-RT qPCR method, the fate of our population of eight enteroviruses in activated sludge was assessed. The range of decay among genotypes was very wide, and the extent of decay was sludge-dependent. The relative sensitivities of some genotypes to decay varied with the sludge samples, though overall our

results suggest that CVA9 and CVB1 will be readily depleted in activated sludge, while E25 will persist quite well.

Virus decay in activated sludge was mainly attributable to microbial inactivation and to a lesser extent to chemical inactivation.

Other studies have found adsorption to solids to be one of the main mechanisms of virus decay in sludge. This study however, found adsorption to have a negligible impact on the infectivity loss of enteroviruses in activated sludge. Infectivity loss was the greatest in presence of microbially active solids, and some chemical inactivation was observed to a lesser extent in absence of solids or in presence of sterilized solids. However, we also found that some adsorption to the solids occurred, and that enteroviruses can retain infectivity for several days on the sludge. This suggests that solids can pose a risk if not well treated prior to disposal.

Inactivation by free chlorine is genotype-dependent and extracellular polymeric substances (EPS) can provide protection to enteroviruses

We exposed our population of eight enteroviruses to disinfection by different doses of free chlorine and used the ICC-RTqPCR method to assess inactivation of each genotype. The range of inactivation among genotypes was very wide, with CVB5 and CVB3 being the least susceptible to chlorine, while E30 was the most sensitive to disinfection. Furthermore, CVB5 and E25, and to a lesser extent CVB3, were found to gain protection to disinfection through exposure to EPS extracted from the activated sludge, suggesting that activated sludge exposure prior to chlorination may reduce the inactivation of these genotypes.

Inactivation of enteroviruses in Lake Geneva is season-dependent

Using an environmental chamber that allows safe exposure of enteroviruses to the lake environment, we assessed the inactivation of our population of enteroviruses in the Lake Geneva in winter at one depth and in spring at two depths. Inactivation was greater in spring than in winter for four of the genotypes, although two genotypes were inactivated to a greater extent in winter. Inactivation was also greater at lower depth in spring, where temperature was higher. The differences observed could not be explained by direct effect of temperature and might be due to indirect effect of temperature, nutrients or light.

Inactivation of enteroviruses is genotype-specific, but the relative sensitivities of the genotype is season-dependent

A wide range of inactivation rates was found among genotypes, both in winter and in spring. However, the patterns across genotypes were not the same in both seasons. For both winter and spring, the inactivation was found to be mainly due to microbial activity, with the controls showing no inactivation.

Figure 5.1 shows a simulation of the fate of our population of eight enteroviruses, starting at equal concentrations in the sewage and undergoing activated sludge treatment and chlorination before being exposed to the Lake Geneva. This simulation has been performed using the outcomes of specific experiments (see details in the figure caption), nonetheless it highlights how each treatment can shape the population and change the prevalence of the genotypes. In our experiments, CVB3, CVB4, CVB5 and E25 are the predominant genotypes after being exposed to all processes. CVB3 is highly dominant after exposure to the lake in winter, representing more than 80% of the remaining enterovirus population. These proportions might change under different conditions, namely a different sludge, a higher chlorine dose, and different lake conditions. CVB5 has been a genotype of interest in water and sewage treatment due to its high resistance to chlorine disinfection. Our results highlight that CVB3 and E25 might also be genotypes of interest when looking at full treatment trains and persistence in the environment.

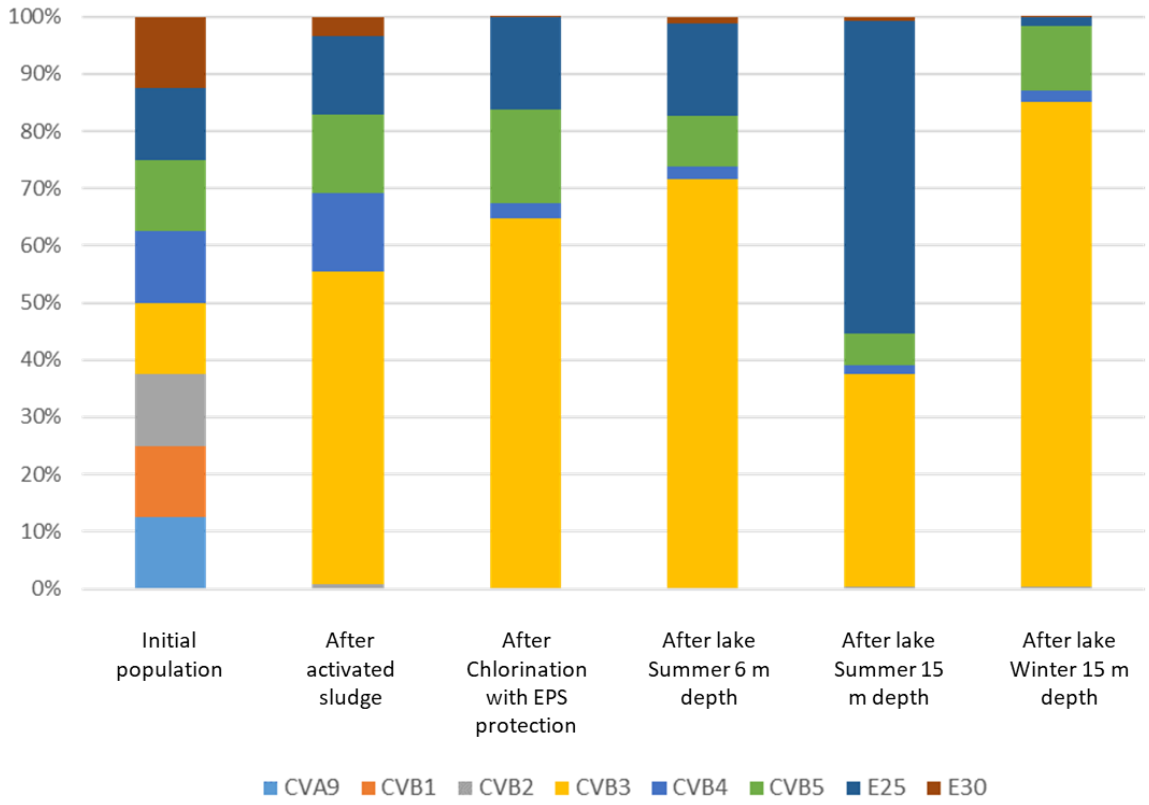


Figure 5.1: Simulation of the change in composition of an enterovirus population subjected to activated sludge treatment followed by chlorination, and exposure to the lake for five days. The initial population is assumed to consist of all eight genotypes studied at equal concentrations. Inactivation rate constants from

the supernatant of one single sludge (sludge 1 in chapter 3) were used to estimate the decay in infectious virus concentrations after 6 hours' exposure to the sludge. Inactivation after exposure to 0.3 mg.min.L^{-1} were used (Chapter 3) and since no data was collected for inactivation of CVB1 by free chlorine, a medium value of inactivation was used. In chlorine inactivation, protection by EPS was assumed for CVB5, E25 and CVB3 and was estimated with the decay rates calculated in Chapter 3. Inactivation after five days was used for the lake exposure and inactivation in winter and in spring at both tested depths is simulated.

5.2 Implications of this work

This thesis has shown the high variability that exists among enterovirus genotypes exposed to common engineered and natural stressors, and highlighted patterns of susceptibility of some genotypes to each of them. These findings have implications for several fields:

- For the prediction of virus abatement and adjustment of treatment trains, for example in wastewater reuse. Disinfectant dosages need to be adjustedto ensure that the water is safe for further use. The results of this study show that we cannot consider the enteroviruses as a homogeneous ensemble, since the fate of the population will depend its composition and can thus vary. However, monitoring them all at the same time is very difficult and costly. Selecting the most resistant genotypes for each treatment and evaluating the treatment dose needed for their abatement would allow tests to be simplified whilst simultaneously ensuring the safety of the end users. This study highlights some genotypes that are persistent through the different treatments, and if these results were confirmed by other studies, they could form a basis to chose conservative indicators for treatment monitoring.

- For the estimation of health risks linked to the use of recreational water. The estimation of risks linked to water use can be estimated by Quantitative Microbial Risk Assesment (QMRA). The risk is estimated by combining data on the concentration of pathogen in the water, their intake, and the dose-response caused by the pathogen. Data on enterovirus concentration in the environment is scarce and difficult to obtain due to extremely low concentrations and consequent variability in their quantification. One option to overcome this issue is to couple data of discharge concentration to persistence data and to hydrodynamic models that estimate the distribution of pathogens over time in the water body. Studies of persistence of enteroviruses in natural environments include a limited number of genotypes, and few studies perform actual in-situ measurements of virus decay. This study presents new inactivation data specifically generated in-situ for a large number of genotypes, and points out which genotypes might be more or less persistent. The difference in persistence observed in this study among genotypes can have a big impact on the estimation of the risks. This data,

if validated by other studies, could be integrated in risk models that estimate the fate of enterovirus in water bodies and the potential risks for arising from recreational water use.

- For further study of the mechanisms of enterovirus inactivation. This study has compared an important number of enterovirus genotypes, and pointed out variability in their sensitivities to different treatments. This could give basis for investigating what causes the differences in sensitivities to different processes, through selection of only few genotypes of high and low sensitivity and exploration of the differences that give rise to the contrasting inactivation rates. For example, it could be interesting to investigate the structural or compositional features shared by genotypes that are resistant to microbial degradation or chlorine.

5.3 Current limitations and future development needs

Many things are still needed in order to make accurate predictions for the fate of enteroviruses in the environment and through engineered processes.

The need to expand the diversity of the enterovirus population considered: this study has highlighted significant differences in decay among the eight genotypes studied when exposed to different stressors. However, we only studied one strain per genotype, and there might be variability in the response of other strains when exposed to the same treatments. In order to select for the most persistent genotypes for a given treatment, repetitions should be made including different strains. Furthermore, a selection of eight enteroviruses has been made for this study, but some ubiquitous genotypes have been discarded for technical reasons and might be of interest. Other genotypes of particular interest for their prevalence in certain regions or the severity of diseases that they cause could also be considered.

The need for more environmental persistence data: this study presents persistence data for eight enteroviruses in Lake Geneva during two seasons, but only three measurement campaigns were performed. In order to obtain a more realistic overview of the persistence of enteroviruses in the lake, more measurement campaigns should be undertaken. Specifically, collection of data during spring, at higher water temperature would be of great interest, as well as having more data for each season. A bigger dataset with a comprehensive uncertainty level could then be introduced in hydrodynamic models to estimate the concentration of enteroviruses in Lake Geneva.

Understand the mechanisms of inactivation: in order to build predictive models, understanding how a genotype will behave when exposed to a given stressor is necessary. For this, mechanistic studies are required, to understand how stressors act on the viruses and what creates the disparity of responses. This study has

shown that microbially mediated inactivation plays a big role in activated sludge, but the exact mechanism remains to be unraveled.

Work on methods to estimate inactivation: the method used in this study has proven instrumental to quantify the inactivation of our selected population of enteroviruses through the different processes considered. It has limitations though, the main one being the difficulty to apply the method to unknown samples as the qPCR primers specificity has only been vetted on our laboratory population. It can also be applied to a limited range of concentrations of virus. The matter of infectivity is crucial, and raises many questions: are we completely underestimating the infectious concentration of enteroviruses in our samples due to the cell culture limitations? Is the infectivity measured in laboratory representative of the infectivity in a human host? If we use molecular methods to estimate inactivation, the risk is to over-scale our treatment (and the costs) by overestimating the risks. Finding a way to relate molecular methods such as deep sequencing, to the infectivity measured on cells, or other ways to measure virus infectivity, using genotypes that can be propagated might be a way to avoid both pitfalls. Moving towards enteroids to culture currently unculturable viruses would also be beneficial.

Validate experimental findings in situ: In this study, we added high concentrations of enterovirus to our matrices of interest. Future work should focus on the viruses present in situ to validate the genotype-specific trends observed herein. This requires efficient concentration methods. Comparing what is found in the sewage to what is found clinically will also inform about outbreaks and genotypes of potential interest.

Appendix A : Chapter 2

Table A.1: Accession numbers of enterovirus genotypes used to create the consensus sequences

CVA9	CVB1	CVB2	CVB3	CVB4	CVB5	E3	E6	E7	E11	E13	E25	E30
KM201659	LN854562	HF948083	KJ489414	DQ480420	KX139461	AY302553	JQ929657	MH043135	AJ577590	HF948099	KX139459	JN797616
JN996514	JN797615	FJ525912	AY752944	KC558560	MG845894	FJ766334	HM852755	MH043136	DQ092796	AM236977	HQ897677	KY131965
JN996499	MG845887	FJ525950	MG451802	KC558561	MG451803	MF101535	HM852754	MH043134	KX527626	AM711067	FJ525937	KF920600
HQ897672	FR798000	HG793667	JN979570	MG451808	MG845890	AJ849942	MG451809	MH043133	KJ830693	HG793714	FJ525917	KF920598
HQ897680	FJ525916	AM711056	KJ818297	KF460439	KT285015	MG451804	KF931635	MG451810	HQ897669	AM236975	AM711086	JN797614
HQ897676	KU189247	KF938915	JX946655	FJ525949	HG793678	KU189249	HQ897675	MG451805	GU393781	AM711009	HG793730	KT809220
HQ897652	AM711081	KU560981	KU189250	HQ897655	HF948276	AM236931	HG793697	MG451806	KJ830687	AJ537609	HF948110	KT809196
FJ525918	HG793666	KY865775	KU189248	AF160065	HF948059	AM236930	KX683352	JN996506	KJ830688	KU189243	HF948111	KT809212
FJ525939	HG793665	KY866387	KJ400861	KU189251	HF948066	JX009121	KX683354	HQ897671	KJ830683	HF948101	KU561054	KC539428
HG793656	HF948082	JX009107	HG793668	HG793670	HF948041	HF948092	FN691461	HG793699	KU189254	KU561029	KC893471	KY987033
HG793657	JQ239014	KC893529	HF948085	AM711064	HF948067	KX842456	KU561006	AJ241426	HG793702	AY227344	KC893488	KY986982
HG793663	JQ239015	JN034251	HF948086	GQ352391	HF948039	KU561001	KC183943	AM236932	HG793709	AH014807	JX009115	KY986976
HG793658	KU560979	JN034242	HF948084	AM943370	KU560996	KF601701	KC852176	HF948093	HG793703	DQ317212	KC880339	KM015258
HF948081	KF577980	JN034245	HF948088	HG793672	KF460441	KY866612	KC880340	KU561016	KP090646	EU372165	MF459669	KX277966
KC893537	KY866341	JN034218	KC893530	HF948090	KF134012	KY866127	KM598863	KC893491	KC893514	EU372168	KF484510	KT897955
KJ361510	KY865869	JN034217	KC862305	HF948089	KF850475	KY866071	KP262024	AM492473	KF154121	EU372059	KY866650	KF709448
KF906544	AH014511	JN034244	KF437812	JX009117	KY866446	KY865916	KY866567	JQ780694	KY866634	DQ317209	KY866139	KJ361511
KU560977		KJ867446	KY866644	KF752598	KY866172	KY865828	KY865981	AF521310	KY866391	DQ317210	KY865919	KU645936
KY865833		KJ867450	KY866002	KY866031	KY866244		KY865755		KY865759	AY518355	HQ897665	KU645939
			KY866000	KY865915	JX009100		KC183931		KY865898	AY697441	AY342708	KU645940

Table A.2: Specific primers used in the study: sequence, region of attachment and length of amplicon

Primer pair Name	Sequence	Region	Length of the amplicon
CVA9	F - 5’ ATG CAG ACT AGG CAC GT 3’ R - 5’ CGC ATT TGC ACC ATC TG 3’	VP1	136
CVB1	F - 5' GAA AAT TTC CTG TGC CGG T 3' R - 5' GGG TTG TTG TGC ACT CGT TA 3'	VP1	148
CVB2	F – 5’ TCA AGA TCA GAG TCC AG 3’ R – 5’ GAT GGA TCC TGT GCG CT 3’	VP1	187
CVB3	F - 5’ GAG ACT GGG CAC ACA TC 3’ R - 5’ TAC TCT GTG AAA TAA ACA CA 3’	VP1	100
CVB4	F - 5' CGY TAT GCA GAG TGG GT 3' R - 5' TAT GTG AAC ATT TCC ATT TT 3'	VP1	34
CVB5	F - 5' ATG CAA ACC AGG CAC GT 3' R - 5' GTG CTT GTT ATC ACA AA 3'	VP1	199
E3	F - 5’ ACA ACC TAT AAG TCG GC 3’ R - 5’ ACC ATT TGC CGG GTG TT 3’	VP1	40
E6	F – 5’ ACY CCA GAC AAA ATG TA 3’ R – 5’ ACT TCC ACG TCA AAY CT 3’	VP1	70
E7	F – 5’ TAC ATG TCR TGG ACC ATA AA 3’ R – 5’ AAT GTG AYY TCC ATG TC 3’	VP1	109
E11	F - 5' TAC CAC TCG AGA TCA GA 3' R - 5' TCT CAT CTG CAC CAT GCG 3'	VP1	112
E13	F – 5’ GGT AAC GCA TAC AGC AGC TTT TA 3’ R – 5’ ACA TTC CCT GAT TTC TCA TA 3’	VP1	184
E25	F – 5’ TGG AAG ATC AAC ACG CG 3’ R – 5’ GTG AGC ACH GGG GTG TC 3’	VP1	112
E30	F – 5’ AGT GAC ACA ATG CAG AGA CG 3’ R – 5’ TCA AAT CTC ATG TAT GTA TGT GAA CAT 3’	VP1	175

Table A.3: DNA standards (Gene blocks) sequences and PCR efficiencies based on DNA standards or viral RNA. Efficiencies were determined based on a single qPCR run.

