TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät für Chemie

Institut für Wasserchemie und Chemische Balneologie

Lehrstuhl für Analytische Chemie und Wasserchemie

Rapid concentration and detection methods for enteric viruses in water

Sandra Hess

Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. Martin Elsner
Prüfer der Dissertation:	1. Priv.-Doz. Dr. Michael Seidel
	2. Prof. Dr. Sabrina Schreiner

Die Dissertation wurde am 07.07.2021 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 27.10.2021 angenommen.

To my dad Thomas and my husband Cory

Acknowledgement

This thesis was developed between March 2015 and February 2018 at the Institute of Hydrochemistry of the Technical University Munich under the supervision of Mr. PD Dr. Michael Seidel. The thesis was funded by the joint BMBF project METAWATER – New metagenomics and molecular based tools for European scale identification and control of emergent microbial contaminants in irrigation water.

I want to thank Mr. Prof. Dr. Reinhard Nießner for the opportunity to become a PhD student at his institute. I appreciated the constructive discussions and his expert opinion regarding my research.

My special thanks go furthermore to Mr. PD Dr. Michael Seidel for giving me the opportunity to do my PhD work in this interesting field of research under his supervision. I appreciated the good working conditions, the well-equipped laboratory and the opportunity to take part at conferences. Furthermore, I want to thank Mr. Seidel for his generous scientific support and his encouragement, which highly contributed to the success of this work as well as to my personal development.

My special thanks go to one of my closest and best friends Katharina Zirngibl for her scientific as well as personal support. I appreciated the great times we spent together and all the conversations we had.

Furthermore, I want to thank my colleagues Dr. Dennis Elsäßer, Dr. Andreas Kunze, Dr. Carolin Hartmann, Dr. Jonas Bemetz, Jessica Beyerl, Dr. Anna Neumann and Susanne Mahler for the good teamwork and time at the IWC. I also thank all further co-workers as well as PhD students for the pleasant working environment during my time at the institute.

I appreciated the great working environment inside of the METAWATER project. My special thanks go to Marta Rosinol, Maria Hellmér and Immaculada Amorós for the good teamwork and constructive discussions. Furthermore, I want to thank Prof. Dr. Sabrina Schreiner-Gruber, Dr. Lars Jurzik and Florian Kubek for their generous cooperation and support.

Finally, I want to thank my parents, especially my dad Dr. Thomas Schäfer, and my husband Cory Hess. I am thankful for your support, your trust and your believe in me. Thank you for encouraging me when necessary, which brought me to where I am right now.

Parts of this thesis were submitted for publishing or already published.

Hess, S.; Niessner, R.; Seidel, M. Quantitative detection of human adenovirus from river water by monolithic adsorption filtration and quantitative PCR. Journal of Virological Methods, 2021, 292, 114128.
https://doi.org/10.1016/j.jviromet.2021.114128.

Table of Content

I. Introduction

Pandemics, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), showcase the risk that viruses can pose to human health and how the uncontrolled transmission of them can impact our daily lives as well as the world economy. While viruses are most commonly transmitted by direct person-to-person contact, other indirect routes of transmission are often overlooked. The most important and unpredictable one is water, which has a direct impact on human health if contaminated by viruses. However, data on viral waterborne diseases is still fragmented, either because research is focusing only on particular countries or certain pathogens and because viruses have not been the focus of water quality regulations to date (Bertrand et al., 2012).

Each year more than 250 million diseases and up to 20 million deaths occur in the human population due to an exposure to waterborne pathogens. These pathogens can be found in water used for drinking as well as recreational purposes and they may also be present in water employed in agricultural procedures, such as the irrigation of crops and the processing of food (Bosch, 1998; Bosch et al., 2008; Malik et al., 2012). One of the greatest risks arises from water which is contaminated with fecally derived pathogens of humans and animals (Wilkes et al., 2009; Zamxaka et al., 2004). For this reason, the World Health Organization (WHO) established microbial quality standards, which define potential pathogens indicating the pollution of water by feces in order to ensure the safety of water resources (WHO, 2017). Among these pollutants are potential pathogenic bacteria, such as pathogenic *E. coli*, *Salmonella enterica*, *Campylobacter jejuni* and viruses such as adenoviruses, enteroviruses, rotaviruses, and noroviruses (Seidel et al., 2016).

Despite the fact that microbial standards are often met, waterborne diseases still occur (Gerba et al., 1979; Lipp et al., 2001). Concurrently, intense research of different aquatic environments showed that fecally derived viruses, also known as enteric viruses, can even be found in water which does not contain indicator organisms (Baggi et al., 2001; Espinosa et al., 2009; Gantzer et al., 1998; Hauri et al., 2005; Hot et al., 2003; Jiang and Chu, 2004; Jurzik et al., 2010; Morens et al., 2004; Skraber et al., 2004a; Skraber et al., 2004b). Furthermore, the appearance and diversity of detected enteric viruses was often closely related to the pattern of human infections and, in some cases, also connected with epidemic outbreaks (Fumian et al., 2010; He et al., 2012; Karmakar et al., 2008; Montazeri et al., 2015). The most prevalently found type of human enteric viruses in environmental water is human adenovirus (HAdV) (Jiang et al., 2006), which is known to be one of the leading causes of recreationally associated waterborne disease worldwide (Sinclair et al., 2009). Due to its high occurrence, HAdV is widely used for the detection of fecal pollution and has been proposed to be added as fecal indicator for aquatic environments as well as for bathing water quality (Albinana-Gimenez et al., 2006; Fong and

Lipp, 2005; Hundesa et al., 2006; Miagostovich et al., 2008; Pina et al., 1998; Puig et al., 1994; Tong and Lu, 2011; Wyn-Jones et al., 2011).

Although the associated risk is known, neither HAdV nor other enteric viruses have yet been included in water quality regulations. The main reason for this is their complex and difficult detection in water due to a small load and size on one hand and the lack of affordable methods on the other hand (Maier et al. 2008). Thus, virus analysis is currently only performed in outbreak situations or when an outbreak is suspected (Maunula et al., 2009).

Traditional culture-based techniques have long been the gold standard for virus detection. Particularly in outbreak situations where time is a crucial factor in protecting human health, these methods are not applicable as detection can take up to several days and counts only culturable viruses. Replacing them with molecular approaches such as quantitative polymerase chain reaction (qPCR) has drastically improved sensitivity and reduced turnaround times to just a few hours. However, even with qPCR, direct quantification of viruses in water is often still not possible due to concentration levels below its detection limit (Calgua et al., 2013; van Heerden et al., 2003; van Heerden et al., 2004; Vergara et al., 2016). For this reason, prior concentration steps are required to increase virus concentration and to facilitate detection using qPCR. The most commonly applied technique for virus concentration is VIRus Adsorption Elution (VIRADEL), which has been recommended by governmental organizations for its application in water (Berg et al., 1984). However, VIRADEL carries high capital costs of up to 250 EUR/sample, and its reproducibility can be poor (Ikner et al., 2012). A less known, but far more cost-efficient method, which is based on a similar principle as VIRADEL, is monolithic adsorption filtration (MAF). MAF was developed by Peskoller et al. in 2009 at the Institute of Hydrochemistry of the Technical University Munich and since then, has already been successfully applied for the concentration of different pathogens from various water matrices (Elsaesser et al., 2018; Pei et al., 2012; Wunderlich et al., 2016). MAF is a laboratory developed, non-commercial method, and, due to its easy and affordable production process, carries costs of around 1 EUR/sample. Thus, MAF provides an affordable alternative to previously mentioned methods and allows for a rapid concentration of viruses in sample volumes of up to 10 L with high flow rates and low pressure (Hess et al., 2021). Thereby, MAF represents a promising tool for application in routine virus surveillance of water and provides a solution to move from reactive virus analysis performed in outbreak situations to proactive monitoring of virus transmission by water on a routine basis.

Long-term monitoring of SARS-CoV-2 in wastewater highlighted the potential of the aquatic environment to be leveraged as an early warning system to predict the progress of an outbreak and protect human health (Agrawal et al., 2021). Furthermore, it showed the importance of continuous collection of data on the occurrence and waterborne diffusion of viruses to uncover and better understand the routes of transmission and associated risk level to human health.

With the potential to revolutionize virus concentration in water by offering an affordable and easy-to-implement approach MAF needs to be further optimized for an application in routine virus surveillance. Here, research is needed to optimize the method for enteric viruses and establish a standard operating procedure (SOP) defining each step of the process from sampling to data analysis. To do so, the main transmission routes of enteric viruses by water and the most commonly found enteric viruses in the aquatic environment with the highest relevance to human health need to be identified. Processing conditions for MAF need to be optimized and adjusted to the water and target virus of choice. For an application of the SOP in routine virus surveillance, the established workflow needs to be evaluated at an interlaboratory scale and optimized based on gained results.

The ultimate goal of this thesis was the research on analytical evaluation methods for the detection of HAdV in surface water based on MAF and qPCR to be able to define a standard operating procedure (SOP) for the quantification of viruses in water. This included the evaluation and optimization of MAF to allow for a concentration of HAdV from water at neutral pH as well as a critical examination of qPCR for virus detection. The SOP should improve upon the disadvantages of existing approaches by offering a standardized and easy-to-implement workflow, which combines a short turnaround time with reliable results at an affordable cost and is compatible with field applications. The SOP is developed and evaluated by combining currently practiced protocols for virus concentration, nucleic acid extraction and qPCR detection of three expert laboratories in the area of water research to provide a state-of-the-art workflow. A cascade of different spiking experiments is conducted to evaluate MAF for the concentration of enteric viruses from water. With each step the complexity of the spiking matrix is increased, and the SOP tailored to the application of MAF to concentrate HAdV from surface water.