Primer pair Name	Gene block Sequence	Amplification efficiency for DNA standards	Amplification efficiency for Serial dilutions of viral RNA
CVA9	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTGAGTGACACC**ATGCAGACTAGGCACGT**GAAGAATTACCACACTCGTTCTGAGTCCACTGTGGAAAACTTTCTTGGCAGATCAGCTTGTGTTTATATGGAGGAATACAAGACCACAGATAATGATGTTAACAAGAAATTCGTGGCGTGGCCGATCAACACTAAA**CAGATGGTGCAAATGCG**TAGGAAGCTAAACGATGCACGTTTGGCATGGTTGTCGTCTCTAGGTATCT	0.97	0.98
CVB1	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTATCATCCATA**GAAAACTTCCTGTGCCGAT**CTGCCTGTGTTTATTATGCCACCTACACGAACAACACAGAAAAAGGGTACGCAGAGTGGGTCGTAAACACTAGGCAAGTAGCCCAATTAAGGAGAAAGCTAGAACTGTTCACTTATCTAAGATTTGATTTAGAGTTGACATTTGTGA**TAACGAGCGC****TCAACAACCC**AGCACTGCCAAACGATGCACGTTTGGCATGGTTGTCGTCTCTAGGTATCT	1.11	1.06
CVB2	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTCGTGCGCAACTACCAC**TCAAGATCAGAG****TCCAG**CGTGGAGAACTTTCTGGCGCGATCGGCATGTGTGTTCTACACAACGTACACCAACAGCAAAACTGCAGCCAAAGAGAAAAAGTTTGCAACATGGAAGGTGAGTGTTAGACAAGCCGCACAGTTGAGGAGGAAGTTGGAGTTGTTCACATACTTGCGCTGTGACATCGAGCTCACATTCGTCATCACC**AGCGCACAGGATCCATC**AACTGCCACCAACAACGATGCACGTTTTGGCATGGTTGTCGTCTCTAGGTATCT	0.82	0.98
CVB3	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTCACAGCAGCT**GAGACTGGGCACACATC**CCAAGTTGTTCCAGGTGATACCATGCAAACGCGTCACGTGAAAAATTATCACTCAAGATCTGAGTCAACAATTGAGAACTTCGTATGCAGGTCCGCATGT**GTTTATTTCACAGAGTA**TGAAAACTCAAACGATGCACGTTTGGCATGGTTGTCGTCTCTAGGTATCT	0.89	0.89

CVB4	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTTGTGTGATTTACATCAAATATTCAAGTGC TGAATCCAACAACTTAAAG**CGTTATGCAGAGTGGGT**CATTAACACAAGACAGGTGGCACAGCTGCGACGG**AAAATGG AAATGTTCACATA**CATTCGCTGTGACATGGAATTGACATTTGTCATAACCAGTCACCAGGAAATGTCTACAACGATGCA CGTTTGGCATGGTTGTCGTCTCTAGGTATCT	0.85	0.83
CVB5	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTGTTCCGGCAGACACC**ATGCAAACCAGGC ACGT**GAAGAATTATCACTCGCGATCTGAATCCACAGTAGAGAACTTTCTGTGTAGATCCGCGTGCGTATATTACACAAC CTACAAGAACCATGGCACCGATGGGGACAATTTCGCCTACTGGGTGATTAACACAAGACAGGTTGCGCAATTACGCCG CAAACTAGAAATGTTCACATATGCCAGATTTGATTTAGAGCTCACC**TTTGTGATAACAAGCAC**ACAAGAACAATCCACC AACGATGCACGTTTGGCATGGTTGTCGTCTCTAGGTATCT	0.88	0.89
E25	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTCGCTTCACTAAC**TGGAAGATCAACACGC G**CCAAGTTGTCCAGCTAAGGCGCAAACTGGAGATGTTCACATACATTAGGTTTGATGTGGAAATCACATTTGTAATCAC AAGCACTCAGGGGACATCAACCCAAACAAACACA**GACACCCCCGTGCTCAC**ACACCAAGTGATGTAACGATGCACGTT TGGCATGGTTGTCGTCTCTAGGTATCT	0.96	0.97
E30	ATTGGCGCCAGGCGCTCCGCTACCTTGCCCTAAACGAAGATCGAAAGTTTCACAGGTGGTACCG**AGTGACACAATGCA GACACG**GCACGTGGTCAACTACCACACCAGATCAGAATCGTCAATAGAGAACTTTATGGGTAGAGCGGCGTGTGTGT ACATTGCTCAGTACGCCACAGAGAAGGTCAATGACGAGTTGGACAGGTACACCAACTGGGAGATAACAACCAGGCAA GTGGCACAATTAAGGCGGAAACTGGAA**ATGTTCACATACATGAGATTTGA**CCTTGAGATCACGTAACGATGCACGTTT GGCATGGTTGTCGTCTCTAGGTATCT	0.86	0.87

Table A.4: Competition experiment: difference in average log(Δgc(24h)) between single genotype and mixed samples, along with t-test statistics (p-value, confidence intervals and significance of the difference).

Genotype	MPN	p-value	Difference in log(Δgc(24h))	95% CI min Difference in log(Δgc(24h)	95% CI max Difference in log(Δgc(24h))	Significant difference (α=0.05)
CVA9	1000	0.302	0.084	-0.178	0.347	
CVA9	5000	0.016	0.253	0.113	0.394	*
CVB1	1000	0.077	0.096	-0.026	0.218	
CVB1	5000	0.397	0.096	-0.291	0.484	
CVB2	1000	0.650	0.049	-0.349	0.447	
CVB2	5000	0.683	0.033	-0.264	0.330	
CVB3	1000	0.167	-0.074	-0.222	0.075	
CVB3	5000	0.655	0.075	-0.549	0.700	
CVB4	1000	0.270	-0.643	-2.478	1.191	
CVB4	5000	0.284	-0.146	-0.579	0.287	
CVB5	1000	0.445	-0.240	-1.336	0.855	
CVB5	5000	0.189	0.227	-0.271	0.725	
E25	1000	0.099	0.576	-0.266	1.419	
E30	1000	0.043	0.153	0.012	0.294	*
E30	5000	0.032	0.609	0.128	1.089	*

Table A.5: ICC-RTqPCR calibration curves parameters for individual and pooled curves, and associated statistical parameters

				Pooled calibration curve		**ANCOVA analysis**	
Genotype	Calibration curve repeat	Slope	Intercept	Pooled slope	Pooled intercept	p value slope difference (α=0.05)	p value intercept difference (α=0.05)
CVA9	SC1	1.428	0.262	1.062	2.041	0.090	1.03E-09
	SC2	1.037	2.151				
	SC3	0.919	3.040				
CVB1	SC1	0.998	2.511	1.086	2.204	0.927	0.058
	SC2	1.054	2.497				
	SC3	1.130	1.860				
CVB2	SC1	1.397	0.200	1.234	1.150	0.546	4.95E-06
	SC2	1.159	1.224				
	SC3	1.475	1.082				
CVB3	SC1	1.398	-0.744	1.204	0.290	0.178	2.71E-08
	SC2	1.079	1.165				
	SC3	1.158	0.422				
CVB4	SC1	1.190	0.755	1.110	1.669	0.782	6.91E-07
	SC2	1.323	0.998				
	SC3	1.075	2.433				
CVB5	SC1	1.346	0.442	1.246	1.281	0.996	8.10E-06
	SC2	1.351	0.875				
	SC3	1.318	1.695				
E25	SC1	1.318	0.246	1.495	0.015	0.607	2.44E-13
	SC2	1.479	-1.825				
	SC3	1.054	2.466				
E30	SC1	0.872	3.642	1.030	3.232	0.115	6.04E-11
	SC2	1.203	2.096				
	SC3	0.816	4.213				

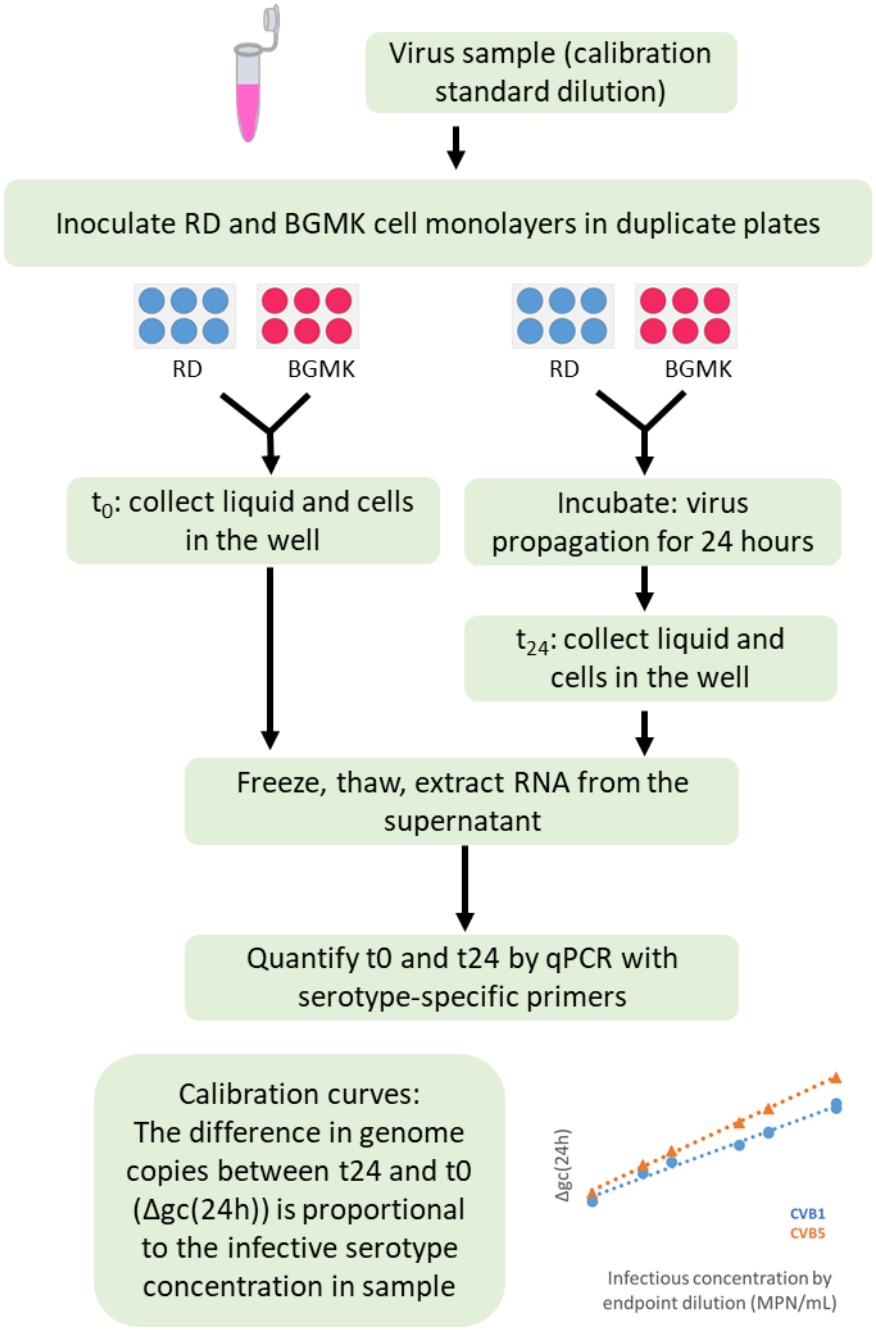


Figure A.1: detailed ICC-RTqPCR protocol for the creation of calibration curves.

Run 1

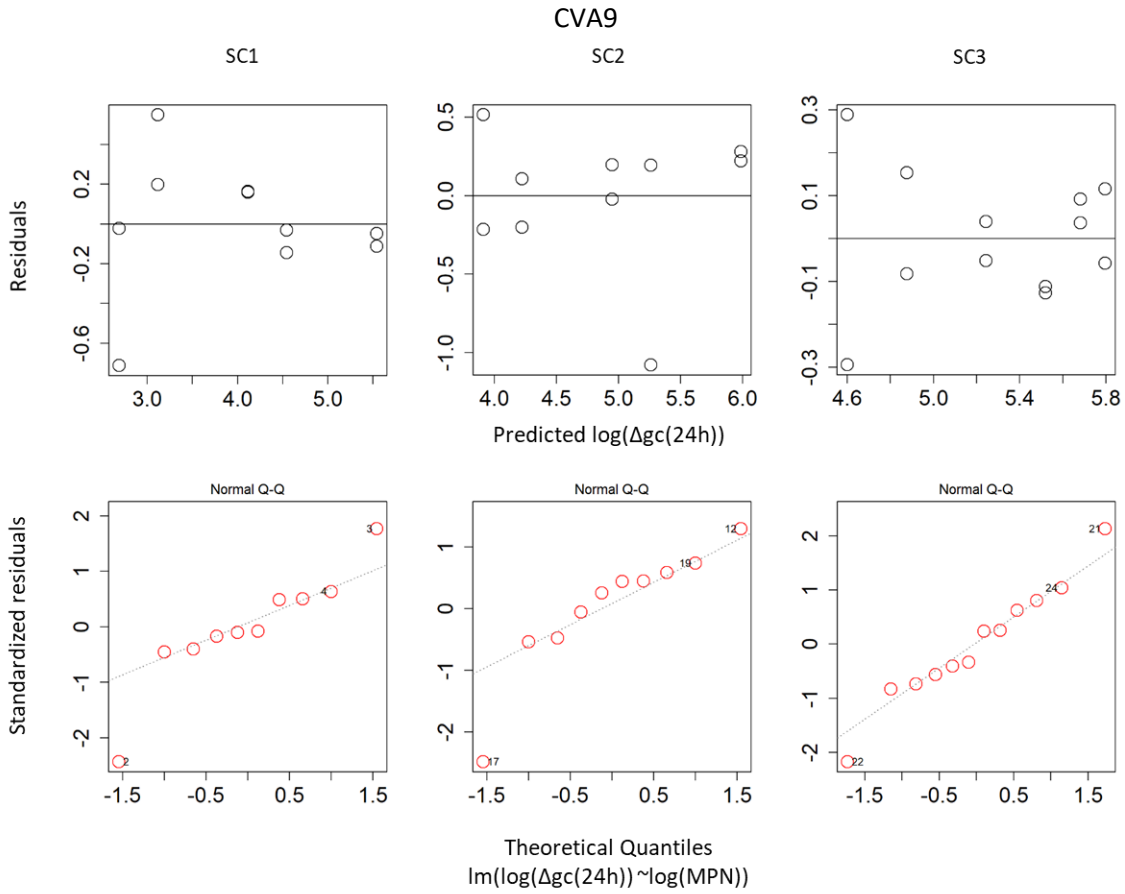
	CVB1		CVB2		CVB3		CVB4		CVB5		CVA9		E3		E6		E7		E11		E13		E25		E30	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
CVB1	7.5	7.5	12.7	12.7			22.2	22.6	7.2	7.3	20.0	23.5							23.2			9.8	20.1	19.9		
CVB2			11.6	11.7																						
CVB3		16.3	12.4	16.8	10.4	10.4								22.5									19.0			
CVB4			10.9	10.7			3.5	3.5	17.7	16.2	17.1	16.7	19.3	19.0											13.1	13.3
CVB5	11.6	12.1		12.5	5.4	5.6	25.2		3.4	3.4	16.9	18.4											20.5	18.3		
CVA9			12.0	11.2					14.4	14.3	2.5	2.5	21.5									7.8				21.1
E3	20.9		12.9	15.7	12.4	12.8							2.5	2.5									17.7	17.5		
E6			**1.0**	**1.0**	15.5						22.0		12.4	12.2	12.1	12.2	9.9	10.0					19.0		15.1	14.0
E7											21.9		16.9	17.0			4.5	4.4							16.6	18.0
E11				13.8	12.7				11.1	11.2	17.3	17.8	14.7	14.8	**3.2**	**3.4**	10.7	10.6	5.0	5.1						
E13							10.1	10.1									5.6	5.6			19.1	19.0			17.8	14.9
E25	18.1								17.3	17.2	21.1												6.3	6.2		
E30							24.0	25.5				22.5													6.7	6.7

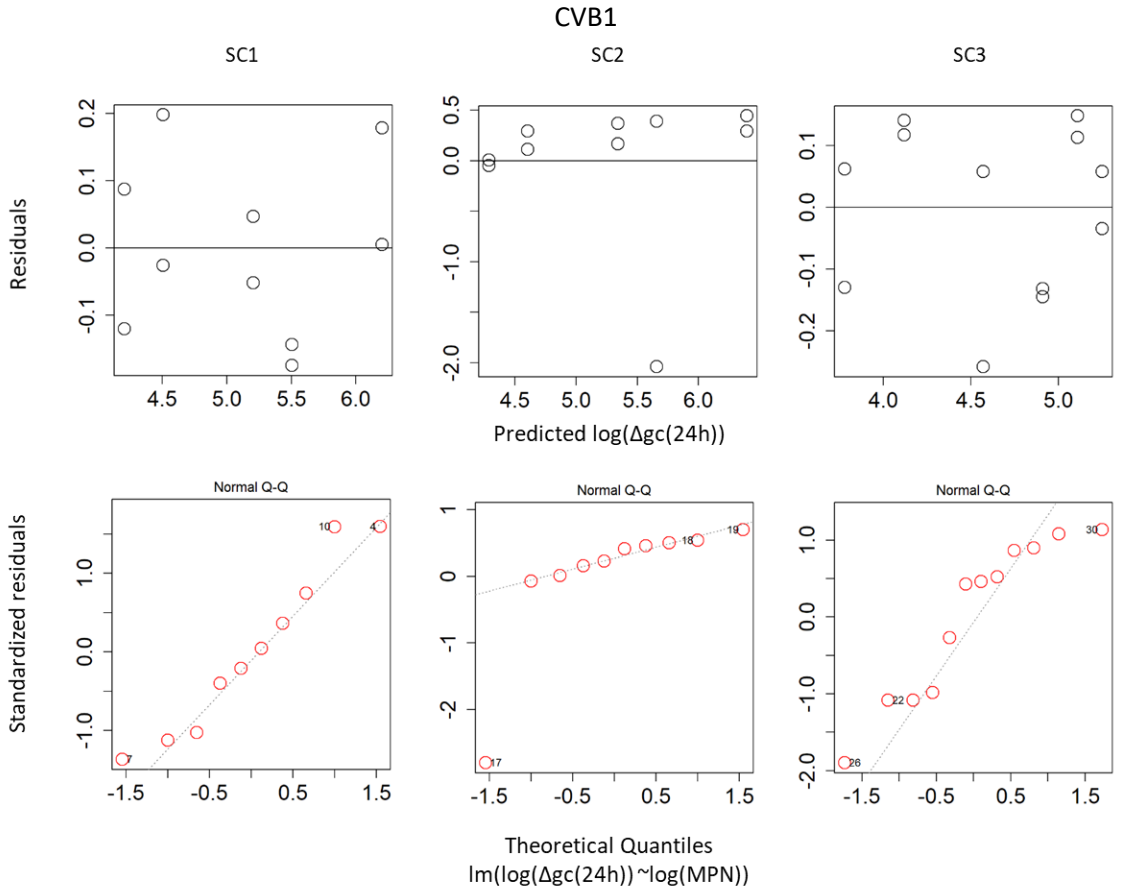
Run 2

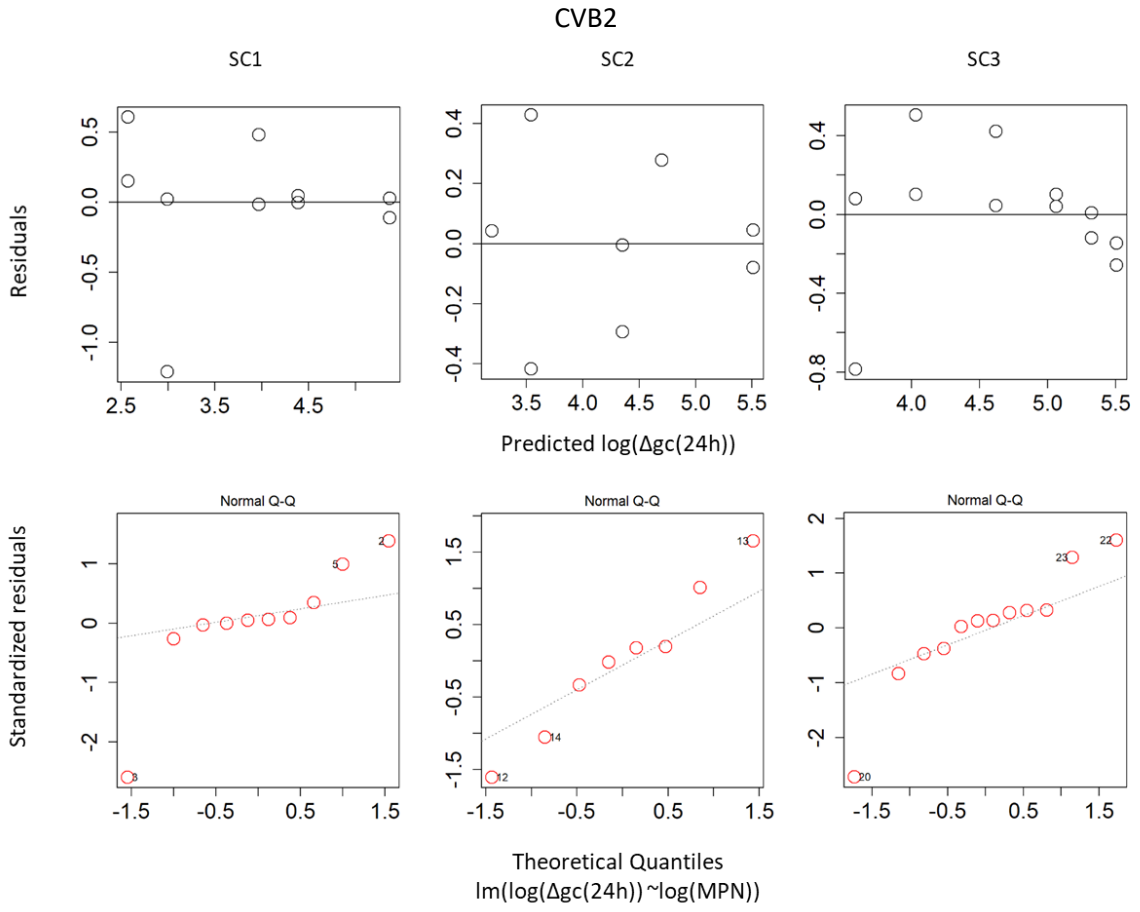
CVB1	10.5	10.6	14.0	13.7			22.2		8.1	8.4													19.3			
CVB2			11.8	11.7																						
CVB3					13.0	13.0																				
CVB4				11.4			5.5	5.5		18.7	18.8	17.8	21.3	21.3											14.3	14.1
CVB5	9.6	10.2	13.2		5.1	4.5			4.9	5.0	19.9	19.3												16.8		
CVA9			12.6	12.7					11.6	11.9	2.5	2.5		19.6											18.1	
E3					12.0	12.5						23.6	3.0	2.5									19.1	19.0		
E6			**3.0**	**3.1**									13.9	14.7	14.1	13.7	9.6	9.7					17.4		14.1	14.2
E7													18.7	20.7			5.4	5.5								
E11						12.8			12.5	12.3	17.9	18.6	17.2	17.1	**3.4**	**3.3**	10.6	10.5	6.9	6.8						
E13							9.8	9.8									5.5	5.4			20.2	19.8			14.2	16.5
E25									16.0	15.9													8.2	8.2		
E30												23.6													8.6	8.7

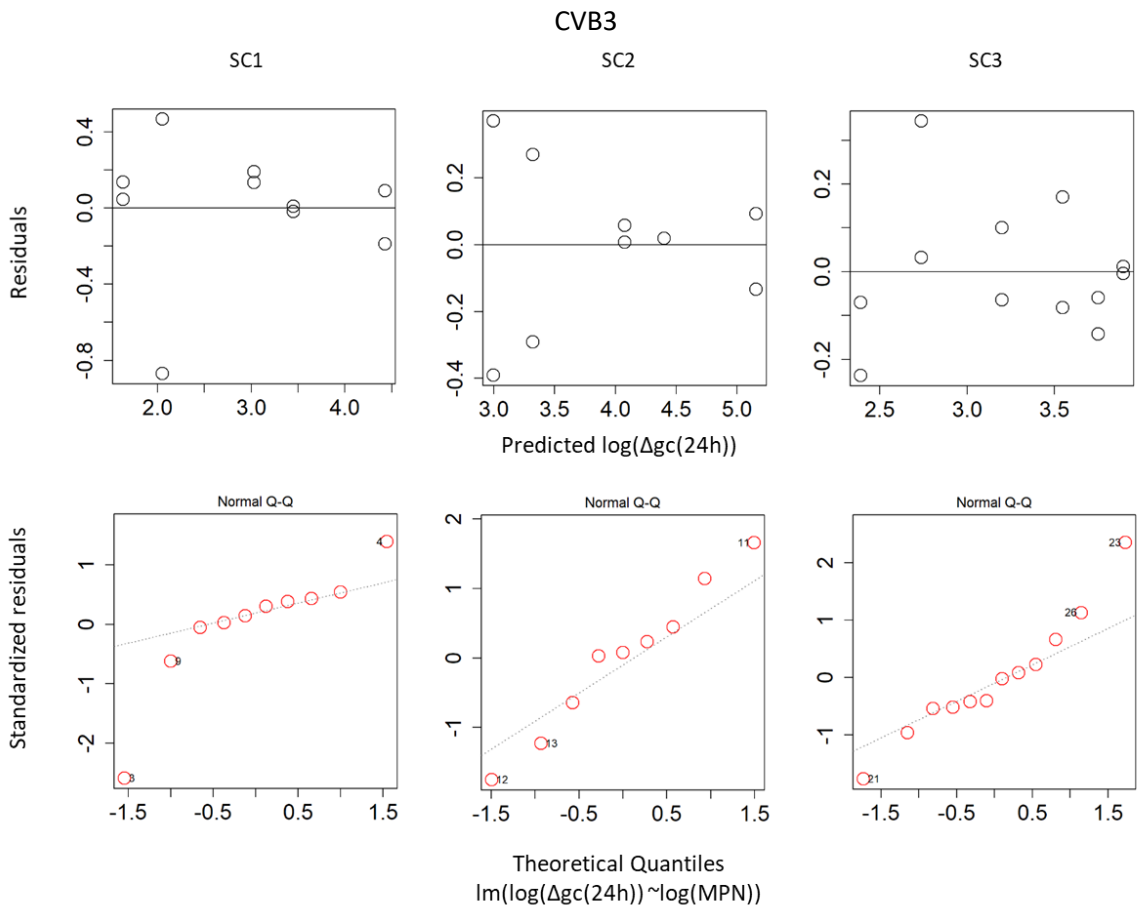
Cq <5 | Cq 5-10 | Cq 10-15 | Cq 15 -20 | Cq 20 -25 | Cq 25 -30

Figure A.2: ΔCq (amplification of specific genotype – amplification of non-specific genotype). Top panel: MFqPCR run 1 (five-fold dilution of pre-amplified sample). Bottom panel: MFqPCR run 2 (20-fold dilution of pre-amplified sample). Each column corresponds to a primer pair, and each row to an enterovirus genotype tested. Differences lower than 3.9 ΔCq are in red, indicating non-specificity of the primers. Colored cells indicate Cq values from primer pairs amplifying their target genotype, and the color indicates the Cq range.

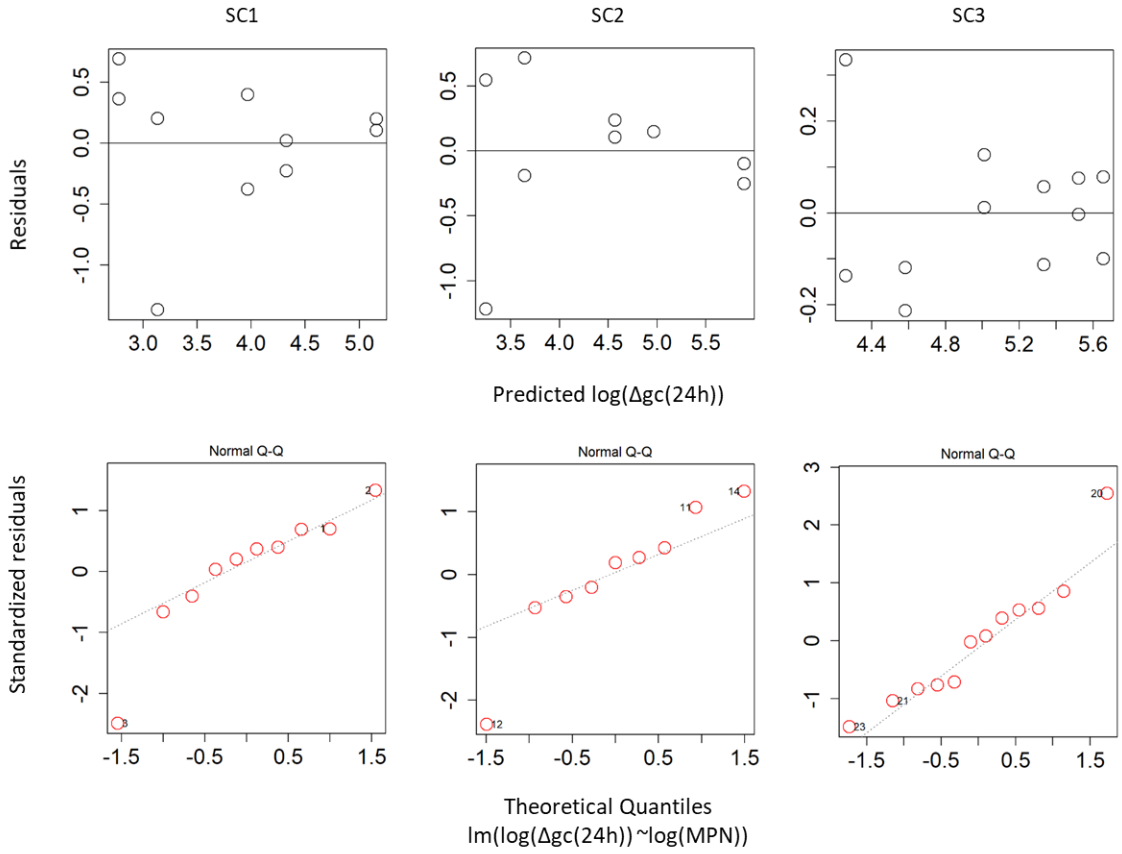

CVA9
SC1
SC2
SC3
Residuals
Predicted log(Δgc(24h))
Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(Δgc(24h)) ~log(MPN))


CVB1
SC1
SC2
SC3
Residuals
Predicted log(Δgc(24h))
Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(Δgc(24h)) ~log(MPN))