First, the potential of the SOP to be applied at an interlaboratory scale is examined. Here, the performance of MAF using monoliths with a negatively charged hydroxyl surface (MAF-OH) is compared to skimmed milk flocculation (SMF), an alternative concentration method, which, similar to MAF-OH, also requires sample pre-conditioning to allow for virus adsorption. Targets of concentration are laboratory model viruses HAdV35 and murine norovirus (MNV) representing one DNA and one RNA enteric virus, respectively. Spiking experiments are carried out in artificially contaminated Evian mineral water with a well-defined, highly controlled and consistent water chemistry. Furthermore, different qPCR reagents are evaluated for detection to improve costs of the total workflow.

Based on gained results the SOP is optimized for the concentration of HAdV by MAF in the next step. For the first time, MAF using positively charged monoliths exposing diethyl aminoethyl groups on their surface (MAF-DEAE) should be applied to enable a direct concentration of HAdV without the need of water sample pre-conditioning. Proof-of-principle

experiments are conducted in comparison to MAF-OH using tap water as spiking matrix with a less defined and controlled water chemistry than mineral water. The laboratory model virus HAdV5 is applied as target of concentration due to a limited availability of HAdV35.

In a final step, MAF-DEAE is evaluated for concentrating HAdV directly from surface water. Experiments are carried out in river water as representative matrix with the highest complexity due to an undefined water chemistry. The influence of MAF-DEAE on the limit of detection (LOD) in qPCR is determined for HAdV5 and the most predominantly found waterborne serotype HAdV41. The performance of MAF-DEAE is evaluated for each serotype and compared to SMF. In addition, the impact of different sample volumes on the recovery rate by MAF-DEAE is examined for HAdV5 only due to a limited availability of HAdV41. Gained results are used to tailor the workflow to MAF-DEAE for the concentration of HAdV from river water on one hand and to highlight further points of improvement of the SOP on the other hand.

Figure 1 gives a general overview of the experimental structure.

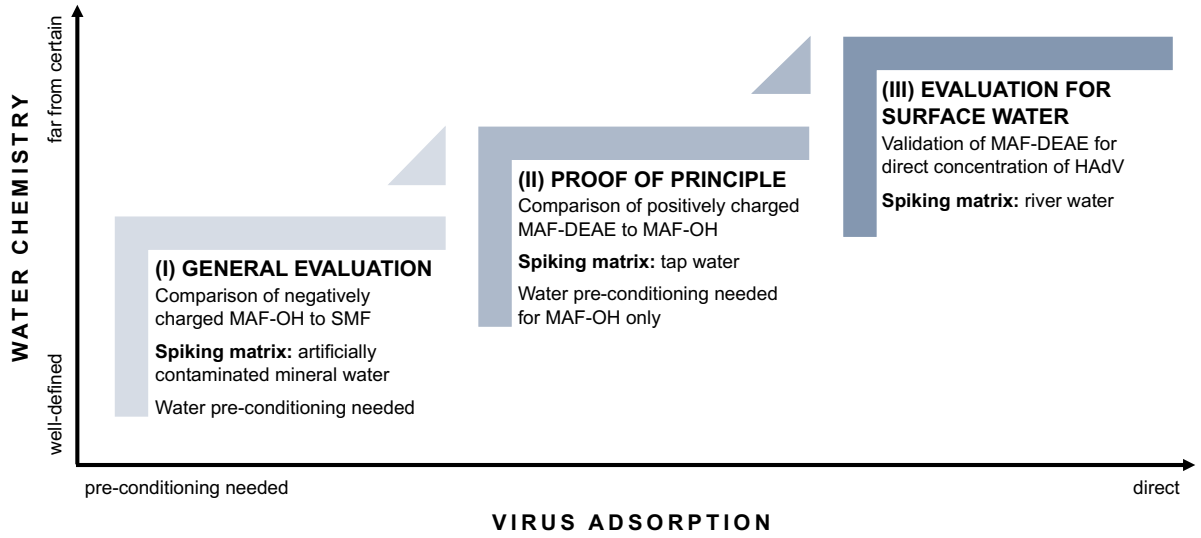


Figure 1: Overview of the different steps to establish a virus adsorption method by MAF for the concentration of HAdV from surface water. (I) General evaluation of negatively charged MAF-OH in comparison to SMF. Spiking experiments are conducted in artificially contaminated mineral water with a well-defined, highly controlled and consistent water chemistry. Water samples are pre-conditioned to allow for adsorption of enteric viruses HAdV35 and murine norovirus (MNV). (II) Proof-of-principle experiments to verify the direct concentration of HAdV by positively charged MAF-DEAE. Performance of MAF-DEAE is compared to MAF-OH using tap water as spiking matrix with a less defined and controlled water chemistry than mineral water. To allow for virus adsorption water samples are pre-conditioned or directly seeded with HAdV5 for experiments with MAF-OH or MAF-DEAE, respectively. (III) Evaluation of MAF-DEAE for concentrating HAdV5 and HAdV41 directly from surface water. River water is used as spiking matrix representative for surface water with the highest complexity due to an undefined water chemistry. No pre-conditioning of water samples is necessary as MAF-DEAE allows for a direct adsorption of HAdV.

II. Fundamentals

1. Health related relevance of viruses in water

Apart from the air we breathe, water is the most essential component for human survival. While this mainly refers to the purpose of hydration, water plays furthermore a substantial role in our daily routine ranging from personal hygiene and food preparation to recreational activities. Thus, we are exposed directly and indirectly to water sources in many ways. Depending on the region, the season and the purpose of use, these include drinking as well as groundwater, but also surface, irrigation and wastewater. Apart from consumption by humans, the aquatic environment moreover represents a habitat for different microbes. Some of the most harmful microbes to human health when present in water represent enteric viruses, which are, among all viruses, the most commonly found type of viruses in water. Viruses are characterized by their type of genome (RNA or DNA), symmetry of capsid, presence of a lipid layer and size. They are categorized into families (-viridae), which are further differentiated into subfamilies (-virinae), followed by genus (-virus), subgenus (-virus) and finally species, which may comprise different serotypes (International Committee on Taxonomy of Viruses Executive Committee, 2020). Enteric viruses include viruses from a wide spectrum of different genera. Thus, they are often genetically diverse, species specific and highly infectious within species. However, all enteric viruses have in common that they enter the human host through the intestinal tract. They can cause a wide range of diseases, including acute gastroenteritis, conjunctivitis, myocarditis and infectious hepatitis (Dhopeshwarkar et al., 1957; Naidu and Viswanathan, 1957; Viswanathan and Sidhu, 1957).

Enteric viruses can be categorized as following:

- viruses inducing localized inflammation at any level of the intestinal tract resulting in acute gastroenteritis, such as rotaviruses, caliciviruses, adenoviruses, astroviruses
- viruses multiplying at any level of the intestinal tract. They lead to a few enteric symptoms before causing diseases of clinical relevance at a distant site, such as measles virus, polioviruses, coxsackieviruses, enteroviruses, hepatitis A and E
- viruses spreading to the intestinal tract at later stages of systemic disease, generally in an immunocompromised host, such as human immunodeficiency virus (HIV), cytomegalovirus

In general, acute enteric symptoms such as nausea, vomiting, abdominal pain, fever and acute diarrhea can be caused by a variety of viral, bacterial and parasitic pathogens. However, in contrast to infections with bacterial or parasitic pathogens, those with viral agents cannot be treated with antibiotics and, if left untreated, may lead to death. To date, there are more than 200 identified human serotypes known to be primarily transmitted from person to person via

the fecal-oral route, either directly or indirectly by water (AWWA, 2006; Feachem et al., 1981; Feachem, 1983; Rao, 2013; Williams et al., 1986).

Enteric viruses almost exclusively enter the aquatic environment through sewage contaminated with the feces of infected individuals, which can carry loads of up to 10^{11} virions per gram of stool. Once present, enteric viruses use water as a vehicle for transmission, where they can survive for long periods of time and travel great distances, either directly through the water itself, or on the surface of contaminated foods being shipped from one location to another (Berg, 1967; Lipp and Rose, 1997). These include shellfish, ready-to-eat-foods such as salads, but also raw materials like soft fruits and vegetables, which are preserved frozen (Bertrand et al., 2012).

It has been found that domestic sewage commonly contains levels between 5.000 to 100.000 PFU/L of enteric viruses (Okoh et al., 2010; Rao and Melnick, 1986; Rodriguez-Lazaro et al., 2012; Seidel et al., 2016). Although sewage treatment, dilution and natural inactivation can reduce the viral load by 10 to 1000 folds, no current treatment process exists which can provide virus-free wastewater effluents (Bosch et al., 2006; Rao and Melnick, 1986; Vantarakis and Papapetropoulou, 1999). The reason for this is that viral inactivation is highly variable between virus and matrix types as well as treatment procedures. Consequently, determination of the most resistant virus for a particular treatment in a certain matrix is commonly not feasible with the result that there is no single treatment policy applicable for every virus in every matrix (Bertrand et al., 2012). Thus, treated discharge in general still carries a remaining load between 50 to 100 PFU/L of enteric viruses and represents the main contamination source of aquatic environments when recirculated into surface water (Maalouf et al., 2010). Further sources of surface water contamination are meteorological runoffs, including heavy rains or melting snow, and agricultural runoffs (Formiga-Cruz et al., 2005; Melnick, 1984). As a result, contaminated surface water can pose a significant health risk by introducing enteric viruses to human and animal populations. Common cases of this are, among others, when contaminated water is used as a direct source for drinking purposes, the irrigation of crops, processing of foods, production of ice, and finally, for recreational activities (Abbaszadegan et al., 2003; Rodriguez-Lazaro et al., 2012). Moreover, when applied for irrigation, enteric viruses can also reach groundwater aquifers by infiltrating soils due to their extremely small size (Fout et al., 2003; Gibson and Schwab, 2011b). An illustrational overview of common routes for viral contamination of surface water is shown in Figure 2.