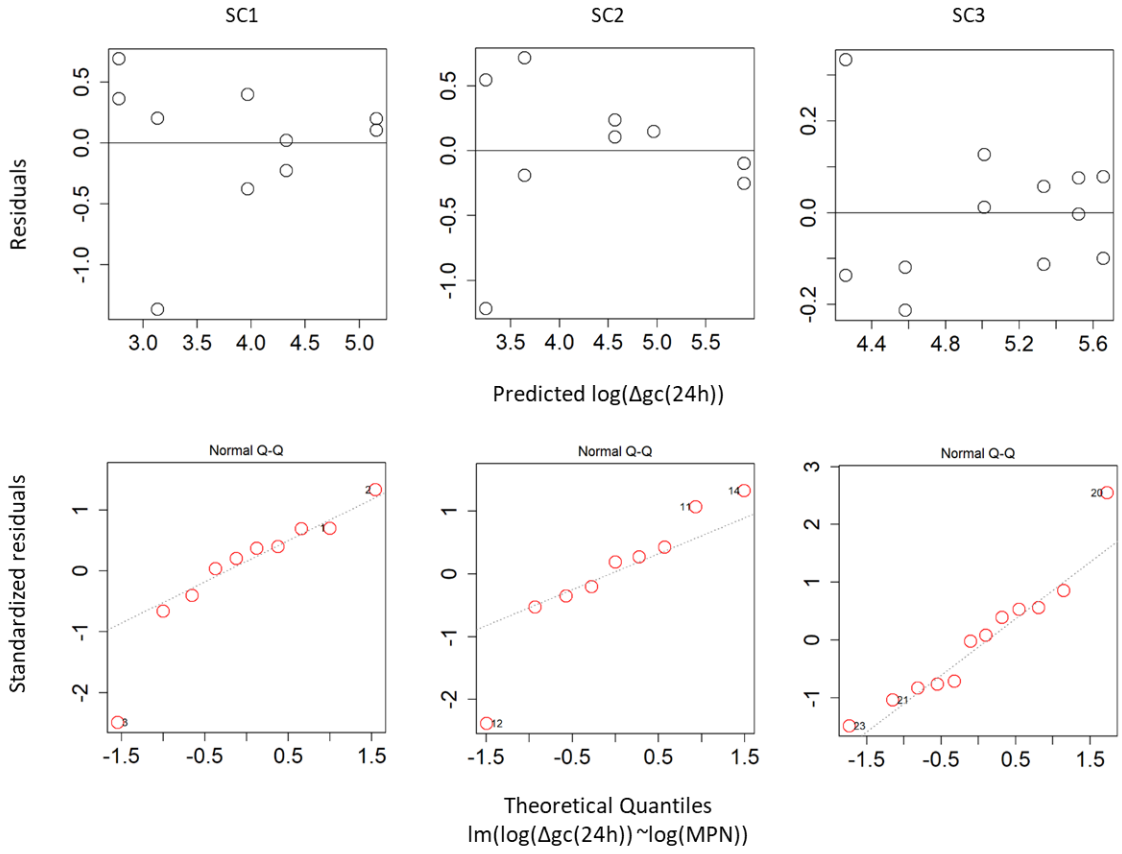

CVB2
SC1
SC2
SC3
Residuals
Predicted log(Δgc(24h))
Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(Δgc(24h)) ~log(MPN))


CVB3
SC1
SC2
SC3
Residuals
Predicted log(Δgc(24h))
Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(Δgc(24h)) ~log(MPN))

CVB4


SC1
SC2
SC3
Residuals
Predicted log(Δgc(24h))
Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(Δgc(24h)) ~log(MPN))

CVB5


SC1
SC2
SC3
Residuals
Predicted log(Δgc(24h))
Normal Q-Q
Standardized residuals
Theoretical Quantiles
lm(log(Δgc(24h)) ~log(MPN))

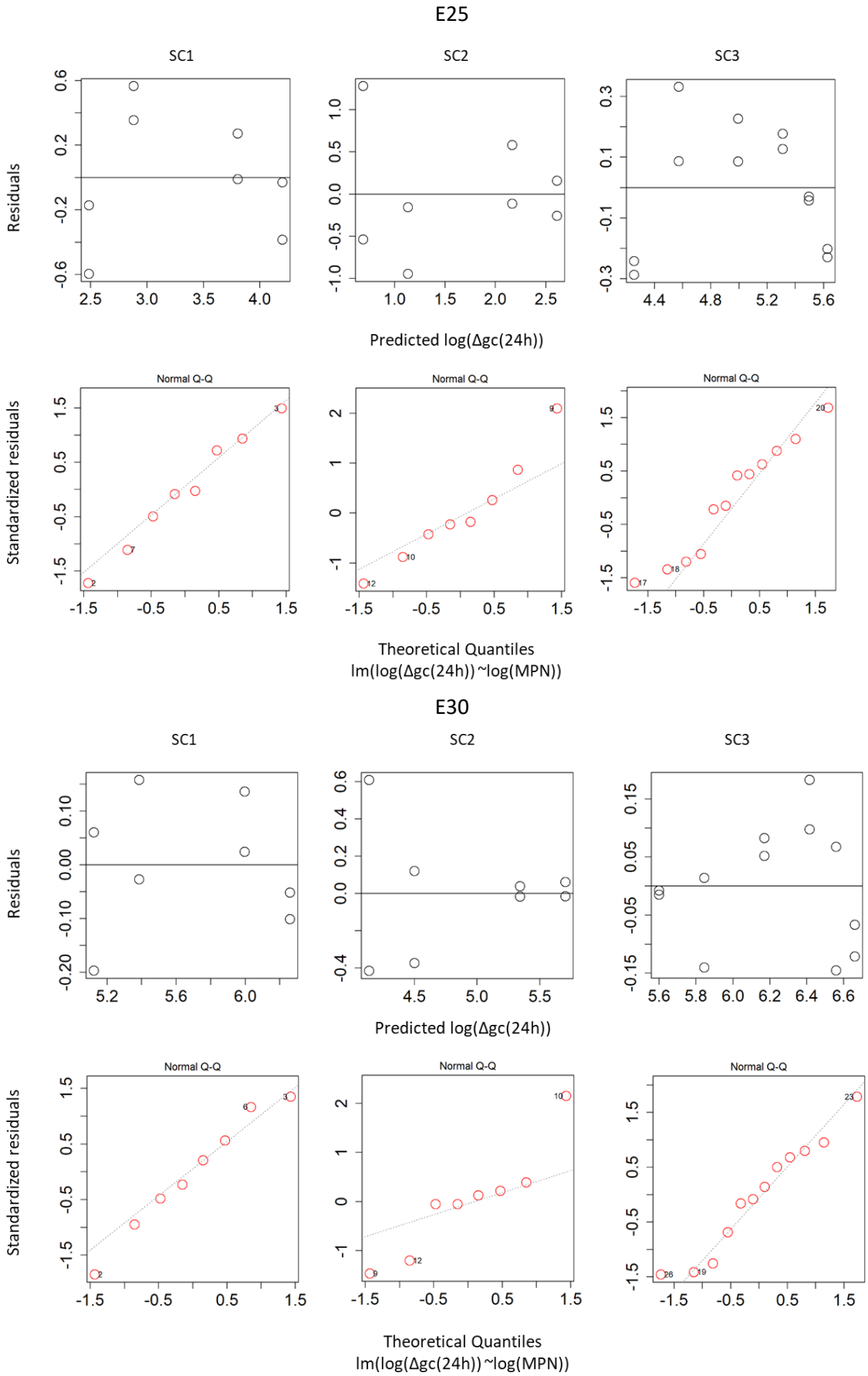


Figure A.3: Regression residuals of the ICC-RTqPCR calibration curves fits and linearity of the residuals

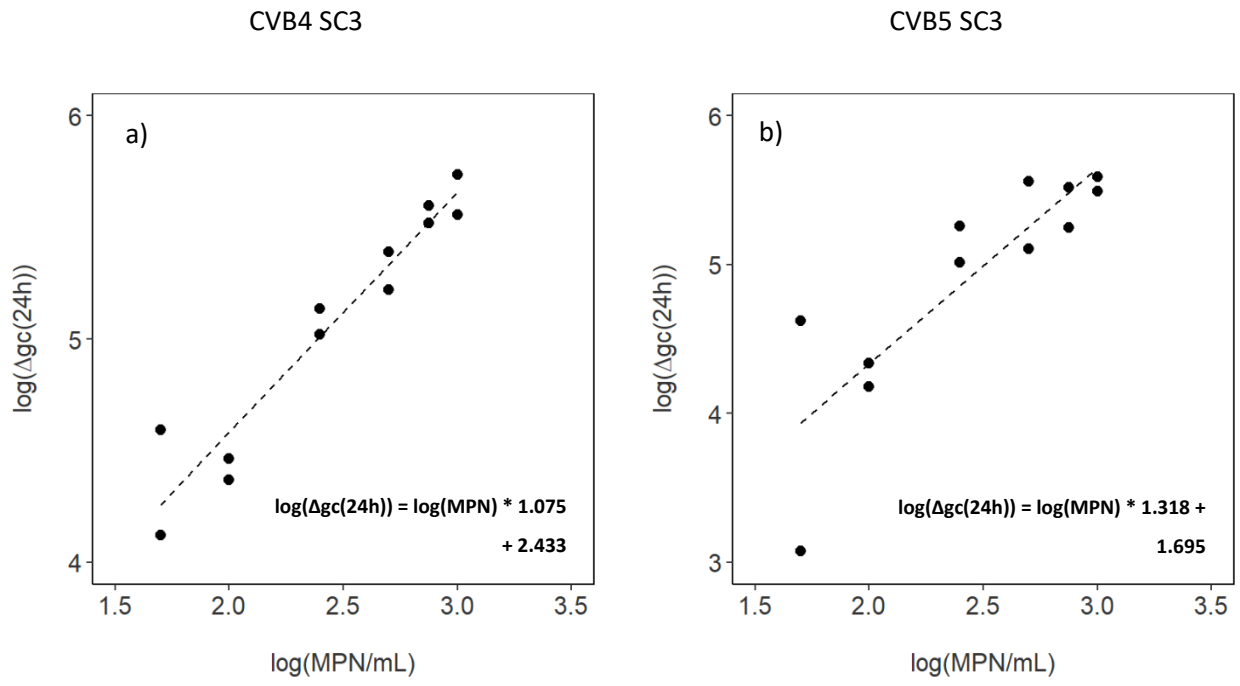


Figure A.4: Calibration curves of the third repeat for CVB4 and CVB5

ITEM TO CHECK	IMPORTANCE	CHECKLIST	COMMENT
EXPERIMENTAL DESIGN			
Definition of experimental and control groups	**E**	**Y**	Material and Methods
Number within each group	**E**	**Y**	Material and Methods
SAMPLE			
Description	**E**	Y	Material and Methods
Microdissection or macrodissection	**E**	N	Not Applicable
Processing procedure	**E**	Y	Material and Methods
If frozen - how and how quickly?	**E**	Y	Material and Methods
If fixed - with what, how quickly?	**E**	N	Not Applicable
Sample storage conditions and duration (especially for FFPE samples)	**E**	Y	Material and Methods
NUCLEIC ACID EXTRACTION			
Procedure and/or instrumentation	**E**	Y	Material and Methods
Name of kit and details of any modifications	**E**	Y	Material and Methods
Details of DNase or RNAse treatment	**E**	N	Not Applicable
Contamination assessment (DNA or RNA)	**E**	N	Not Done
Nucleic acid quantification	**E**	N	Not Done
RNA integrity method/instrument	**E**	N	Not Done
Inhibition testing (Cq dilutions, spike or other)	**E**	Y	Material and Methods
REVERSE TRANSCRIPTION			
Complete reaction conditions	**E**	Y	Material and Methods
Amount of RNA and reaction volume	**E**	Y	Material and Methods
Priming oligonucleotide (if using GSP) and concentration	**E**	Y	Material and Methods
Reverse transcriptase and concentration	**E**	Y	Material and Methods
Temperature and time	**E**	Y	Material and Methods
qPCR TARGET INFORMATION			
Sequence accession number	**E**	Y	Supplementary Information
Amplicon length	**E**	Y	Supplementary Information
In silico specificity screen (BLAST, etc)	**E**	N	Not Provided
Location of each primer by exon or intron (if applicable)	**E**	N	Not Applicable
qPCR OLIGONUCLEOTIDES			
Primer sequences	**E**	Y	Supplementary Information
Location and identity of any modifications	**E**	N	Not Applicable
qPCR PROTOCOL			
Complete reaction conditions	**E**	Y	Material and Methods
Reaction volume and amount of cDNA/DNA	**E**	Y	Material and Methods
Primer, (probe), Mg++ and dNTP concentrations	**E**	Y	According to kit manufacturer, Material and Methods
Polymerase identity and concentration	**E**	Y	Material and Methods
Buffer/kit identity and manufacturer	**E**	Y	According to kit manufacturer, Material and Methods
Additives (SYBR Green I, DMSO, etc.)	**E**	Y	According to kit manufacturer, Material and Methods
Complete thermocycling parameters	**E**	Y	Material and Methods
Manufacturer of qPCR instrument	**E**	Y	Material and Methods
qPCR VALIDATION			
Specificity (gel, sequence, melt, or digest)	**E**	N	Not Provided
For SYBR Green I, Cq of the NTC	**E**	N	Not Provided
Standard curves with slope and y-intercept	**E**	Y	Results
PCR efficiency calculated from slope	**E**	Y	Results and Supplementary Information
r2 of standard curve	**E**	N	Results
Linear dynamic range	**E**	N	Not Done
Evidence for limit of detection	**E**	Y	Results
If multiplex, efficiency and LOD of each assay.	**E**	N	Not Applicable
DATA ANALYSIS			
qPCR analysis program (source, version)	**E**	Y	Material and Methods
Cq method determination	**E**	Y	Material and Methods
Outlier identification and disposition	**E**	N	Not Done
Results of NTCs	**E**	Y	Material and Methods
Justification of number and choice of reference genes	**E**	N	Not Applicable
Description of normalisation method	**E**	N	Not Applicable
Number and concordance of biological replicates	D	Y	Results
Number and stage (RT or qPCR) of technical replicates	**E**	Y	Material and Methods
Repeatability (intra-assay variation)	E	N	Not Done
Statistical methods for result significance	**E**	Y	Material and Methods
Software (source, version)	E	Y	Material and Methods

Figure A.5: MIQE checklist for authors, reviewers and editors

Appendix B : Chapter 3

Sludge sample

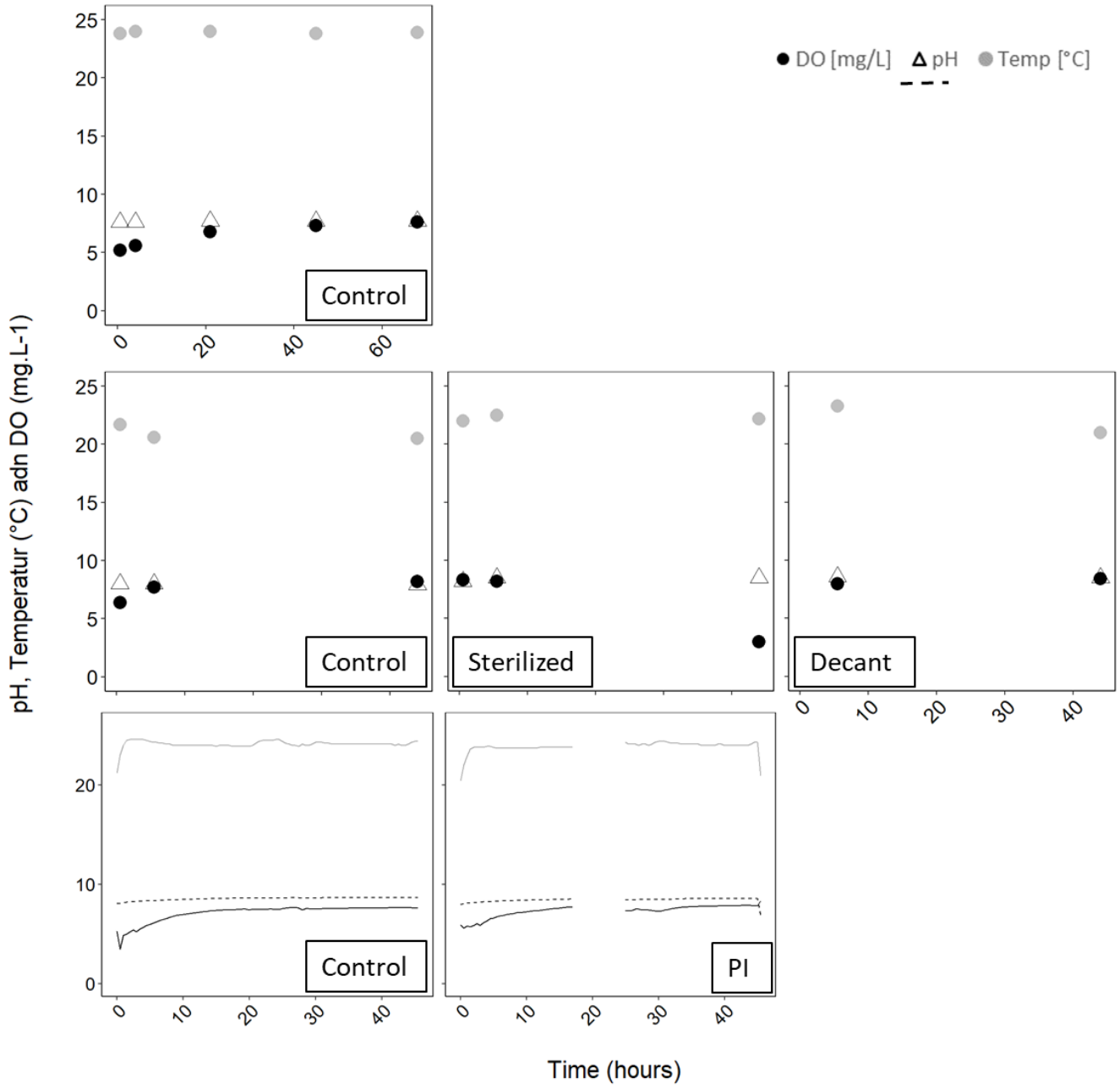


Figure B.1: Chemical characterization of the different sludge samples: dissolved oxygen (DO), pH and Temperature.

Sludge sample

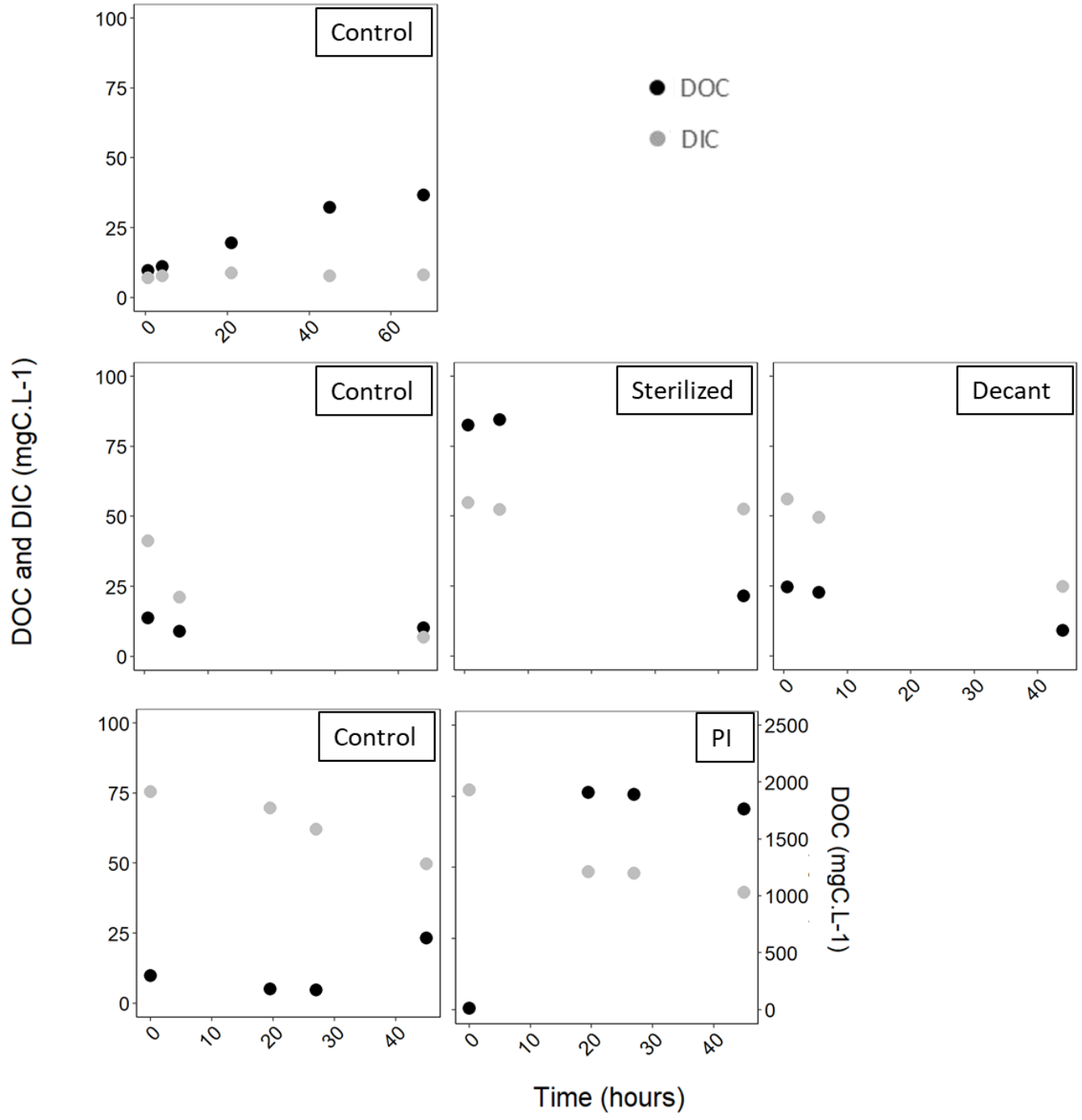


Figure B.2: Chemical characterization of the different sludge samples: Carbon species: dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC)

Sludge sample

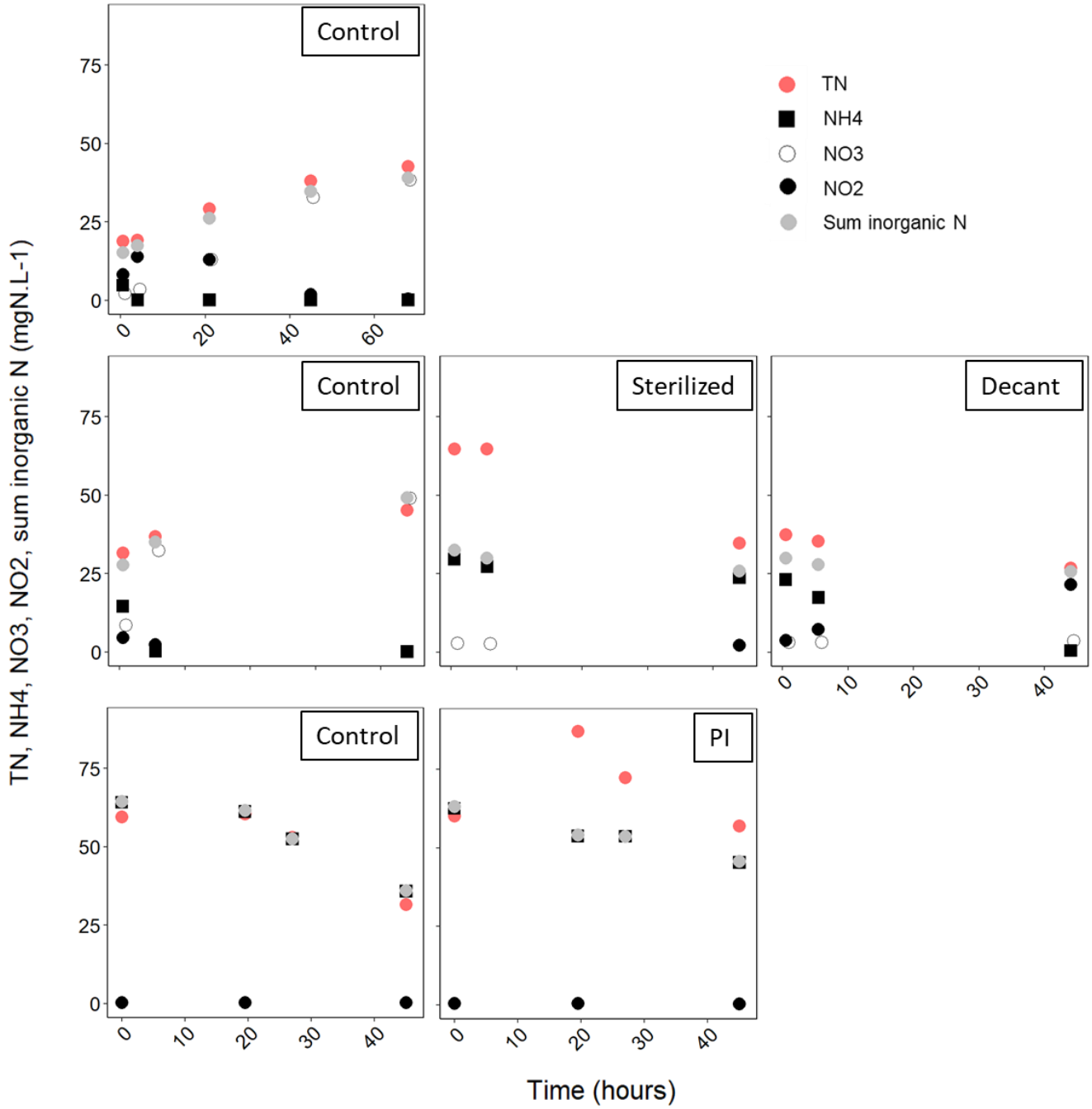


Figure B.3: Chemical characterization of the different sludge samples: Nitrogen species: nitrate (NO3), nitrite (NO2), ammonium (NH4) and total nitrogen (TN).

Table B.1: Total suspended solids (TSS) concentration at the start and the end of each sludge experiment.

	TSS start of experiment (g.L-1)	TSS end of experiment (g.L-1)
Sludge 1	1.4	0.9
Sludge 2	1.4	0.9
Sludge 3	2.4	1.1

Table B.2: Results of the post-hoc pairwise analysis to compare the decay of different genotypes in the super-natant and solids fraction of sludge 1

	CVA9	CVB1	CVB2	CVB3	CVB4	CVB5	E25	E30
CVA9		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
CVB1	<0.01		0.21	0.39	0.28	0.69	0.01	0.14
CVB2	<0.01	0.21		<0.01	<0.01	<0.01	<0.01	<0.01
CVB3	<0.01	0.39	<0.01		1.00	1.00	0.58	0.98
CVB4	<0.01	0.28	<0.01	1.00		0.99	0.73	1.00
CVB5	<0.01	0.69	<0.01	1.00	0.99		0.26	0.84
E25	<0.01	0.01	<0.01	0.58	0.73	0.26		1.00
E30	<0.01	0.14	<0.01	0.98	1.00	0.84	1.00	

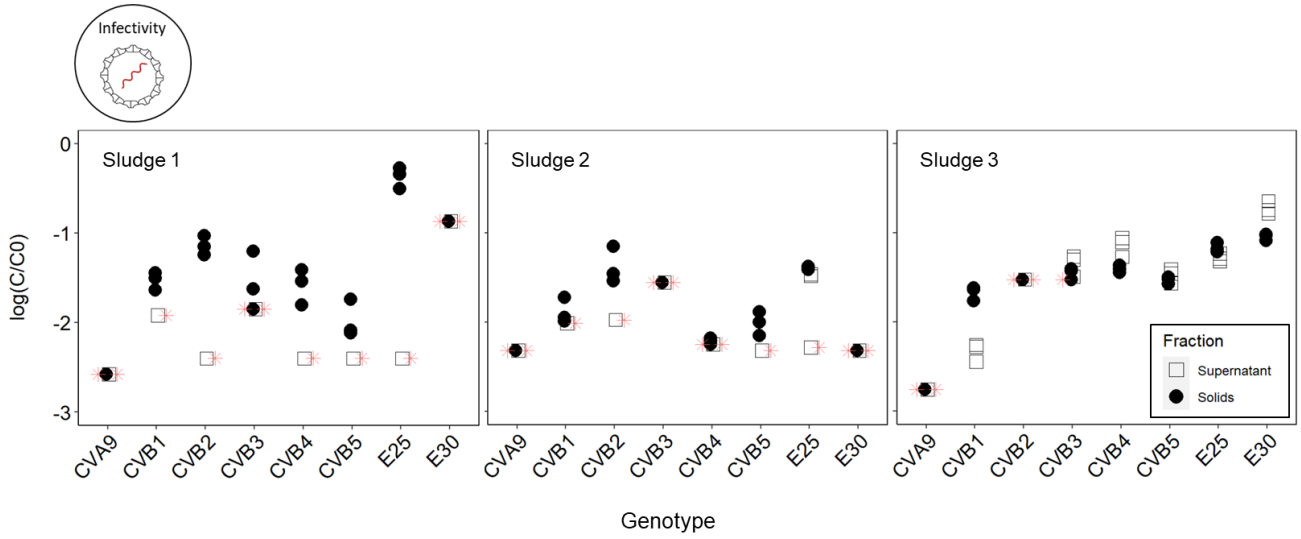


Figure B.4 : log infectivity loss in solids and supernatant fractions in the three activated sludge samples. Red stars indicate concentration <LOQ.

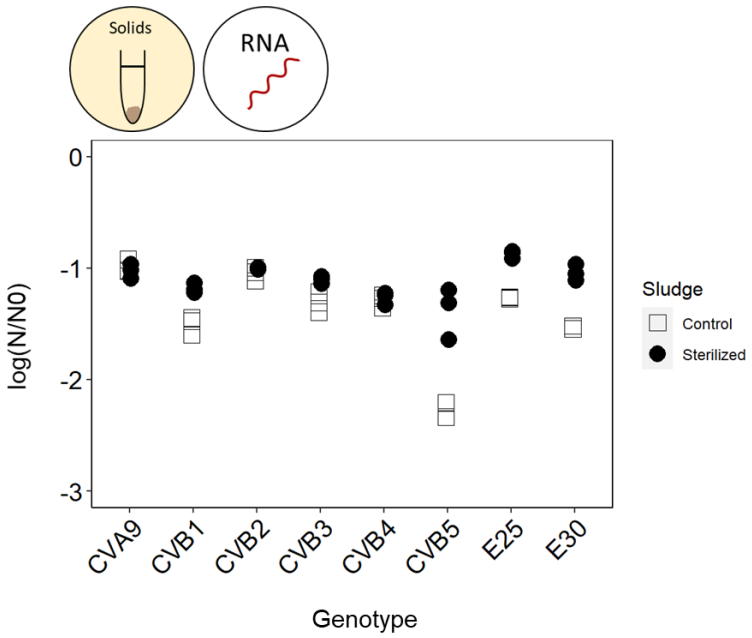


Figure B.5: Adsorption capacity of heat sterilized sludge. Genome copies on the solids after 0.5 hours for control (white) and sterile (black) reactors.

Table B.3: Results of post-hoc pairwise analysis to compare the inactivation of eight genotypes by chlorine. The comparison was done separately for two different chlorine doses (CT).

A) CT= 0.3 mg.min.L^{-1}

	CVA9	CVB2	CVB3	CVB4	CVB5	E25	E30
CVA9		0.45	<0.01	1.00	<0.01	0.01	<0.01
CVB2	0.45		<0.01	0.39	<0.01	<0.01	<0.01
CVB3	<0.01	<0.01		<0.01	1.00	0.11	<0.01
CVB4	1.00	0.39	<0.01		<0.01	0.01	<0.01
CVB5	<0.01	<0.01	1.00	<0.01		0.16	<0.01
E25	0.01	<0.01	0.11	0.01	0.16		<0.01
E30	<0.01	<0.01	<0.01	<0.01	<0.01	0.000	

B) CT= 1 mg.min.L^{-1}

	CVA9	CVB2	CVB3	CVB4	CVB5	E25	E30
CVA9		0.43	<0.01	1.00	<0.01	0.65	1.00
CVB2	0.43		<0.01	0.55	<0.01	0.03	0.39
CVB3	<0.01	<0.01		<0.001	0.89	<0.01	<0.01
CVB4	1.00	0.55	<0.01		<0.01	0.53	1.00
CVB5	<0.01	<0.01	0.89	<0.001		<0.01	<0.01
E25	0.65	0.03	<0.01	0.53	<0.01		0.71
E30	1.00	0.39	<0.01	1.00	<0.01	0.71	

Appendix C : Chapter 4

Table C.1: Changes in the volume inside the chamber during the field experiments, with the type of sample considered, time of the experiment, depth of mooring and experiment considered.