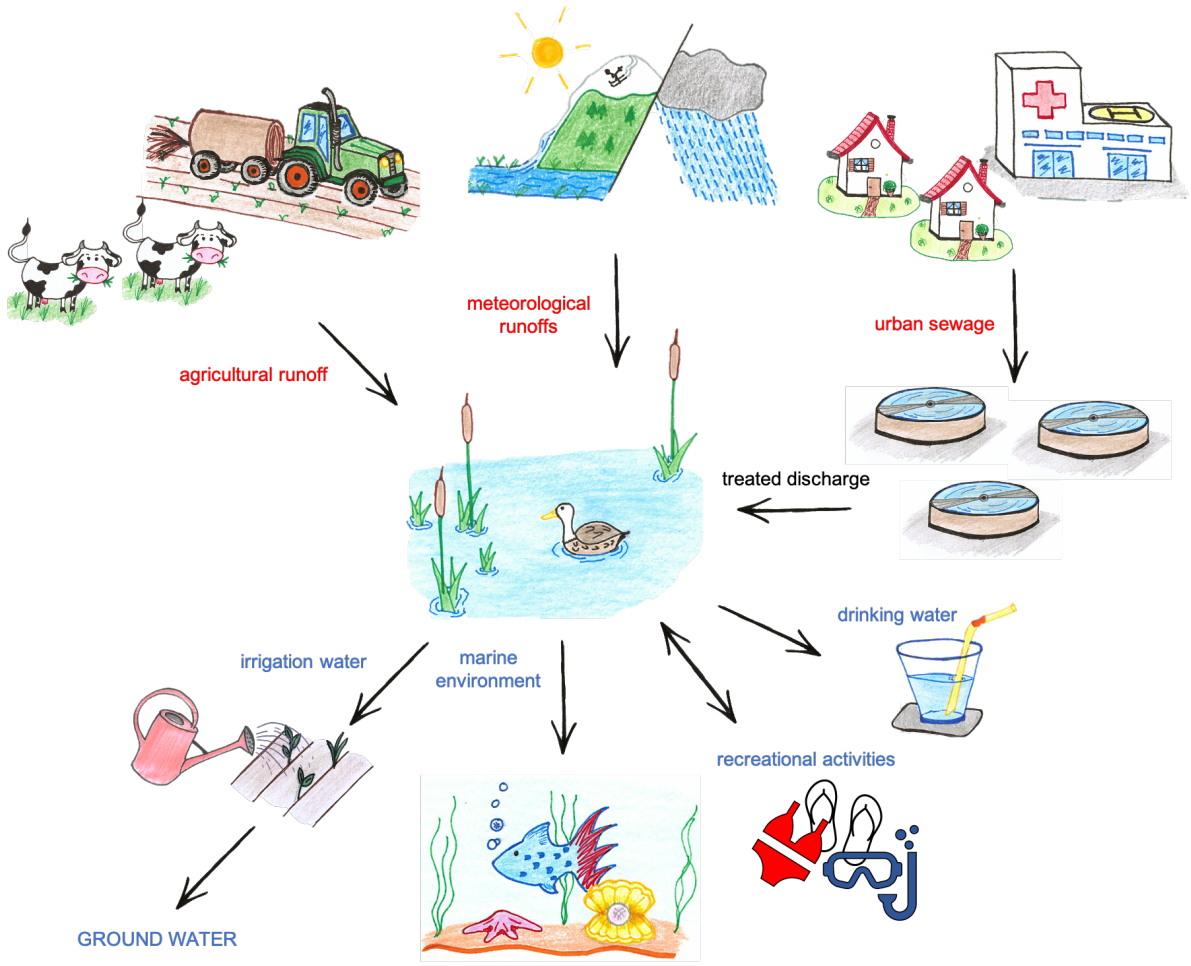


Figure 2: Graphical illustration of common routes for viral contamination of surface water.

Large scale sewage contamination of the aforementioned water supplies can lead to massive outbreaks of waterborne viral diseases in the human population. An example of the impact that an uncontrolled transmission of viruses can have represents the still ongoing pandemic of SARS-CoV-2. SARS-CoV-2 causes coronavirus disease 2019 (COVID-19) with first outbreaks detected in China in December 2019. From China the virus was globally spread until the WHO finally declared SARS-CoV-2 as a pandemic in March 2020 (WHO, 2020). Due date, COVID-19 has been reported in over 213 countries with more than 179 million confirmed cases including 3.8 million deaths. The pandemic has led to nationwide lockdowns over months and strict contact restrictions between people in many countries to prevent further spread of the disease (Kataki et al., 2021). The SARS-CoV-2 RNA genome is excreted by feces and urine of infected individuals leading to its presence in wastewater (Chen et al., 2020; Ren et al., 2019; Foladori et al., 2020). Long-term monitoring of SARS-CoV-2 RNA in wastewater highlighted once more the important role that the aquatic environment plays as an early warning system for disease outbreaks, particularly in case of asymptomatic patients (Agrawal et al., 2021).

Nonetheless, the risk level associated to contaminated water supplies depends in general on whether a minimal quantity of present enteric viruses can cause infections (Jiang et al., 1987).

It has been shown experimentally that only a few virions can lead to an infection and that the risk increases with increasing ingested doses (Melnick, 1957; Sato et al., 1981; Ward et al., 1986). However, the actual risk for an individual to contract an infection when being exposed to water contaminated with low viral loads has not yet been determined (Borchardt et al., 2003). The main reason for this is, that data on waterborne viral diseases is fragmented (Bertrand et al., 2012). While enteric viruses are not part of standard water quality regulations, their presence and prevalence in the aquatic environment is often only assessed after an outbreak or when an outbreak is suspected (Maunula et al., 2009). Collected data has shown that the viral composition in water depends on the geographical region and the season (Myrmel et al., 2006; Nordgren et al., 2009). In regions with a temperate climate, peaks of human enteric virus infections and consecutive excretions are typically found in the summer and early fall months, which coincides with an increase in water-based recreational activities (Kocwa-Haluch, 2001; Nairn and Clements, 1999; Sedmak et al., 2003). On the other hand, the presence of enteric viruses is usually evenly spread throughout the year in tropical climates with a slightly higher prevalence during rainy seasons (Fong and Lipp, 2005).

A general overview of the prevalence in relation to seasonality and geographical regions of the most commonly found enteric viruses in sewage is given in Table 1.

Table 1: Overview of seasonality and geographical prevalence of enteric viruses in sewage.

Virus	Seasonality	Geographical area	Reference
Astrovirus	yes	independent	Girones et al., 2010
Coronavirus (SARS-CoV-2)	yes	independent	Agrawal et al., 2021
Enterovirus	yes	temperate climate	Girones et al., 2010
	no	tropical climate	Fong and Lipp, 2005
Human adenovirus	no	independent	Pina et al., 1998
Hepatitis A virus	yes	endemic areas	Pina et al., 2000; Vaidya et al., 2002
Hepatitis E virus	no	developing countries	Pina et al., 2000
Human rotavirus	yes	independent	Girones et al., 2010
Norovirus	yes	independent	Anon, 2011; Girones et al., 2010; Kozyra et al., 2004

Outbreaks of viral diseases in the human population can lead to shifts in the actual viral composition found in aquatic environments due to higher excretion levels of a certain enteric virus type in sewage (Girones et al., 2010; Sedmak et al., 2003). However, among all enteric viruses, human adenovirus (HAdV) and norovirus (NoV) have been shown to possess the highest prevalence in water and have been identified as the two primary causes of waterborne illnesses worldwide (Leclerc et al., 2002; Pina et al., 1998; Pusch et al., 2005; van Heerden et

al., 2005; Wyn-Jones et al., 2011). As a result, the United States Environmental Protection Agency (USEPA) added both viruses to the Contaminant Candidate List (CCL) for drinking water, which highlights contaminants known or anticipated to be present in public water systems not currently subjected to USEPA drinking water regulations (Chapron et al., 2000; Dongdem et al., 2009; Grabow et al., 2001). Due to their importance and influence on human health, this work and all following sections will focus on HAdV and NoV only. A detailed description of other enteric viruses as well as pathogens and their impact on water environments can be found in *Microbial aspects*, chapter 7 of the *Guidelines for drinking-water quality* released by the WHO in 2017 (WHO, 2017).