Type of Sample	Initial volume (mL)	Final Volume (mL)	Time of experiment (hours)	Depth of mooring (m)	Experiment (season)
Methylene blue	20	22	120	20	preliminary
Methylene blue	20	18	48	2	1 (winter)
Virus	20	21	48	15	1 (winter)
Virus	20	21.5	48	15	1 (winter)
Methylene blue	20	20	48	2	2 (winter)
Methylene blue	20	19.8	120	2	2 (winter)
Virus	20	20.8	48	15	2 (winter)
Virus	20	21.1	48	15	2 (winter)
Virus	20	22	120	15	2 (winter)
Virus	20	21.2	120	15	2 (winter)
Virus	20	21.2	120	15	2 (winter)
Virus	20	21.2	48	15	3 (spring)
Virus	20	21.6	48	15	3 (spring)
Virus	20	20.4	48	15	3 (spring)
Virus	20	20.6	48	6	3 (spring)
Virus	20	20	48	6	3 (spring)
Virus	20	19.8	48	6	3 (spring)
Virus	20	22	120	15	3 (spring)
Virus	20	22.6	120	15	3 (spring)
Virus	20	21.8	120	6	3 (spring)
Virus	20	21.9	120	6	3 (spring)

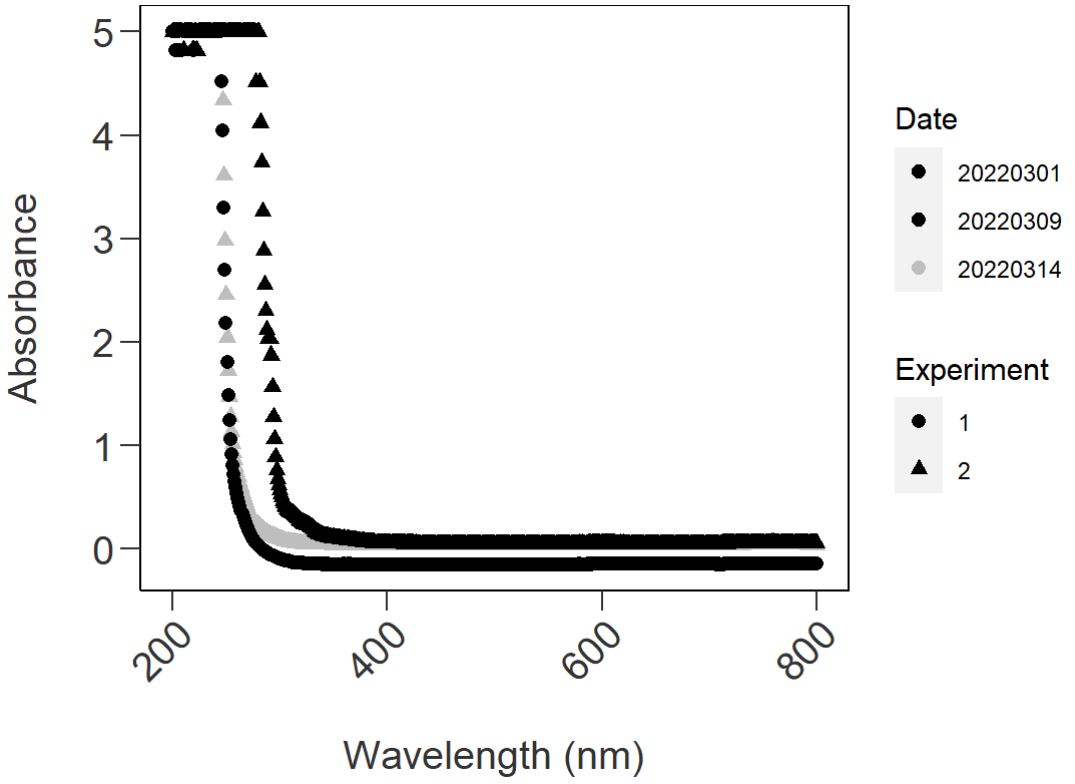


Figure C.1: Absorbance spectrum of the surface water at the start of experiment 1 and 2 and during experiment 2.

Table C.2: Physical-chemical parameters measured in the lake during the three experiments.

Date (experiment)	Temperature	Hour of the day measured	Conductivity (ms.cm-1)	Turbidity (FTU)	pH	DO (mg.L-1)	O_2 saturation (%)	Depth (m)
01.03.2022 (1)	6.9	10:10	0.2	0.2	7.8	10.3	85.1	15
03.03.2022 (1)	6.9	9:30	0.2	0.2	7.8	9.6	79.6	15
09.03.2022 (2)	6.9	15:55	0.2	0.2	7.8	10.1	83.1	15
11.03.2022 (2)	7.0	13:40	0.2	0.3	7.8	9.6	79.6	15
14.03.2022 (2)	7.1	14:15	0.2	0.5	7.8	9.0	74.9	15
15.06.2022 (3)	19	19:10	0.3	not measured	8.7	5.2	55.8	6
16.06.2022 (3)	19.5	05:10	0.3	not measured	8.7	5.0	55.0	6
18.06.2022 (3)	17.7	05:30	0.2	not measured	8.7	5.6	56.9	6
15.06.2022 (3)	12	19:11	0.2	not measured	8.4	5.9	55.0	15
16.06.2022 (3)	13.9	05:11	0.2	not measured	8.6	5.7	54.0	15
18.06.2022 (3)	13.2	06:25	0.2	not measured	8.5	5.9	56.6	15

Table C.3: Results of the one sample t-test to determine if there is significant inactivation of each genotype at 15 m depth in winter.

Genotype	Time	p value	CI95 lower	CI95 upper
CVA9	48	0.33	-0.34	0.16
CVA9	120	0.01	0.08	0.25
CVB1	48	0.03	-0.67	-0.09
CVB1	120	0.04	-2.44	-0.22
CVB2	48	0.71	-0.39	0.30
CVB2	120	0.01	0.06	0.14
CVB3	48	0.03	-1.17	-0.09
CVB3	120	0.00	-0.38	-0.29
CVB4	48	0.78	-0.49	0.40
CVB4	120	0.03	-1.01	-0.13
CVB5	48	0.00	-0.63	-0.30
CVB5	120	0.00	-0.67	-0.55
E25	48	0.74	-0.64	0.51
E25	120	0.00	-1.61	-1.29
E30	48	0.02	0.13	0.78
E30	120	0.02	-1.11	-0.24

Table C.4: Results of the one sample t-test to determine if there is significant inactivation of each genotype at 15 m depth in spring. CVA9 after five days is not included because both replicates were <LOQ, impeding the testing, but it is significantly different from 0.

Genotype	Time	p value	CI95 lower	CI95 upper
CVA9	48	0.14	-0.27	0.08
CVB1	48	0.00	-0.62	-0.44
CVB1	120	0.07	-1.64	0.28
CVB2	48	0.04	0.04	0.46
CVB2	120	0.64	-4.26	3.86
CVB3	48	0.00	-1.19	-0.93
CVB3	120	0.01	-1.26	-0.72
CVB4	48	0.00	-1.03	-0.61
CVB4	120	0.18	-4.75	2.75
CVB5	48	0.00	-1.22	-1.01
CVB5	120	0.03	-1.95	-0.47
E25	48	0.08	-1.52	0.20
E25	120	0.09	-0.62	0.16
E30	48	0.76	-0.68	0.58
E30	120	0.37	-2.02	2.57

Table C.5: Results of the one sample t-test to determine if there is significant inactivation of each genotype at 6 m depth in spring. CVA9 after five days is not included because both replicates were <LOQ, impeding the testing, but it is significantly different from 0.

Genotype	Time	p value	CI95 lower	CI95 upper
CVA9	48	0.15	-2.21	0.67
CVB1	48	0.00	-0.74	-0.55
CVB1	120	0.20	-7.53	4.59
CVB2	48	0.45	-0.54	0.35
CVB2	120	0.04	-2.33	-0.30
CVB3	48	0.00	-1.46	-0.86
CVB3	120	0.04	-1.68	-0.27
CVB4	48	0.03	-1.71	-0.22
CVB4	120	0.08	-2.68	0.55
CVB5	48	0.00	-1.58	-1.00
CVB5	120	0.03	-2.15	-0.42
E25	48	0.06	-1.71	0.08
E25	120	0.00	-1.05	-1.00
E30	48	0.16	-0.29	0.90
E30	120	0.34	-1.20	1.57

Table C.6: Results of the t-test pairwise analysis to compare the inactivation of genotypes at 15 m depth in spring and in winter

Genotype	Time	p value	CI95 lower	CI95 upper
CVA9	120	0.00	-2.57	-2.40
CVB3	120	0.00	-0.73	-0.59
CVB3	48	0.09	-0.94	0.09
CVB4	120	0.19	-1.25	0.38
CVB4	48	0.01	-1.22	-0.34
CVB5	120	0.00	-0.75	-0.45
CVB5	48	0.00	-0.82	-0.49
E25	48	0.08	-1.29	0.11
CVB1	120	0.15	-0.43	1.72
E25	120	0.00	1.05	1.39
E30	120	0.01	0.36	1.54

Table C.7: Results of the t-test pairwise analysis to compare the inactivation of genotypes at 6m depth and at 15 m depth in spring.

Genotype	Time	p value	CI95 lower	CI95 upper
CVB2	120	0.08	-0.30	2.53
CVA9	48	0.12	-0.26	1.61
CVB1	48	0.02	0.03	0.20
CVB1	120	0.24	-1.29	2.87
CVB3	48	0.25	-0.11	0.32
CVB3	120	0.79	-0.27	0.24
CVB4	48	0.47	-0.35	0.64
CVB4	120	0.86	-1.32	1.45
CVB5	48	0.07	-0.02	0.37
CVB5	120	0.51	-0.31	0.46
E25	48	0.61	-0.64	0.96
E25	120	0.00	0.66	0.93

References

1. Pons-salort, M., Parker, E. P. K. & Grassly, N. C. The epidemiology of non-polio enteroviruses : recent advances and outstanding questions. *Curr. Opin. Infect. Dis.* **28**, 479–487 (2015).
2. Tapparel, C., Siegrist, F., Petty, T. J. & Kaiser, L. Infection , Genetics and Evolution Picornavirus and enterovirus diversity with associated human diseases. *Infect. Genet. Evol.* **14**, 282–293 (2013).
3. Benschop, K. S. M. *et al.* Molecular epidemiology and evolutionary trajectory of emerging echovirus 30, Europe. *Emerg. Infect. Dis.* **27**, 1616–1626 (2021).
4. Bonadonna, L. & LaRosa, G. A Review and Update on Waterborne Viral Diseases Associated with Swimming Pools. *Int. J. Environ. Res. Public Health* (2019) doi:10.3390/ijerph16020166.
5. Sinclair, R. G., Jones, E. L. & Gerba, C. P. Viruses in recreational water-borne disease outbreaks : a review. *J. Appl. Microbiol.* **107**, 1769–1780 (2009).
6. Viral Zone. https://viralzone.expasy.org/.
7. Simmonds, P. *et al.* Recommendations for the nomenclature of enteroviruses and rhinoviruses. *Arch. Virol.* **165**, 793–797 (2020).
8. Hyypiä, T., Hovi, T., Knowles, N. J. & Stanway, G. Classification of enteroviruses based on molecular and biological properties. *J. Gen. Virol.* **78**, 1–11 (1997).
9. Oberste, M. S. *et al.* Typing of human enteroviruses by partial sequencing of VP1. *J. Clin. Microbiol.* **37**, 1288–1293 (1999).
10. ICTV, International Committee on Taxonomy of Viruses. https://ictv.global/report/chapter/picornaviridae/picornaviridae/enterovirus.
11. Baggen, J., Thibaut, H. J., Strating, J. R. P. M. & Van Kuppeveld, F. J. M. The life cycle of non-polio enteroviruses and how to target it. *Nat. Rev. Microbiol.* **16**, 368–381 (2018).
12. Racaniello, V. R. One hundred years of poliovirus pathogenesis. *Virology* **344**, 9–16 (2006).
13. Ooi, M. H., Wong, S. C., Lewthwaite, P., Cardosa, M. J. & Solomon, T. Clinical features, diagnosis, and management of enterovirus 71. *Lancet Neurol.* **9**, 1097–1105 (2010).
14. Yi, E. & Shin, Y. Enterovirus 71 infection and vaccines. *Clin Exp Vaccine Res* **6**, 4–14 (2017).
15. González-sanz, R., Casas-alba, D., Launes, C. & Muñoz-almagro, C. Molecular epidemiology of an enterovirus A71 outbreak associated with severe neurological disease , Spain , 2016. 1–9 (2016).
16. Messacar, K. *et al.* A cluster of acute flaccid paralysis and cranial nerve dysfunction temporally associated with an outbreak of enterovirus D68 in children in Colorado, USA. *Lancet* **385**, 1662–1671 (2015).
17. Holm-Hansen, C. C., Midgley, S. E. & Fischer, T. K. Global emergence of enterovirus D68: A systematic review. *Lancet Infect. Dis.* **16**, e64–e75 (2016).
18. Melnick, J. L. & Rennick, V. Infectivity Titers of Enterovirus as Found in Human Stools. *J. Med. Virol.* **5**, 205–220 (1980).
19. Sobsey, M. D. & Meschke, J. S. Virus survival in the environment with special attention to survival in sewage droplets and other environmental media of fecal or respiratory origin. *Rep. World Heal. Organ. Geneva, Switz.* 70 pages (2003).
20. Tani, N. *et al.* Enteric virus levels in river water. *Water Res.* **26**, 45–48 (1992).
21. Moce, L., Lucena, F. & Jofre, J. Enteroviruses and Bacteriophages in Bathing Waters. **71**, 6838–6844 (2005).
22. Lee, S. & Kim, S. Detection of infectious enteroviruses and adenoviruses in tap water in urban areas in Korea. **36**, 248–256 (2002).

23. Payment, P., Tremblay, M. & Trudel, M. Relative resistance to chlorine of poliovirus and coxsackievirus isolates from environmental sources and drinking water. *Appl. Environ. Microbiol.* **49**, 981–983 (1985).
24. D'Alessio, D. J., Minor, T. E., Allen, C. I., Tsiatis, A. A. & Nelson, D. B. A Study of the proportions of swimmers among well controls and children with enterovirus-like illness shedding or not shedding an Enterovirus. *Am. J. Epidemiol.* **113**, (1981).
25. Benschop, K. S. M. *et al.* VIRO-TypeNed, systematic molecular surveillance of enteroviruses in the Netherlands between 2010 and 2014. *Eurosurveillance* **21**, 1–9 (2016).
26. Benschop, K. S. M. *et al.* Polio and measles down the drain: Environmental enterovirus surveillance in the Netherlands, 2005 to 2015. *Appl. Environ. Microbiol.* **83**, 1–12 (2017).
27. Delogu, R. *et al.* Poliovirus and Other Enteroviruses from Environmental Surveillance in Italy , 2009 – 2015. *Food Environ. Virol.* **10**, 333–342 (2018).
28. Hovi, T., Stenvik, M. & Rosenlew, M. Relative abundance of enterovirus serotypes in sewage differs from that in patients: Clinical and epidemiological implications. *Epidemiol. Infect.* **116**, 91–97 (1996).
29. Brouwer, L., Moreni, G., Wolthers, K. C. & Pajkrt, D. World-wide prevalence and genotype distribution of enteroviruses. *Viruses* (2021) doi:10.3390/v13030434.
30. Sedmak, G., Bina, D. & MacDonald, J. Assessment of an Enterovirus Sewage Surveillance System by Comparison of Clinical Isolates with Sewage Isolates from Milwaukee, Wisconsin, Collected August 1994 to December 2002. *Appl. Environ. Microbiol.* **69**, 7181–7187 (2003).
31. Pons-salort, M. *et al.* The seasonality of nonpolio enteroviruses in the United States : Patterns and drivers. *PNAS* **115**, (2018).
32. Brinkman, N. E., Fout, G. S. & Keely, S. P. Retrospective Surveillance of Wastewater To Examine Seasonal Dynamics of Enterovirus Infections. *mSphere* **2**, (2017).
33. Bosch, A., Pintó, R. M. & Abad, F. X. Survival and Transport of Enteric Viruses in the Environment. *Viruses in Foods* 151–187 (2006) doi:10.1007/0-387-29251-9_6.
34. Rao, V. C., Seidel, K. M., Goyal, S. M., Metcalf, T. G. & Melnick, J. L. Isolation of enteroviruses from water, suspended solids, and sediments from Galveston Bay: Survival of poliovirus and rotavirus adsorbed to sediments. *Appl. Environ. Microbiol.* **48**, 404–409 (1984).
35. Lucena, F., Bosch, A., Jofre, J. & Schwartzbrod, L. Identification of viruses isolated from sewage, riverwater and coastal seawater in Barcelona. *Water Res.* **19**, 1237–1239 (1985).
36. Matossian, A. M. & Garabedian, G. A. Virucidal action of seawater. *Am. J. Hyg.* **85**, (1967).
37. Lo, S., Gilbert, J. & Hetrick, F. Stability of Human Enteroviruses in Estuarine and Marine Waters. *Appl. Environ. Microbiol.* **32**, 245–249 (1976).
38. Nasser, A. M., Zaruk, N., Tenenbaum, L. & Netzan, Y. Comparative survival of Cryptosporidium , coxsackievirus A9 and Escherichia coli in stream , brackish and sea waters. *Water Sci. Technol.* 91–96 (2003).
39. Yates, M. V, Gerba, C. P. & Kelley, L. M. Virus persistence in ground water. *Appl. Environ. Microbiol.* **31**, 778–781 (1985).
40. Boehm, A. B., Silverman, A. I., Schriewer, A. & Goodwin, K. Systematic review and meta-analysis of decay rates of waterborne mammalian viruses and coliphages in surface waters. *Water Res.* **164**, 114898 (2019).
41. Olive, M., Gan, C., Carratalà, A. & Kohn, T. Control of Waterborne Human Viruses by Indigenous Bacteria and Protists Is Influenced by Temperature , Virus Type , and Microbial Species. *Appl. Environ. Microbiol.* **86**, 1–12 (2020).
42. Lycke, E., Magnusson, S. & Lund, E. Studies on the nature of the virus inactivating capacity of sea water. *Arch. Gesamte Virusforsch.* **17**, 409–413 (1965).

43. Shuval, I. & Katzenelson, E. Detection and inactivation of enteric viruses. *EPA US Reports* (1977).
44. Cliver, D. O. & Hermann, J. E. Proteolytic and Microbial inactivation of Enteroviruses. *Water Res.* **6**, 797–805 (1972).
45. Akin, E. W., Hill, W. F., Cline, G. B. & Benton, W. H. The loss of poliovirus 1 infectivity in marine waters. *Water Res.* **10**, 59–63 (1976).
46. Kohn, T. & Nelson, K. L. Sunlight-Mediated Inactivation of MS2 Coliphage via Exogenous Singlet Oxygen Produced by Sensitizers in Natural Waters. *Environ. Sci. Technol.* **41**, 192–197 (2007).
47. Silverman, A. I., Peterson, B. M., Boehm, A. B., McNeill, K. & Nelson, K. L. Sunlight inactivation of human viruses and bacteriophages in coastal waters containing natural photosensitizers. *Environ. Sci. Technol.* **47**, 1870–1878 (2013).
48. Smith, E. M., Gerba, C. P. & Melnick, J. L. Role of sediment in the persistence of enteroviruses in the estuarine environment. *Appl. Environ. Microbiol.* **35**, 685–689 (1978).
49. Labelle, R. L. & Gerba, C. P. Influence of estuarine sediment on virus survival under field Influence of Estuarine Sediment on Virus Survival Under Field Conditions. *Appl. Environ. Microbiol.* **39**, 749–755 (1980).
50. Liew, P. F. & Gerba, C. P. Thermostabilization of enteroviruses by estuarine sediment. *Appl. Environ. Microbiol.* **40**, 305–308 (1980).
51. Lipp, E. K. *et al.* The Effects of Seasonal Variability and Weather on Microbial Fecal Pollution and Enteric Pathogens in a Subtropical Estuary. *Estuaries* **24**, 266 (2001).
52. Shirasaki, N., Matsushita, T., Matsui, Y. & Marubayashi, T. Effect of aluminum hydrolyte species on human enterovirus removal from water during the coagulation process. *Chem. Eng. J.* **284**, 786–793 (2016).
53. Asami, T., Katayama, H., Torrey, J. R., Visvanathan, C. & Furumai, H. Evaluation of virus removal efficiency of coagulation-sedimentation and rapid sand filtration processes in a drinking water treatment plant in Bangkok, Thailand. *Water Res.* **101**, 84–94 (2016).
54. Dullemont, Y. J. *et al.* Removal of microorganisms by slow sand filtration. in *Recent Progress in Slow Sand and Alternative Biofiltration Processes*.
55. Anderson, W. B., DeLoyde, J. L., Van Dyke, M. I. & Huck, P. M. Influence of design and operating conditions on the removal of MS2 bacteriophage by pilot-scale multistage slow sand filtration. *AQUA, Water infrastructure, Ecosyst. Soc.* **November**, (2009).
56. Canh, V. D., Furumai, H. & Katayama, H. Removal of pepper mild mottle virus by full-scale microfiltration and slow sand filtration plants. *npj Clean Water* **2**, 1–7 (2019).
57. Yasui, M., Iso, H., Torii, S., Matsui, Y. & Katayama, H. Applicability of pepper mild mottle virus and cucumber green mottle mosaic virus as process indicators of enteric virus removal by membrane processes at a potable reuse facility. *Water Res.* **206**, 117735 (2021).
58. Adham, S. S., Trussel, R. S., Gagliardo, P. F. & Trussell, R. R. Rejection of MS-2 virus by RO membranes.pdf. *J. Am. Water Work. Assoc.* **90**, (1988).
59. Gerba, C. P., Betancourt, W. Q., Kitajima, M. & Rock, C. M. Reducing uncertainty in estimating virus reduction by advanced water treatment processes. *Water Res.* **133**, 282–288 (2018).
60. Soller, J. A., Eftim, S. E. & Nappier, S. P. Direct Potable Reuse Microbial Risk Assessment Methodology: Sensitivity Analysis and Application to State Log Credit Allocations. *Water Res.* **128**, 286–292 (2018).
61. Kelly, S. M. & Sanderson, W. W. The effect of sewage treatment on viruses. *Sewage Ind. Waste.* **31**, 683–689 (1959).
62. Hewitt, J., Leonard, M., Greening, G. E. & Lewis, G. D. Influence of wastewater treatment

process and the population size on human virus profiles in wastewater. *Water Res.* **45**, 6267–6276 (2011).
63. Costán-Longares, A., Mocé-Llivina, L., Avellón, A., Jofre, J. & Lucena, F. Occurrence and distribution of culturable enteroviruses in wastewater and surface waters of north-eastern Spain. *J. Appl. Microbiol.* **105**, 1945–1955 (2008).
64. Qiu, Y. *et al.* Assessment of human virus removal during municipal wastewater treatment in Edmonton, Canada. *J. Appl. Microbiol.* **119**, 1729–1739 (2015).
65. Kohn, T., Decrey, L. & Vinneras, B. Chemical disinfectants. in *Global Water Pathogen Project* (ed. J.B. Rose and B. Jiménez-Cisneros (eds), Michigan State University, UNESCO: East Lansing, M.) (2017).
66. Sobsey, M. D., Fuji, T. & Shields, P. A. Inactivation of hepatitis A virus and model viruses in water by free choline and monochloramine. *Water Sci. Technol.* **20**, 385–391 (1988).
67. Gerba, C. P., Gramos, D. M. & Nwachuku, N. Comparative Inactivation of Enteroviruses and Adenovirus 2 by UV Light. *Appl. Environ. Microbiol.* **68**, 5167–5169 (2002).
68. Black, S., Thurston, J. A. & Gerba, C. P. Determination of Ct values for chlorine of resistant enteroviruses. *J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng.* **44**, 336–339 (2009).
69. Cromeans, T. L., Kahler, A. M. & Hill, V. R. Inactivation of adenoviruses, enteroviruses, and murine norovirus in water by free chlorine and monochloramine. *Appl. Environ. Microbiol.* **76**, 1028–1033 (2010).
70. Wati, S., Robinson, B. S., Mieog, J., Blackbeard, J. & Keegan, A. R. Chlorine inactivation of coxsackievirus B5 in recycled water destined for non-potable reuse. *J. Water Health* **17**, 124–136 (2019).
71. Shirasaki, N., Matsushita, T., Matsui, Y. & Koriki, S. Suitability of pepper mild mottle virus as a human enteric virus surrogate for assessing the efficacy of thermal or free-chlorine disinfection processes by using infectivity assays and enhanced viability PCR. *Water Res.* **186**, 116409 (2020).
72. Liu, O. C. & McGowan, F. Effect of chlorination on human enteric viruses in partially treated water from the Potomac river estuary. *Environ. Prot. Agency Water supply programs Div. Northeast. water supply Lab.* (1971).
73. Meister, S., Verbyla, M. E., Klinger, M. & Kohn, T. Variability in Disinfection Resistance between Currently Circulating Enterovirus B Serotypes and Strains. *Environ. Sci. Technol.* **52**, 3696–3705 (2018).
74. Torii, S. *et al.* Impact of the heterogeneity in free chlorine, uv254, and ozone susceptibilities among coxsackievirus b5 on the prediction of the overall inactivation efficiency. *Environ. Sci. Technol.* **55**, 3156–3164 (2021).
75. Torii, S. *et al.* Genotype-dependent kinetics of enterovirus inactivation by free chlorine and ultraviolet (UV) irradiation. *Water Res.* **220**, 118712 (2022).
76. EPA USA. Guidance Manual for compliance with the filtration and disinfection requirements for public water systems using surface water sources. (1991).
77. WHO. World Health Organisation: Guidelines for drinking water quality, Third Edition, Vol. 1. Recommendations. **1**, 1–564 (2004).
78. Black & Veatch Corporation. *White's Handbook of Chlorination and Alternative Disinfectants, 5th Edition*. (Wiley, 2010).
79. Kitajima, M., Iker, B. C., Pepper, I. L. & Gerba, C. P. Relative abundance and treatment reduction of viruses during wastewater treatment processes - Identification of potential viral indicators. *Sci. Total Environ.* **488–489**, 290–296 (2014).

80. La Rosa, G., Pourshaban, M., Iaconelli, M. & Muscillo, M. Quantitative real-time PCR of enteric viruses in influent and effluent samples from wastewater treatment plants in Italy. *Ann. Ist. Super. Sanita* (2010) doi:10.4415/ANN-10-03-07.
81. Katayama, H. *et al.* One-year monthly quantitative survey of noroviruses , enteroviruses , and adenoviruses in wastewater collected from six plants in Japan. *Water Res.* **42**, 1441–1448 (2008).
82. Naughton, C. & Rousselot, O. Activated Sludge. in *Global Water Pathogen Project* (ed. J.B. Rose and B. Jiménez-Cisneros (eds), Michigan State University, UNESCO: East Lansing, M.) (2017).
83. Lodder, W. J. & De Roda Husman, A. M. Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands. *Appl. Environ. Microbiol.* **71**, 1453–1461 (2005).
84. Katayama, H. *et al.* One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan. *Water Res.* **42**, 1441–1448 (2008).
85. Gerba & C. Type and strain dependence of enterovirus adsorption to activated sludge, soils and estuarine sediments. *Water Research* (1980) doi:10.1016/0043-1354(80)90176-1.
86. Haun, E., Ulbricht, K., Nogueira, R. & Rosenwinkel, K.-H. Virus elimination in activated sludge systems : from batch tests to mathematical modeling. *Water Sci. Technol.* **70**, 1115–1121 (2014).
87. Kelly, S., Sanderson, W. W. & Neidl, C. Removal of Enteroviruses from Sewage by Activated Sludge. *J. Water Pollut. Control Fed.* **33**, 1056–1062 (1961).
88. Chaudhry, R. M., Nelson, K. L. & Drewes, J. E. Mechanisms of Pathogenic Virus Removal in a Full-Scale Membrane Bioreactor. *Environ. Sci. Technol.* **49**, 2815–2822 (2015).
89. Malina, J. F., Ranganathan, K. R., Sagik, B. P. & Moore, B. E. Poliovirus inactivation by activated sludge. *J. Water Pollut. Control Fed.* **47**, 2178–2183 (1975).
90. Knowlton, D. R. & Ward, R. L. Characterization of virucidal agents in activated sludge. *Appl. Environ. Microbiol.* **53**, 621–626 (1987).
91. Kim, T.-D. & Unno, H. The roles of microbes in the removal and inactivation of viruses. *Water Sci. Technol.* **33**, 243–250 (1996).
92. Nakajima, M. *et al.* Adhesion and releasing of Poliovirus to activated sludge of wastewater purifying plants. *Water Sci. Technol.* 117–121 (1866).
93. Guellil, A. *et al.* Hydrolysis of wastewater colloidal organic matter by extracellular enzymes extracted from activated sludge flocs. *Water Sci. Technol.* **43**, 33–40 (2001).
94. Frolund, B., Griebe, T. & Nielsen, P. H. Enzymatic activity in the activated-sludge floc matrix. *Appl. Microbiol. Biotechnol.* **43**, 755–761 (1995).
95. Goel, R., Mino, T., Satoh, H. & Matsuo, T. Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. *Water Res.* **32**, 2081–2088 (1998).
96. Tao, Z. *et al.* One-year Survey of human enteroviruses from sewage and the factors affecting virus adsorption to the suspended solids. *Sci. Rep.* **6**, 1–9 (2016).
97. Olive, M., Moerman, F., Fernandez-Cassi, X., Altermatt, F. & Kohn, T. Removal of Waterborne Viruses by Tetrahymena pyriformis Is Virus-Specific and Coincides with Changes in Protist Swimming Speed. *Environ. Sci. Technol.* **56**, 4062–4070 (2022).
98. Beychok, M. R. Performance of surface-aerated basins. *Chem. Eng. Prog. Symp. Ser.* **67**, 322–339 (1971).
99. Waldman, P., Meseguer, A., Lucas, F., Moulin, L. & Wurtzer, S. Interaction of Human Enteric Viruses with Microbial Compounds: Implication for Virus Persistence and Disinfection

Treatments. *Environ. Sci. Technol.* **51**, 13633–13640 (2017).
100. Robinson, C. M., Jesudhasan, P. R. & Pfeiffer, J. K. Bacterial lipopolysaccharide binding enhances virion stability and promotes environmental fitness of an enteric virus. *Cell Host Microbe* **15**, 36–46 (2014).
101. Gerba, C. P. *Biological Wastewater Treatment: Principles, Modelling and Design*. (IWA Publishing, 2008).
102. Masters, G. M. & Ela, W. *Introduction to Environmental Engineering and Science*. (Prentice Hall, 2008).
103. Wigginton, K. R., Pecson, B. M., Sigstam, T., Bosshard, F. & Kohn, T. Virus Inactivation Mechanisms: Impact of Disinfectants on Virus Function and Structural Integrity. *Environ. Sci. Technol.* (2012) doi:10.1021/es3029473.
104. Torrey, J. Differences in Viral Disinfection Mechanisms as Revealed by Quantitative Transfection of Echovirus 11 Genomes. *Appl. Environ. Microbiol.* **85**, 1–14 (2019).
105. Nuanualsuwan, S. & Cliver, D. O. Infectivity of RNA from inactivated poliovirus. *Appl. Environ. Microbiol.* **69**, 1629–1632 (2003).
106. Alvarez, M. E. & O'Brien, R. T. Effects of chlorine concentration on the structure of poliovirus. *Appl. Environ. Microbiol.* **43**, 237–239 (1982).
107. Zhong, Q. *et al.* Resistance of Echovirus 11 to ClO2 Is Associated with Enhanced Host Receptor Use, Altered Entry Routes, and High Fitness. *Environ. Sci. Technol.* **51**, 10746–10755 (2017).
108. Sigstam, T. *et al.* Subtle differences in virus composition affect disinfection kinetics and mechanisms. *Appl. Environ. Microbiol.* **79**, 3455–3467 (2013).
109. Maurer, C. P. *et al.* Adenovirus , enterovirus and thermotolerant coliforms in recreational waters from Lake Guaíba beaches , Porto Alegre , Brazil. *J. Water Health* **13**, 1123–1129 (2015).
110. Wurtzer, S., Prevost, B., Lucas, F. S. & Moulin, L. Detection of enterovirus in environmental waters : A new optimized method compared to commercial real-time RT-qPCR kits. *J. Virol. Methods* **209**, 47–54 (2014).
111. Bisseux, M., Debroas, D., Mirand, A. & Archimbaud, C. Monitoring of enterovirus diversity in wastewater by ultra-deep sequencing : An effective complementary tool for clinical enterovirus surveillance. *Water Res.* **169**, (2020).
112. Tao, Z. *et al.* Science of the Total Environment Detection of enteroviruses in urban sewage by next generation sequencing and its application in environmental surveillance. *Sci. Total Environ.* **728**, (2020).
113. Gerba, C. P., Betancourt, W. Q. & Kitajima, M. How much reduction of virus is needed for recycled water: A continuous changing need for assessment? *Water Res.* **108**, 25–31 (2017).
114. Lee, H. K. & Jeong, Y. S. Comparison of total culturable virus assay and multiplex integrated cell culture-PCR for reliability of waterborne virus detection. *Appl. Environ. Microbiol.* **70**, 3632–3636 (2004).
115. Payment, P. & Trudel, M. Immunoperoxidase method with human immune serum globulin for broad-spectrum detection of cultivable human enteric viruses: Application to enumeration of cultivable viruses in environmental samples. *Appl. Environ. Microbiol.* **50**, 1308–1310 (1985).
116. Reynolds, K. a, Gerba, C. P. & Pepper, I. a N. L. Detection of infectious enteroviruses by an integrated cell culture-PCR procedure . Detection of Infectious Enteroviruses by an Integrated Cell Culture-PCR Procedure. *Appl. Environ. Microbiol.* **62**, 1424–1427 (1996).
117. Lee, S. H., Lee, C., Lee, K. W., Cho, H. B. & Kim, S. J. The simultaneous detection of both enteroviruses and adenoviruses in environmental water samples including tap water with an