1.1. Human Adenovirus (HAdV)

Human Adenovirus (HAdV) is one of the most prevalently found types of human enteric viruses in water and is known to be a leading cause of recreationally associated waterborne disease worldwide (Jiang et al., 2006; Sinclair et al., 2009). HAdV belongs to the genus *Mastadenovirus* and is classified into seven groups (A to G) based on physical, chemical and biological properties, which are further divided into serotypes. It is a medium-sized (90 - 100 nm), non-enveloped virus, which is extremely tolerant to a wide range of environmental conditions and, due to its viral structure, shows a high resistance to most employed disinfection methods as opposed to other human enteric viruses (Calgua et al., 2013; Gerba et al., 2002; Thompson et al., 2003; Thurston-Enriquez et al., 2003). The reason for its high resistance to degradation is based on an excellent DNA repair mechanism contributed to the viral double stranded DNA (dsDNA) genome (Beck et al., 2014; Russell, 2009). The genome of HAdV is approximately 35 kilobases (kb) in length and enclosed in an icosahedral shaped nucleocapsid composed of 252 capsomers. The majority of these capsomers have a six-fold symmetry (hexons), while only the twelve particle corners show a five-fold symmetry (pentons). Each corner has an antenna-like fiber composed of glycoproteins, which vary in length among serotypes and represent the most important receptor-binding structures of HAdV.

Adenoviral infections in humans occur throughout the year and can lead to upper respiratory tract syndromes (acute respiratory diseases, pneumonia, pharyngoconjunctival fever), as well as gastrointestinal (gastroenteritis), ophthalmologic (epidemic keratoconjunctivitis, pharyngoconjunctival fever), genitourinary diseases (cervicitis, urethritis, hemorrhagic cystitis) (Carter, 2005; Fox et al., 1977). Commonly, adenoviruses cause a mild, self-limiting acute infection with infants and children being most susceptible. However, in neonates as well as immunosuppressed patients, adenoviruses can cause fulminant fatal pneumonia, hepatitis and encephalitis.

HAdV is transmitted by exposure to infected individuals via the inhalation of aerosolized droplets and by fecal-oral spread, including contact with recreational marine water, swimming pools, freshwater or tap water (Artieda et al., 2009; Bofill-Mas et al., 2010; Love et al., 2014;

van Heerden et al., 2005; Wyn-Jones et al., 2011). The most predominantly found serotypes in aquatic environments are 40 (HAdV40) and 41 (HAdV41) (Haramoto et al., 2007), which are most commonly associated with gastroenteritis and primarily cause infections in children (Levidiotou et al. 2009; Nakanishi et al., 2009; Reynolds, 2004; Svraka et al., 2007).

Given its robust structure, HAdV withstands gastric and biliary secretions in the human intestinal tract during digestion, which enables HAdV to replicate in the gut. Therefore, excreted levels can reach up to 10^{11} virions per gram of stool in infected individuals, which is often higher than those found for other enteric viruses (Matthes-Martin et al., 2013). Thus, HAdV is commonly present in raw sewage, surface and ground water (Fong et al., 2010; Jurzik et al., 2010; Puig et al., 1994; Rodriguez et al., 2012; Tani et al., 1995). An overview of HAdV occurrence in raw sewage and various water environments in different countries is provided in Table 2.

Table 2: Overview of HAdV occurrence in raw sewage and various water in different countries displayed as a percentage of positive samples with average concentration.

Country	Positive samples (%)	Average concentration range (GU/L)	Reference
Raw Sewage			
Brazil	64.2	$1.7 \times 10^2 – 2.3 \times 10^4$	Prado et al., 2011
Germany	100	$1.0 \times 10^7 – 1.7 \times 10^8$	Hamza et al., 2011
Italy	96.0	3.3×10^9	La Rosa et al., 2010
Japan	100	1.1×10^5	He and Jiang, 2005
New Zealand	100	5.2×10^4	Hewitt et al., 2013
Spain	Not reported	$0.38 - 3.9 \times 10^7$	Bofill-Mas et al., 2006
Sweden	Not reported	$3.3 – 1.9 \times 10^5$	Hellmer et al., 2014
USA	100	Not reported	Bibby and Peccia, 2013
Treated Wastewater			
Brazil	100	$50 – 1.3 \times 10^7$	Schlindwein et al., 2010
Germany	100	$1.5 \times 10^4 – 1.7 \times 10^5$	Hamza et al., 2011
Ghana	80	Not reported	Silverman et al., 2013
Italy	90	6.0×10^8	Carducci and Verani, 2013
Norway	92	2.2×10^1	Grondahl-Rosado et al., 2014
Surface water			
Brazil	96	Not reported	Fongaro et al., 2012
Germany	97	$9.1 \times 10^1 - 5.6 \times 10^4$	Hamza et al., 2011
Hungary	56	1.9×10^4	Kern et al., 2013

Country	Positive samples (%)	Average concentration range (GU/L)	Reference
Japan	61.1	3.2×10^3 - 1.4×10^5	Haramoto et al., 2010
South Africa	35	1.2×10^1 - 4.7×10^3	Chigor et al., 2012
Taiwan	34.3	2.8×10^3	Tao et al., 2016
Spain	100	1.24×10^4	Albinana-Gimenez et al., 2009
USA	24.1	< 500	Xagoraraki et al., 2007
Drinking water source - tap water			
Brazil	100	1.0×10^7	Garcia et al., 2012
Ghana	16.6	Not reported	Gibson et al., 2011
Japan	39.0	Not reported	Haramoto et al., 2012
Drinking water source - surface water			
Norway	90.4	Not reported	Grondahl-Rosado et al., 2014
South Africa	10 - 30	Not reported	Van Heerden et al., 2003
West Africa	9.1	Not reported	Verheyen et al., 2009
Drinking water source - ground water			
France	11.7	Not reported	Ogorzaly et al., 2010
Spain	66.7	7.36	Albinana-Gimenez et al., 2009
USA	32.0	Not reported	Futch et al., 2010

GU/L: genomic unit per liter

Due to is high resistance, HAdV might even be detected in water meeting quality standards for treatment and disinfection (Baggi et al., 2001; Dongdem et al., 2009; Heerden et al., 2005; Pina et al., 1998; Vergara et al., 2016). Thus, HAdV has become one of the most widely used enteric viruses for the detection of fecal pollution in environmental waters and was proposed to be added as a fecal indicator for aquatic environments and bathing water quality (Albinana-Gimenez et al., 2006; Hundesa et al., 2006; Miagostovich et al., 2008; Pina et al., 1998; Puig et al., 1994; Tong and Lu, 2011; Wyn-Jones et al., 2011).

1.2. Norovirus (NoV)

Norovirus (NoV) belongs to the family *Caliciviridae*. In contrast to HAdV, norovirus (NoV) is a non-enveloped virus with a positive-sense single-stranded RNA (ssRNA +) genome of 7.1 - 7.7 kb in length, which is enclosed in an icosahedral capsid of 30 – 35 nm in diameter. NoV is the most common enteric virus in terms of total number of cases and is the leading cause of waterborne acute gastroenteritis (Ahmed et al., 2014; Payne et al., 2013). The NoV is divided into seven genogroups (GI-GVII), whereof only GI, GII and to a small extend also GIV cause infections in humans leading to gastroenteritis associated with nausea, stomach pain, vomiting

and/or diarrhea as well as fever, chills, headaches and muscle pain. NoV is highly contagious, and infections occur worldwide with seasonality in temperate climates, where most of the outbreaks are reported during colder months (Katayama et al., 2008). NoV is primarily transmitted via the fecal-oral route by the ingestion of contaminated water, the consumption of contaminated food or by direct person-to-person contact. Its suitability for transmission by water is based on an inherent persistence and a high resistance to inactivation of the viral particle (Atmar et al., 2008). Although the underlying mechanism is still not clear, it is assumed that conformational changes either by forming a stable protein coat or by aggregation of viral particles enable NoV to survive in aquatic environments for more than a year (Seitz et al., 2011). Therefore, NoV can basically be detected in any type of water that has been in contact with contaminated feces, which usually contain up to 10^{11} virions per gram of stool (Graham et al., 1994; Richards et al., 2015; White et al., 1986). The most commonly found genogroups in water are GI and GII, while GII is responsible for the majority of NoV outbreaks (Siebenga et al., 2009). Table 3 gives an overview of NoV occurrence in raw sewage and various water in different countries.

Table 3: Overview of NoV occurrence in raw sewage and various water in different countries displayed as a percentage of positive samples with average concentration.

Country	Positive samples (%)	Average concentration range (GU/L)	Reference
Raw Sewage			
France	88	6.0×10^7	da Silva et al., 2007
Ireland	95	7.4×10^4	Flannery et al., 2013
Italy	100	$6.8 \times 10^5 – 6.8 \times 10^7$	La Rosa et al., 2010
Japan	100	$2.4 \times 10^3 – 1.9 \times 10^6$	Haramoto et al., 2006
Netherlands	100	$5.1 \times 10^3 – 8.5 \times 10^5$	Lodder and Husman, 2005
New Zealand	82	2.5×10^4	Wolf et al., 2010
Spain	98	3.4×10^9	Perez-Sautu et al., 2012
Sweden	100	9.4×10^6	Nordgren et al., 2009
Switzerland	97	2.7×10^5	Masclaux et al., 2013
USA	100	$1.3 \times 10^5 – 4.0 \times 10^8$	Simmons et al., 2011
Treated Wastewater			
Brazil	Not reported	$1.8 \times 10^2 – 1.1 \times 10^3$	Victoria et al., 2010
France	14	3.0×10^6	da Silva et al., 2007
Germany	15 - 53	$1.8 \times 10^4 – 9.7 \times 10^5$	Pusch et al., 2005
Ireland	22	2.9×10^4	Flannery et al., 2013

Country	Positive samples (%)	Average concentration range (GU/L)	Reference
Italy	65	6.3×10^5	Carducci and Verani, 2013
Japan	92	8.1×10^2	Katayama et al., 2008
Netherlands	100	$9.0 \times 10^2 – 7.5 \times 10^3$	Lodder and Husman, 2005
Spain	100	Not reported	Perez-Sautu et al., 2012
Sweden	100	5.0×10^6	Nordgren et al., 2009
Surface water			
Brazil	Not reported	$1.1 \times 10^2 – 3.7 \times 10^3$	Victoria et al., 2010
Germany	32	$9.4 – 2.7 \times 10^4$	Hamza et al., 2009
Japan	44	6.1×10^2	Haramoto et al., 2005
Netherlands	100	$4.0 - 4.9 \times 10^3$	Lodder and Husman, 2005
New Zealand	17	2.0×10^2	Wolf et al., 2010
Singapore	48	1.0×10^2	Liang et al., 2015
Seawater			
Hong Kong	100	$5.3 \times 10^2 – 3.7 \times 10^3$	Yang et al., 2012
Italy	12	$7.6 – 2.4 \times 10^2$	La Rosa et al., 2009
USA	1	1.0×10^2	Gentry et al., 2009

GU/L: genomic unit per liter

2. Analysis of enteric viruses in water

Monitoring the microbial quality of water is crucial to ensure safe water supplies and protect human health against severe infections caused by waterborne pathogens. This is of particular importance for drinking water sources. Thus, the WHO established guidelines for drinking water quality, which were adopted by multiple nations worldwide (WHO, 2011). These guidelines focus not only on the drinking water itself, but also include additional measures for water treatment and disinfection as well as maintenance and monitoring of water pipe networks which may have an impact on the microbial quality of drinking water (Bartram, 2009; WHO, 2011). Based on these measures a barrier for microbiological contamination is generated to protect water sources used for drinking water generation (Figueras et al., 2010; Morris, 1996). In Germany, such a multi barrier system is already adapted and contains the following five steps (Brauch, 2010):

1. Measures in drainage basin to control microbial and chemical pollution from different sources including agriculture, industry, residential areas, traffic routes
2. Pre-treatment of surface water

3. Slow sand filtration, soil passage, bank filtration
4. Treatment and disinfection
5. Pipe network maintenance and disinfection

Legal norms and technical regulations are in place to control the implementation of the multi barrier system (Brauch, 2010). However, measures are still subordinated to local circumstances which do not always allow an optimal implementation of all barriers.

With the highest risk to human health arising from fecally derived pathogens when present in drinking water sources, the WHO furthermore defined indicator organisms for fecal contamination of water. These are selected according to the following criteria (Borrego and Figueras, 1997; WHO, 2017):

- Universal presence in high numbers in feces of humans and other animals
- Easy and affordable detection by simple methods
- No growth in natural water

Indicators include bacteria, such as total coliforms, fecal coliforms, *Escherichia coli* (*E. coli*), fecal streptococci and enterococci as well as bacteriophages. Nevertheless, occasional outbreaks of infections related to consumption of contaminated drinking water still occur, although criteria for microbial safety of water are met (Craun et al., 2010; Figueras et al., 2010; Gerba et al., 1979; Lipp et al., 2001). Concurrently, different studies reported the presence of fecally derived viruses in water, while indicator organisms could not be found (Baggi et al., 2001; Espinosa et al., 2009; Gantzer et al., 1998; Hauri et al., 2005; Hot et al., 2003; Jiang and Chu, 2004; Jurzik et al., 2010; Morens et al., 2004; Skraber et al., 2004a; Skraber et al., 2004b). This raised the assumption, that the presence of fecally derived viruses such as enteric viruses does not always correlate with the one of conventional indicator organisms. In contrast to bacteria enteric viruses are highly resistant to environmental stress, disinfection and can persist in water for a long period of time. In addition, they carry a 10 to 10.000 times higher health risk for humans due to smaller infectious doses than bacteria (Haas et al., 1993; Melnick et al., 1980; Schiff et al., 1984; Ward et al., 1986). These factors highlight the importance and need of implementing enteric viruses as indicators into water quality regulations, which would improve the risk assessment of water on one hand and ensure its microbial safety to human health on the other hand.

Most detection methods for enteric viruses in water were adopted from clinical diagnostics (Jiang, 2006). However, direct analysis became challenging since their sensitivity of 1 to 1.000 viral particles/µL is often not optimized for the low viral loads found in aquatic environments. Thus, detection is combined with prior concentration steps to increase viral concentrations by reducing the total sample volume from the scope of hundreds or thousands of liters to milliliters

or even microliters. Thereby, an increase in viral concentration of up to 6 log-steps can be achieved, which facilitates the detection of 1 viral particle in 90 m^3 as recommended by the WHO for rotavirus (Kunze et al., 2015; WHO, 2011). It was shown that the application of fast and efficient concentration methods can drastically increase the sensitivity for downstream detection and allow for an easy monitoring of the microbial quality of water (Kunze et al., 2015). Such a monitoring system does not only find application in controlling the microbial quality of drinking water directly. It can furthermore be leveraged to enable a sustainable generation of safe water when being implemented early on in the process of water treatment and monitor the microbial quality of raw water used for drinking water generation. Furthermore, it can also be used to determine the reduction efficiency of drinking water treatment plants (Kunze et al., 2015).

Depending on the point of implementation in the water treatment process a number of concentration steps are required to achieve the necessary sensitivity for detection. The number of steps depends on the type of water and its level of viral contamination. Water of high quality, such as ground and drinking water, often requires several thousand-fold of concentration due to initial sample volumes, which may exceed 100 L. This is reduced to 100 - 1000 mL in a first step (primary concentration) and then further decreased to 5 - 20 mL in a second step to allow for virus detection (secondary concentration) (Hamza et al., 2009; Haramoto et al., 2005; Jiang et al., 2007; Sobsey, 1982). In contrast, water with higher degrees of viral contamination, such as surface water, commonly needs a 1000-fold of concentration with initial sample volumes of approximately 10 L. Here, a single concentration step is often sufficient to allow for virus detection. In raw sewage, viruses can commonly be analyzed directly in sample volumes of 20 – 50 mL without upstream concentration steps.

Requirements for concentration methods are high. At the best, they are applicable for a wide range of water types and quantities, provide a small volume of concentrate and achieve a high and reproducible recovery of all enteric viruses present in the initial water sample. At the same time, they prevent the co-concentration of components which could interfere with viral detection such as dissolved particles and suspended materials (Ikner et al., 2011; Ikner et al., 2012; Cashdollar and Wymer, 2013). Moreover, the ideal concentration method is easy to implement and efficient regarding time and costs (Block and Schwartzbrod, 1989; Wyn-Jones and Sellwood, 2001).

To date, no method exists which fulfills all of these requirements. Therefore, choosing the best fitting concentration method depends on the type of virus and water, its level of contamination and processing volume as well as the available budget and time.

2.1. Concentration of HAdV and NoV from surface water

As previously discussed, particularly surface water represents the major source of introducing enteric viruses to humans when contaminated with feces. Due to the associated risk for human health, this section focusses on the most commonly applied concentration methods within this water matrix and highlights their performance for HAdV and NoV concentration.

2.1.1. Virus concentration by adsorption-elution

Adsorption-elution methods are some of the earliest and to date, still the most commonly used approaches for virus concentration from water. These methods achieve concentration by the adsorption of the virus to a solid matrix while the water is removed. The virus is then eluted in a smaller volume, which in turn increases its initial concentration (Pepper and Gerba, 2015). Adsorption and elution are based on electrostatic and hydrophobic interactions between the solid matrix and the virus. Their strength depends mainly on the net surface charge of the virus and can be controlled by pH and ionic strength conditions (Gerba, 1984). The net surface charge is a serotype specific characteristic of a virion, which is determined by the functional groups of the viral coating proteins. Depending on their protonation state, virions can have a positive, negative or neutral net charge. Figure 3 gives an overview of the different protonation states in relation to the surrounding pH.

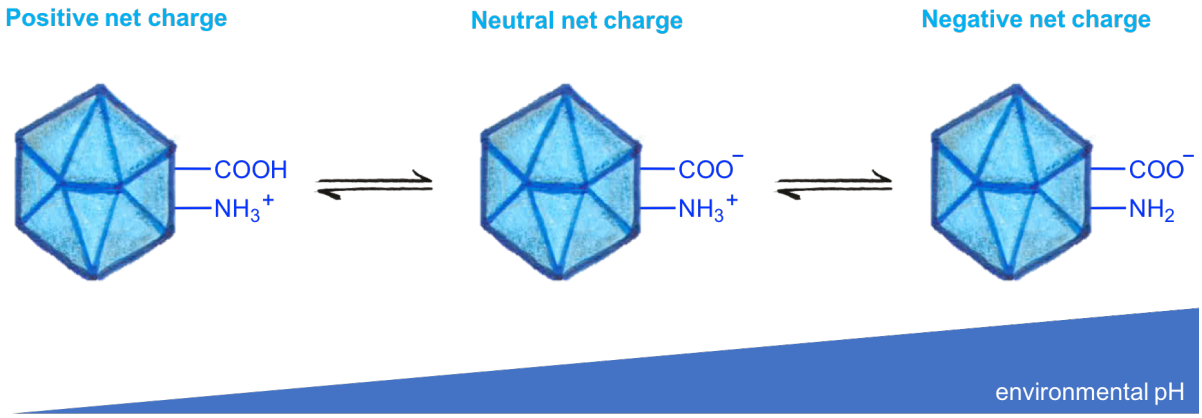


Figure 3: Schematic illustration of different protonation states of functional groups on the viral surface in relation to the surrounding pH. The equilibrium between the H_3O^+ concentration and the carboxyl and amino functional groups changes with the environmental pH. The total net charge of the virus is determined by the superposition of the protonated and unprotonated states of its surface functional groups (Michen and Graule, 2010; modified).