integrated cell culture-multiplex-nested PCR procedure. *J. Appl. Microbiol.* **98**, 1020–1029 (2005).
118. Blackmer, F., Reynolds, K. A., Gerba, C. P. & Pepper, I. L. Use of integrated cell culture-PCR to evaluate the effectiveness of poliovirus inactivation by chlorine. *Appl. Environ. Microbiol.* **66**, 2267–2268 (2000).
119. Chapron, C. D., Ballester, N. A., Fontaine, J. H., Frades, C. N. & Margolin, A. B. Detection of Astroviruses , Enteroviruses , and Adenovirus Types 40 and 41 in Surface Waters Collected and Evaluated by the Information Collection Rule and an Integrated Cell Culture-Nested PCR Procedure. *Appl. Environ. Microbiol.* **66**, 2520–2525 (2000).
120. Grimm, A. C., Cashdollar, J. L., Williams, F. P. & Fout, G. S. Development of an astrovirus RT-PCR detection assay for use with conventional, real-time, and integrated cell culture/RT-PCR. *Can. J. Microbiol.* **50**, 269–278 (2004).
121. Jiang, Y. *et al.* Detection of infectious hepatitis A virus by integrated cell culture / strand-specific reverse transcriptase-polymerase chain reaction. *J. Appl. Microbiol.* **97**, 1105–1112 (2004).
122. Mayer, B. K., Ryu, H. & Abbaszadegan, M. Treatability of U.S. Environmental Protection Agency contaminant candidate list viruses: Removal of coxsackievirus and echovirus using enhanced coagulation. *Environ. Sci. Technol.* **42**, 6890–6896 (2008).
123. Ryu, H., Schrantz, K. A., Brinkman, N. E. & Boczek, L. A. Applicability of integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR) for the simultaneous detection of the four human enteric enterovirus species in disinfection studies. *J. Virol. Methods* **258**, 35–40 (2018).
124. Antona, D. *et al.* Surveillance of enteroviruses in France, 2000-2004. *Eur. J. Clin. Microbiol. Infect. Dis.* **26**, 403–412 (2007).
125. Bernhard, R., Martin, E., Annette, A., Artur, P. & Elena, T.-L. Epidemiologic Aspects and Laboratory Features of Enterovirus Infections in Western Germany, 2000–2005. *J. Med. Virol.* **79**, 956–962 (2007).
126. Khetsuriani, N., LaMonte-Fowlkes, A., Oberste, M. S. & Pallansch, M. A. Enterovirus Surveillance United States , 1970-2005. *CDC Surveill. Summ.* **55(SS08)**, 1–20 (2006).
127. Bell, E. J. & Cosgrove, B. P. Routine enterovirus diagnosis in a human rhabdomyosarcoma cell line. *Bull. World Health Organ.* **58**, 423–428 (1980).
128. Betancourt, W. Q. & Shulman, L. M. Specific excreted pathogens: environmental and epidemiology aspects poliovirus and other enteroviruses. in *Global Water Pathogen Project* (Rose, J. B., Jimenez, B., Eds.; Michigan State University, UNESCO: East Lansing, MI, 2017).
129. Rodríguez, R. A., Gundy, P. M. & Gerba, C. P. Comparison of BGM and PLC/PRC/5 cell lines for total culturable viral assay of treated sewage. *Appl. Environ. Microbiol.* **74**, 2583–2587 (2008).
130. Chonmaitreel, T., Ford, C., Sanders, C. & Lucia, H. L. Comparison of Cell Cultures for Rapid Isolation of Enteroviruses. *Microbiology* **26**, 2576–2580 (1988).
131. Johnston, S. L. G. & Siegel, C. S. Presumptive Identification of Enteroviruses with RD, HEp-2, and RMK Cell Lines. *J. Clin. Microbiol.* **28**, 1049–1050 (1990).
132. Pérez-Ruiz, M., Navarro-Marí, J. M., Palacios Del Valle, E. & Rosa-Fraile, M. Human rhabdomyosarcoma cells for rapid detection of enteroviruses by shell-vial assay. *J. Med. Microbiol.* **52**, 789–791 (2003).
133. Prim, N. *et al.* Combining Cell Lines to Optimize Isolation of Human Enterovirus From Clinical Specimens : Report of 25 Years of Experience. *J. Med. Virol.* **120**, 116–120 (2013).
134. Schmidt, N. J., Ho, H. H. & Lennette, E. H. Propagation and isolation of group A coxsackieviruses in RD cells. *J. Clin. Microbiol.* **2**, 183–185 (1975).

135. Pepper, I. L., Gerba, C. P. & Gentry, T. J. Introduction to Environmental Microbiology. in *Environmental Microbiology: Third Edition* (2015). doi:10.1016/B978-0-12-394626-3.00001-6.
136. Pickett, B. E. *et al.* ViPR: An open bioinformatics database and analysis resource for virology research. *Nucleic Acids Res.* **40**, 593–598 (2012).
137. Kearse, M. *et al.* Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649 (2012).
138. De Leon, R., Shieh, C., Baric, R. S. & Sobsey, M. D. Detection of Enteroviruses and Hepatitis a Virus. *Proc. 1990 Water Qual. Technol. Conf.* 833–853 (1990).
139. Klymus, K. E. *et al.* Reporting the limits of detection and quantification for environmental DNA assays. *Environ. DNA* 271–282 (2020) doi:10.1002/edn3.29.
140. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (2020).
141. Fox, J. & Weisberg, S. An R Companion to Applied Regression, Third Edition. *Sage, Thousand Oaks CA* (2019).
142. US Environmental Protection Agency. Most Probable Number (MPN) Calculator Version 2.0 - User and System Installation and Administration Manual. 3–4 (2013).
143. Miller, J. N. & Miller, J. C. *Statistics and Chemometrics for Analytical Chemistry*. (Pearson Education Limited, 2005).
144. Ismail, N. S., Olive, M., Fernandez-cassi, X., Bachmann, V. & Kohn, T. Viral Transfer and Inactivation through Zooplankton Trophic Interactions. *Environ. Sci.* (2020) doi:10.1021/acs.est.0c02545.
145. Hu, X. *et al.* Simultaneously Typing Nine Serotypes of Enteroviruses Associated with Hand , Foot , and Mouth Disease by a GeXP Analyzer-Based Multiplex Reverse Transcription-PCR Assay. *J. Clin. Microbiol.* **50**, 288–293 (2012).
146. Bendig, J. W. A., O'Brien, P. S. & Muir, P. Serotype-specific detection of coxsackievirus A16 in clinical specimens by reverse transcription-nested PCR. *J. Clin. Microbiol.* **39**, 3690–3692 (2001).
147. Xiao, X. L. *et al.* Simultaneous detection of enterovirus 70 and coxsackievirus A24 variant by multiplex real-time RT-PCR using an internal control. *J. Virol. Methods* **159**, 23–28 (2009).
148. Nguyen, T. T. T. *et al.* Development of a multiplex polymerase chain reaction assay for simultaneous identification of human enterovirus 71 and coxsackievirus A16. *J. Virol. Methods* **170**, 134–139 (2010).
149. Mayer, B. K., Ryu, H., Gerrity, D. & Abbaszadegan, M. Development and validation of an integrated cell culture-qRTPCR assay for simultaneous quantification of coxsackieviruses, echoviruses, and polioviruses in disinfection studies. *Water Sci. Technol.* **61**, 375–387 (2010).
150. Corre, M. H., Bachmann, V. & Kohn, T. Bacterial matrix metalloproteases and serine proteases contribute to the extra-host inactivation of enteroviruses in lake water. *ISME J.* 1–10 (2022) doi:10.1038/s41396-022-01246-3.
151. Harakeh, S. The behavior of viruses on disinfection by chlorine dioxide and other disinfectants in effluent. *FEMS Microbiol. Lett.* **44**, 335–341 (1987).
152. Larivé, O., Brandani, J., Dubey, M. & Kohn, T. An integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR) method to simultaneously quantify the infectious concentrations of eight environmentally relevant enterovirus serotypes. *J. Virol. Methods* **296**, (2021).
153. Bischel, H. N., Schertenleib, A., Fumasoli, A., Udert, K. M. & Kohn, T. Inactivation kinetics and mechanisms of viral and bacterial pathogen surrogates during urine nitrification. *Environ. Sci.*

Water Res. Technol. **1**, 65–76 (2015).
154. State of Maine Department of Environmental Protection. Notes on activated sludge process control. (2009).
155. Felz, S., Vermeulen, P., van Loosdrecht, M. C. M. & Lin, Y. M. Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). *Water Res.* **157**, 201–208 (2019).
156. Kobayashi, K. & Koike, S. Cellular receptors for enterovirus A71. *J. Biomed. Sci.* **27**, 1–12 (2020).
157. Li, C., Sylvestre, É., Fernandez Cassi, X., Julian, T. R. & Kohn, T. Waterborne virus transport and the associated risks in a large lake. *Revis.*
158. Wait, D. A. & Sobsey, M. D. Comparative survival of enteric viruses and bacteria in Atlantic Ocean seawater. *Water Sci. Technol.* **43**, 139–142 (2001).
159. Shang, Y. *et al.* Factors affecting seasonal variation of microbial community structure in Hulun Lake, China. *Sci. Total Environ.* **805**, 150294 (2022).
160. McFeters, G. a & Stuart, D. G. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. *Appl. Microbiol.* **24**, 805–811 (1972).

Curriculum Vitae

Odile Larivé

Rives de la Morges 7 – 1110 Morges

larive.odile@gmail.com

+41 786472240

---------- EDUCATION ----------

July 2017 - present	**PhD Student,** Laboratory of Environmental Chemistry**,** EPFL. PhD in Environmental Virology.
2012 - 2015	**Master,** Environmental Engineering, EPFL. Focus : Environmental bioprocesses and hydrology. Minor : Social sciences, South-East Asia.
2008 - 2012	**Bachelor,** Environmental Engineering, EPFL. Erasmus exchange program, Universitat Politècnica de València (Spain).
2003 - 2008	**French Baccalauréat, sciences** (secondary diploma), Collège International Beau Soleil, Switzerland.

---------- CORE EXPERIENCE ----------

July 2017 - present

PhD Student, Laboratory of Environmental Chemistry, EPFL.

The goal of my work was to evaluate the diversity of responses of an enterovirus population when exposed to wastewater treatment and to the lake environment.

I first had to develop a method to quantify infectious enteroviruses and design an environmental chamber that allows me to safely expose enteroviruses to the lake environment. I then used those tools to evaluate the effects of wastewater treatment in a lab-built reactor and lake exposure in situ.

This work has implication for predicting the persistence of viruses in the environment and through treatments, and for understanding how enteroviruses differ in their sensitivity to stressors.

I also took active part in the laboratory organization, taking over resource management and orders for the lab during four months when we had no lab technician.

August 2015 - March 2017	**Research Engineer,** Eawag, Dübendorf. Optimization of water recycling systems treating different types of wastewater, Blue Diversion AUTARKY Project. Construction and maintenance of biological reactors, experimental planning, evaluation of water quality and treatment efficiency, method development, writing of reports, resource management.
Feb 2014 May 2014 (4 months)	**Intern,** e-dric.ch, (now Hydrique), Le Mont-sur-Lausanne. Error detection and improvement of flow forecast. Data analysis, correction procedures.

---------- ADDITIONAL EXPERIENCE ----------

Jan - Sept 2021	**Design of an environmental chamber for field experiments with viruses** in collaboration with a mechanical workshop.
Sept 2014 Jan 2015 (5 months)	**Master Thesis,** Eawag, Dübendorf Optimization of biological SBR pilot and modeling to identify the mechanisms limiting biological phosphorus removal at Uster wastewater treatment plant. Eawag, Dübendorf
Feb - June 2013 (5 months)	**Semester Project,** Institute for Work and Health (IST), Lausanne Assessment of biological quality of ecological degreasing products: sampling campaign and air sample analysis.

---------- TECHNICAL SKILLS ----------

Molecular biology techniques: qPCR, PCR, agarose gel electrophoresis, sanger sequencing, primer design.

Software: R statistical software, Geneious, Prism, MS Office, AQUASIM, Matlab.

Data: Design of experiment; data collection, cleaning and analysis; data visualization; statistical analysis; data reporting.

Writing: Scientific articles, posters, conference presentations, OFEV authorization request.

Wet lab skills: cell culture, virus culturing, bacterial plating, lab safety and FOBS 2 and 3 training for biosafety work, building and monitoring biological reactors.

Advanced analytical methods: Flow cytometry, Ion Chromatography, LC-OCD, spectrophotometer,microfluidic qPCR.

Interpersonal and organizational:

- Internal **Leadership Workshop** from the EPFL Post-Doc Association, EPFL.
- **Co-supervised** one master student, oversaw 2 lab technicians and one research assistant who participated in my project.
- **Intercultural**, worked with professionals from multiple countries.
- **Collaborated** successfully with different platforms and services from the campus.
- Initiative and organization: took over **resource management** and orders for the lab.
- Convivial, persevering, interdisciplinary and innovative vision, thorough, good team member.

LANGUAGES

French – Mother tongue

English – Fluent

Spanish – Fluent

German – B1 acquired, B2 in process

PERSONAL INTERESTS

Art: drawing portraits and painting aquarelle landscapes.

Singing in a choir since 2018.

Dance: oriental and disco fox styling.

Hiking in nature and camping.

PERSONAL INFORMATION

Birth: 06.12.1991. Swiss and French citizenship, married, one daughter. Driving license.

SCIENTIFIC ARTICLES

Larivé Odile, Brandani Jade, Dubey Manupriyam, Kohn Tamar. 2021, *Journal of Virological Methods*. An integrated cell culture reverse transcriptase quantitative PCR (ICC-RTqPCR) method to simultaneously quantify the infectious concentrations of eight environmentally relevant enterovirus serotypes

Ziemba Christopher, Larivé Odile, Reynaert Eva, Huisman Theo, Morgenroth Eberhard. 2020. *Science of the Total Environment*. Linking transformations of organic carbon to post-treatment performance in a biological water recycling system.

Ziemba Christopher, Larivé Odile, Deck Svenja, Huisman Theo, Morgenroth Eberhard. 2019. *Water Research X*. Comparing the anti-bacterial performance of chlorination and electrolysis post-treatments in a hand washing water recycling system.

Ziemba Christopher, Larivé Odile, Reynaert Eva, Morgenroth Eberhard. 2018. Chemical composition, nutrient-balancing and biological treatment of hand washing greywater.

Nguyen Mi T., Allemann Lukas, Ziemba Christopher, Larivé Odile, Morgenroth Eberhard, Julian Timothy R. 2017. *Frontiers in Environmental Science*. Controlling Bacterial Pathogens in Water for Reuse: Treatment Technologies for Water Recirculation in the Blue Diversion Autarky Toilet.

CONFERENCES

May 2022 — **Conference presentation**, International Society for Food and Environmental Virology Conference, Santiago de Compostela (Spain).
Shaping of an Enterovirus population by wastewater treatment.

Oct 2018 — **Poster presentation**, International Society for Food and Environmental Virology Conference, Tempe Arizona (USA).
Developing A Method To Specifically And Simultaneously Measure The Concentration Of Infective Enterovirus Serotypes In Environmental Samples.

Oct 2017 — **Conference presentation**, International IWA Conference S2Small, Nantes (France).
Optimizing chlorination and electrolysis dosages for hand washing water recycling post-treatment.