The pH at which the virion has zero or neutral net charge is also known as isoelectric point (IEP) (Parks, 1965). While viruses have a relatively homogeneous surface, changes in pH and ionic strength of the surrounding water may have a high impact on the viral net surface charge. Thus, the IEP is a characteristic parameter of a virion in equilibrium with its environmental water chemistry and provides information about its charge in relation to a certain water matrix. Apart from this, the IEP also controls the adsorption-elution efficiency of a virus to a solid matrix. Most enteric viruses including HAdV and NoV with IEPs of 4.5 and 4.0, respectively, are negatively charged in water, whereas matrices for adsorption can either be positively or

negatively charged (Michen and Graule, 2010; Samandoulgou et al., 2015). While positively charged matrices allow for a direct interaction with viruses, negatively charged matrices require the pre-conditioning of water samples by adjusting pH or ionic strength conditions to enable virus adsorption. Both types have their advantages and disadvantages, which are discussed in more detail in the following sub-sections. However, independently of the used matrix type, the major challenge for matrix-based approaches represents dissolved and colloidal substances present in water, which compete with viruses for adsorption sites. The impact of interference depends on the water's turbidity and chemistry, including pH, presence of salts, proteinaceous materials as well as humic compounds. The result may be variations in viral recoveries in relation to the water type and sampling season. Thus, evaluation and selection of the appropriate matrix in relation to the water type is the first step to achieve a successful concentration.

The second step is choosing the right elution fluid. It has been shown that beef extract buffers (1.5 – 3.0%) at a pH of 9.0 – 9.5 lead to the highest recovery of enteric viruses from solid matrices in most cases, which may even be increased by the addition of glycine (Cashdollar and Dahling, 2006; Dahling, 2002; Fout et al., 1996; Karim et al., 2009; Lambertini et al., 2008; Ma et al., 1994; Melnick et al., 1984). Other elution fluids are based only on amino acids and/or salts (Chang et al., 1981). Aside from inducing virus elution from the adsorption matrix, the high concentration of proteins or salts present in elution fluids may interfere with downstream detection (Abbaszadegan et al., 1993). Thus, elution fluids must be validated for the virus and matrix type as well as for their compatibility with selected detection methods and need to be adjusted accordingly.

An overview of the most commonly applied adsorption-elution methods for the concentration of HAdV and NoV from different water matrices including recoveries and selected workflow conditions relevant to this work is given in Table 4.

Table 4: Overview of the most commonly applied adsorption-elution methods for the concentration of HAdV and NoV from different water matrices.

A. Adenovirus

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	Eluent	Time	Recovery (%)	Reference
VIRADEL									
Positively charged filters									
NanoCeram® filters	41	lake	none	5.9×10^4 GU/L	10.0	1.5% BE 0.05 M Gly pH 9	17.7 min	0.02	Francy et al., 2013
	41	sea	none	1.6×10^7 GU/L 2.5×10^7 GU/L	40.0	3% BE 0.1 M Gly pH 9.5	16.6 min	4.5 3.0	Gibbons et al., 2010
	2	tap	dechlorination	5×10^6 $TCID_{50}$/L	20.0	1% NaPP PB-1 0.05 M Gly pH 9.3	38 min	39.0	Ikner et al., 2011
1 MDS filters	40	tap	pH 7.5	1×10^5 $TCID_{50}$	113.5	1.5% BE 0.05 M Gly pH 9.5	≥ 15 min	26.5	Enriquez and Gerba, 1995
Negatively charged filters									
Filterite	40	sea	1 mM $AlCl_3$ pH 3.5	1×10^5 $TCID_{50}$	113.5	1.5% BE 0.05 M Gly pH 9.5	≥ 15 min	38.0	Enriquez and Gerba, 1995

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	Eluent	Time	Recovery (%)	Reference
	40	tap	0.5 mM $AlCl_3$ pH 3.5	1×10^5 $TCID_{50}$	113.5	1.5% BE 0.05 M Gly pH 9.5	≥ 15 min	36.0	Enriquez and Gerba, 1995
HA membrane	5	lagoon	25 mM $MgCl_2$	1×10^7 $TCID_{50}$/mL	0.5	1 mM NaOH pH 10.5	≥ 5 min	10.0	Rigotto et al., 2009
	n.d.	river	2.5 mM $MgCl_2$	3.9×10^4 GU	1.0	1 mM NaOH pH 11.0	≥ 10 min	3.1 - 5.3	Ahmed et al., 2015
	40	river	250 mM $AlCl_3$, pH 10.8	7.2×10^4 GU/L 7.2×10^5 GU/L	1.3	1 mM NaOH pH 10.8	n.d.	1.03 0.92	Fong et al., 2010
	5	sea	none	1×10^8 $TCID_{50}$/mL	0.5	1 mM NaOH pH 10.5	≥ 5 min	10.0	Rigotto et al., 2009
	n.d.	tap	2.5 mM $MgCl_2$	3.9×10^4 GU	1.0	1 mM NaOH pH 11.0	≥ 10 min	2.4 - 2.8	Ahmed et al., 2015
Glass wool filtration									
	41	ground	pH 7.0	1.6×10^3 GU/L	20.0	3.0% BE 0.5 M Gly pH 9.5	25 min	21.0	Lambertini et al., 2008

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	Eluent	Time	Recovery (%)	Reference
	41	lake	pH 6.5 - 7.0	5.9×10^4 GU/L	10.0	3% BE 0.5 M Gly pH 9.5	20 min	4.7	Francy et al., 2013
	n.d.	river	pH 6.5 - 7.0	1.6×10^5 GU	10.0	3% BE 0.05 M Gly pH 9.5	≥ 15 min	1.3 – 3.0	Ahmed et al., 2016
Monolithic Adsorption Filtration									
Positively charged monoliths									
MAF-DEAE	5	river	none	1.0×10^5 GU/mL	0.1	3.0% BE 0.5 M Gly pH 9.5	< 10 min	63.5	Hess et al., 2021
					0.5			84.0	
					1.0			64.9	
					5.0		15 min	13.0	
					10.0		23 min	14.3	
Negatively charged monoliths									
MAF-OH	2	tap	pH 3.0	3.2×10^2 GU/mL	0.3	3.0% BE 0.5 M Gly pH 9.5	31 min	42.4	Pei et al., 2012

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	Eluent	Time	Recovery (%)	Reference
Skimmed milk flocculation									
	2	river	artificial sea salt, pH 3.5	1.8×10^6 GU/mL	5.0	0.2 M PB-2 pH 7.5	16.5 hrs	25.0 - 95.0	Calgua et al., 2013
	2	sea	pH 3.5	6.9×10^4 GU	10.0	0.2 M PB-2 pH 7.5	16.5 hrs	42.0	Calgua et al., 2008
	35	tap	artificial sea salt, pH 3.5	2.8×10^7 GU	10.0	0.2 M PB-2 pH 7.5	16.5 hrs	66.0	Gonzales-Gustavson et al., 2017

BE: beef extract; Gly: glycine; NaPP: sodium polyphosphate; PB-1: phosphate buffer (3.8 mM Na_2HPO_4, 6.5 mM KH_2PO_4); PB-2: phosphate buffer (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4 1:2 (v/v)); GU: genomic unit; $TCID_{50}$: 50% tissue culture infective dose

B. Norovirus

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	Eluent	Time	Recovery (%)	Reference
VIRADEL									
Positively charged filters									
NanoCeram® filters	NoV (GII)	tap / river	none	9.7×10^4 GU/L	10.0	1.5% BE pH 9.8	> 60 min	29.0 / 18.0	Pang et al., 2012
	NoV	tap / river	none	1.2×10^6 GU/L	10.0	1.5% BE 0.05 M Gly pH 9.0	3 – 122 min	3.6 / 12.2	Karim et al., 2009
	MNV	ground / surface	none	5×10^5 PFU/L	10.0	1.5% BE 0.05 M Gly pH 9.0	> 70 min	30.0 / 6.0	Cashdollar et al., 2013
	NoV (GII)	sea	none	8.8×10^4 GU/L	40.0	3% BE 0.1 M Gly pH 9.5	16.6 min	111.0	Gibbons et al., 2010
1 MDS filters	NoV	tap / river	none	1.2×10^6 GU/L	10	1.5% BE 0.05 M Gly pH 9.0	3 – 122 min	1.2 / 0.4	Karim et al., 2009

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	Eluent	Time	Recovery (%)	Reference
Negatively charged filters									
Filterite	NoV (GI)	irrigation	5 mM $AlCl_3$ pH 3.5	5×10^4 GU/L	10.0	10.0% TPB 0.05 M Gly pH 10.0	n.d.	14.0	El-Senousy et al., 2013
				5×10^5 GU/L				13.0	
				5×10^6 GU/L				16.0	
	NoV (GII)			5×10^4 GU/L				15.0	
				5×10^5 GU/L				15.0	
				5×10^6 GU/L				16.0	
HA membrane	NoV (GII)	tap	5 mM $MgCl_2$	4×10^5 GU/L	2.0	1 mM NaOH	> 20 min	3.0	Victoria et al., 2009
		river		3.3×10^5 GU/L				18	
		sea		1.8×10^5 GU/L				1.0	
	NoV (GI)	river	5 mM $AlCl_3$, pH 3.5	1.0×10^6 GU/L	5.0	Tr alk buffer	n.d.	6.0 – 10.0	De Keuckelaere et al., 2013
	NoV (GII)			1.0×10^7 GU/L				13.0 – 15.0	

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	Eluent	Time	Recovery (%)	Reference
Glass wool filtration									
	NoV (GI)	ground	none	1.1×10^7 GU/L	20.0	3.0% BE 0.5 M Gly pH 9.5	25 min	33.0	Lambertini et al., 2008
	NoV (GII)	ground	none	1.9×10^6 GU/L	20.0	3.0% BE 0.5 M Gly pH 9.5	25 min	16.0	Lambertini et al., 2008
	NoV (GII)	tap	none	1.3×10^3 GU/L	20.0	3.0% BE 0.5 M Gly pH 9.5	25 min	30.0	Lambertini et al., 2008
	NoV (GII)	lake	pH 6.5 – 7.0	4.6×10^3 GU/L	10.0	3.0% BE 0.5 Gly pH 9.5	20 min	2.0	Francy et al., 2013
Monolithic Adsorption filtration									
Negatively charged monoliths (MAF-OH)	MNV	tap	pH 3.0	3.2×10^2 GU/mL	0.3	3.0% BE 0.5 Gly pH 9.5	31 min	42.6	Pei et al., 2011
Skimmed milk flocculation									
	NoV (GII)	river	artificial sea salt, pH 3.5	1.7×10^8 GU/mL	5.0	PB pH 7.5	16.5 hrs	52.0	Calgua et al., 2013

BE: beef extract; Gly: glycine; PB: phosphate buffer (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4 1:2 (v/v)); TBP: tryptose phosphate broth; Tr alk buffer: 0.05 M KH_2PO_4, 1 M NaCl, 0.1% (v/v) Triton X-100, pH 9.2; GU: genomic unit; PFU: plaque forming unit; NoV: human norovirus, MNV: murine norovirus; n.d.: not determined

2.1.1.1. VIRADEL - VIRus ADsorption-ELution

The virus adsorption-elution technique, also known as VIRADEL was developed by Wallis and Melnick in 1967 (Wallis and Melnick, 1967). To date, it is still the most commonly applied method, which is also recommended by the USEPA for the concentration of viruses from water (Berg et al., 1984). VIRADEL uses microporous filters as a solid matrix with negatively or positively charged surfaces (Figure 4).

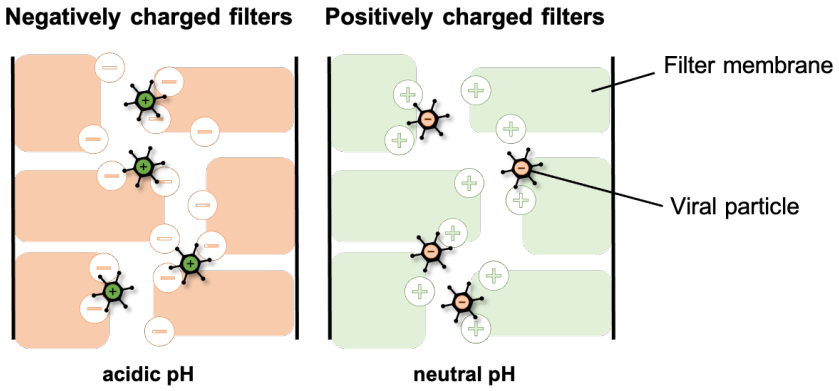


Figure 4: Schematic illustration of microporous filters used for VIRADEL. Left: Negatively charged filters have a negative surface charge leading to adsorption of viral particles at acidic pH. Right: Positively charged filters have a positive surface charge leading to adsorption of viral particles at neutral pH.

Negatively charged filtration

Negatively charged filters have been employed for many years to concentrate HAdV and NoV from different types of water, including sea, tap, surface and wastewater (Beuret, 2003; Bofill-Mas et al., 2010; Haramoto et al., 2004; Haramoto et al., 2009; Victoria et al., 2009). The most widely used negatively charged filters are composed of either glass fiber bound by an epoxy resin (Filterite) or a mixture of cellulose nitrate and cellulose acetate (HA membrane) (Borrego et al., 1991; Haramoto et al., 2009; Victoria et al., 2009). The negative charge of these filters at neutral pH is achieved by esterification or acetylation of the celluloses' glucose molecules with nitro or acetyl groups, respectively. Negatively charged filters have relatively small pore sizes of 0.2 - 0.45 µm and tend to clog with increasing processing volumes in relation to the turbidity of the water sample. To facilitate the processing of larger water volumes, cartridge formats are available, which allow for the concentration of viruses in up to 1000 L, depending on the water type (Asami et al., 2016).

Negatively charged filters show relatively high recoveries and are low in capital costs with expenses of around 18 EUR/filtration (Melnick et al., 1984). However, their major disadvantage is evidenced by the requirement of pre-conditioning of the water sample, either by acidification or by the addition of multivalent cation salts to promote virus adsorption (Haramoto et al., 2004; Haramoto et al., 2005; Haramoto et al., 2010; Preston et al., 1988). An exception represents

seawater, which naturally contains bridging cations enabling the direct adsorption of the virus to the filter surface (Gerba et al., 1978).

Positively charged filtration

In contrast to negatively charged filters, positively charged filters have two key benefits: they allow for a direct adsorption of viruses without the need of water pre-conditioning and enable a fast processing of larger water volumes (>1000 L) due to greater pore sizes of 2.0 µm in average (Karim et al., 2009; Sobsey and Glass 1980). Although these characteristics may be convenient for field applications, their main drawback are high capital costs (Enriquez and Gerba, 1995; Katayama et al., 2002; Lipp et al., 2001; Lukasik et al., 2000). The 1 MDS filter (Cuno, Meriden, CT, USA) is the most commonly applied positively charged filter, which is composed of charge-modified glass and cellulose (Dahling, 2002; Karim et al., 2009; Hill et al., 2009; Sobsey and Glass, 1980). Although this filter was the first one recommended by the USEPA for the VIRADEL process due to its overall performance, its main disadvantage are exorbitantly high costs of approximately 250 EUR/filtration (Berg et al., 1984; Cashdollar and Wymer, 2013). To find a more economical and price competitive alternative to negatively charged filters, the NanoCeram® filter (Argonide, Sanford, FL, USA) was developed. This filter is made out of a thermally bonded blend of micro glass fibers and cellulose, infused with nano alumina fibers in a non-woven matrix and carries costs of around 40 EUR/filtration (Cashdollar and Dahling, 2006; Cashdollar and Wymer, 2013). Comparison studies of NanoCeram® and 1 MDS filters revealed that both filters showed a similar performance for virus concentration from different water (Cashdollar et al., 2013; Chaudhry et al., 2015; Karim et al., 2009; Prevost et al., 2015; Soto-Beltran et al., 2013; Qiu et al., 2015; Ye et al., 2012). Therefore, the USEPA also added NanoCeram® filters to the list of recommended VIRADEL filters with the remark that they are more susceptible to clog than 1 MDS filter (Fout et al., 2010).
NanoCeram® and 1MDS filters have been widely used for the successful concentration of HAdV and NoV from different water, including seeded laboratory studies and detection in swimming pools, drinking and wastewater (Cashdollar et al., 2013; Chapron et al., 2000; Chaudhry et al., 2015; Karim et al., 2009; Pinto et al., 1995).

Independently of the filter type, an inconsistent reproducibility of elution highlights the biggest disadvantage of VIRADEL, which is subject to multiple interferences such as changes in pH, present salts and the composition and loading of dissolved organics of a water sample (Melnick et al., 1984; Sobsey and Glass, 1984; Victoria et al., 2009; Straub and Chandler, 2003). Thus, extensive validation may be required to achieve a satisfying performance, which can be related to high upfront costs depending on the number of filtrations needed.

2.1.1.2. Glass wool filtration

A more economical alternative to VIRADEL can be achieved by glass wool filtration. As implied by its name, glass wool filtration uses glass wool as adsorbent for viruses, which exposes hydrophobic and electropositive sites on its surface. The benefit is a direct adsorption of viruses at a near neutral pH (Figure 5), while, at the same time, diminishing the co-concentration of inhibitors for virus detection (Lambertini et al., 2008; van Heerden et al., 2005).

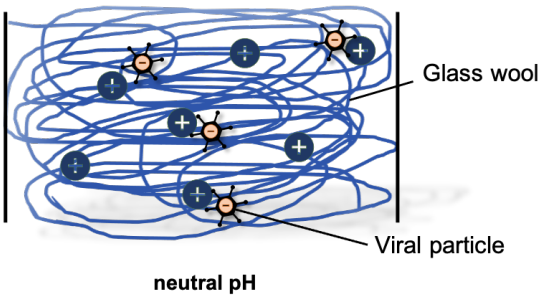


Figure 5: Schematic illustration of virus adsorption to glass wool. Glass wool has a positively charged surface allowing the direct adsorption of viruses at neutral pH.

Moreover, glass wool filtration is easy to implement with low capital costs of less than 1 EUR/filtration (costs of glass wool only; additional costs of around 4 EUR for one-time PVC housing, which can be sanitized and reused). This is mainly attributed to its manual assembly process, in which glass wool is packed in columns by hand at a pre-determined density that can then be applied directly to the water sample (Lambertini et al., 2008). With its easy implementation, cost efficiency and ability to process water samples directly, glass wool filtration fulfills some of the most important requirements for concentration methods, which led to its application in various virus monitoring studies for different water, involving wastewater, seawater, drinking water and groundwater (Calgua et al., 2008; Gantzer et al., 1997; Grabow et al., 2001; van Heerden et al., 2005; Vilaginès et al., 1993; Vivier et al., 2004). However, it has been observed, that the flipside of glass wool filtration are high variances in recovery efficiencies for viruses with reported variations of up to 91% for NoV and 72% for HAdV, which is mainly attributable to missing quality assurance and control of packed columns due to differences in the individual assembly process (Bofill-Mas et al., 2010; Lambertini et al., 2008).

2.1.1.3. Skimmed milk flocculation (SMF)

Another method, which was developed primarily for the enrichment of HAdV from seawater and since then, has been successful applied for the concentration of different viruses from water is skimmed milk flocculation (SMF) (Calgua et al., 2008; Calgua et al., 2013; Bofill-Mas et al., 2010). With costs of around 1 EUR/water sample, SMF is one of the most affordable concentration methods available. In contrast to previously described methods, SMF uses skimmed milk protein flocs as solid matrix for virus adsorption. While this is particularly

beneficial for water samples of high turbidity, such as sewage, where membrane-based approaches may tend to clog, the downside of SMF is a limited compatibility with field application and long turnaround times. The reason for this is attributed to the workflow itself. On one hand, similar to negatively charged filters, SMF requires pre-conditioning of water samples to allow for virus adsorption (Katzenelson et al., 1976). On the other hand, SMF does not enable an in-line processing of water samples as 8 hours of stirring followed by 8 hours of sedimentation is needed for virus adsorption. This limits the total processing volume and results in an extremely long turnaround time of more than 16 hours, independent of the sample volume. Thus, SMF is commonly performed for water samples of up to 10 L only. A schematic illustration of the workflow is given in Figure 6.

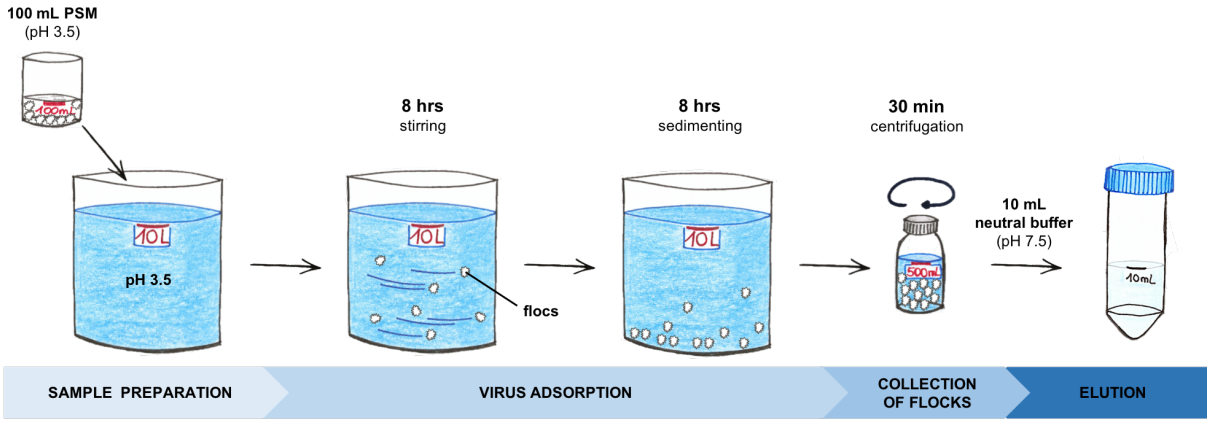


Figure 6: Schematic illustration of the skimmed milk flocculation (SMF) workflow. The water sample of up to 10 L is adjusted to pH 3.5 with a conductivity of 150 µS/cm² and mixed with pre-flocculated skimmed milk (PSM, pH 3.5). Viruses adsorb to flocs under 8 hours of stirring followed by an additional 8 hours of floc sedimentation. The supernatant is discharged and flocs with adsorbed viruses are collected in a remaining volume of around 500 mL by centrifugation for 30 min. The floc pellet is dissolved in up to 10 mL of neutral buffer.

Although SMF can easily be implemented and achieve high recoveries, its reproducibility is inconsistent (Gonzales-Gustavson et al., 2017). Thus, a higher number of replicates may be needed to obtain reliable results, which can be cumbersome due to the long turnaround time.

2.1.1.4. Monolithic adsorption filtration (MAF)

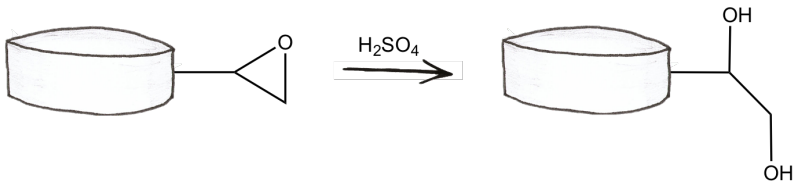
A solution to conquer the disadvantages of previously described methods was presented by the onset of monolithic adsorption filtration (MAF), which was developed by Peskoller et al. in 2009. MAF uses macroporous monoliths without mesopores as a solid matrix for adsorption. These monoliths can easily be manufactured by a self-polymerization reaction of an epoxy-based resin, in which toluene and *tert*-buthyl methyl ether serve as porogen, and, applied in a 60:40 ratio, lead to a defined pore size of 15 – 25 µm. On one hand, this manufacturing process allows for a simple and reproducible production of monoliths with comparable quality with the additional benefit that size and type of pores can easily customized during synthesis without the loss of functionality (Kunze et al., 2015; Bandari et al., 2008). On the other hand, production

is highly affordable with costs of around 1 EUR/monolith. These include only the reagent costs for monolith production (1,11 EUR). Thereby, monoliths for MAF are up to 220 times less expensive than VIRADEL filters and carry comparable costs to glass wool. Further equipment needed to perform MAF includes: disposables with costs of around 0,18 EUR/filtration (syringe, needle), reusables with costs of around 12 EUR (housing, fitting, adapter, O-rings, tubing) as well as a peristaltic pump (costs of around 3.600,00 EUR). Thus, operational costs of MAF per filtration considering only the equipment itself are around 1,40 EUR (monoliths and disposables), while initial costs with one-time expenses are approximately 3.612,00 EUR (reusables and pump). As a pump is commonly needed for every concentration technique based on filtration, initial costs of MAF are low compared to other concentration methods. For example, SMF needs a refridgerated centrifuge, which carries initital costs of approximately 10.000,00 EUR, while ultrafiltration (UF) even requires stainless steel housings with expenses between 5.000,00 to 13.000,00 EUR (Olszewski et al., 2005).

While approaches such as VIRADEL require different membranes for negatively and positively charged filtration, which can be attributed to high costs, monoliths used in MAF can easily be customized and applied for both filtration types. Such an adaption to concentration conditions in relation to the target virus and water type is achieved by chemical modification of epoxy groups on the monolith's surface (Pei et al., 2012). Monoliths with hydroxy groups on their surface (MAF-OH) have already been used for the successful concentration of HAdV and NoV in seeded tap water experiments (Pei et al., 2012). Similar to other negatively charged filters, the main disadvantage of MAF-OH is considered to be the need to pre-condition water samples to allow for virus adsorption. A first breakthrough for a direct concentration of viruses from water was achieved by a recent study, in which positively charged monoliths exposing diethyl aminoethyl groups on their surface (MAF-DEAE) were successfully applied to enrich bacteriophages without water sample pre-conditioning (Elsaesser, 2017). However, these monoliths have not yet been tested for HAdV or NoV.

An overview of synthesis of monoliths used in MAF-OH and MAF-DEAE is displayed in Figure 7. A detailed description of further modifications and their application can be found in Elsaesser, 2017.

A. Negatively charged monoliths exposing hydroxyl groups on their surface (MAF-OH)



B. Positively charged monoliths exposing diethyl aminoethyl groups on their surface (MAF-DEAE)

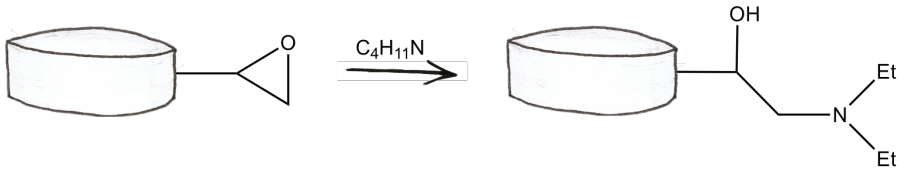


Figure 7: Chemical modification of epoxy groups on monolith's surface. (A) Treatment of epoxy groups with sulfuric acid (H_2SO_4) leads to negatively charged monoliths exposing hydroxyl groups on their surface (MAF-OH). (B) Treatment of epoxy groups with diethylamine ($C_4H_{11}N$) leads to positively charged monoliths exposing diethyl aminoethyl groups on their surface (MAF-DEAE).

After surface modification, the monolith is inserted in a housing, which is directly connected to the water sample. An illustrational overview of the workflow is displayed in Figure 8.

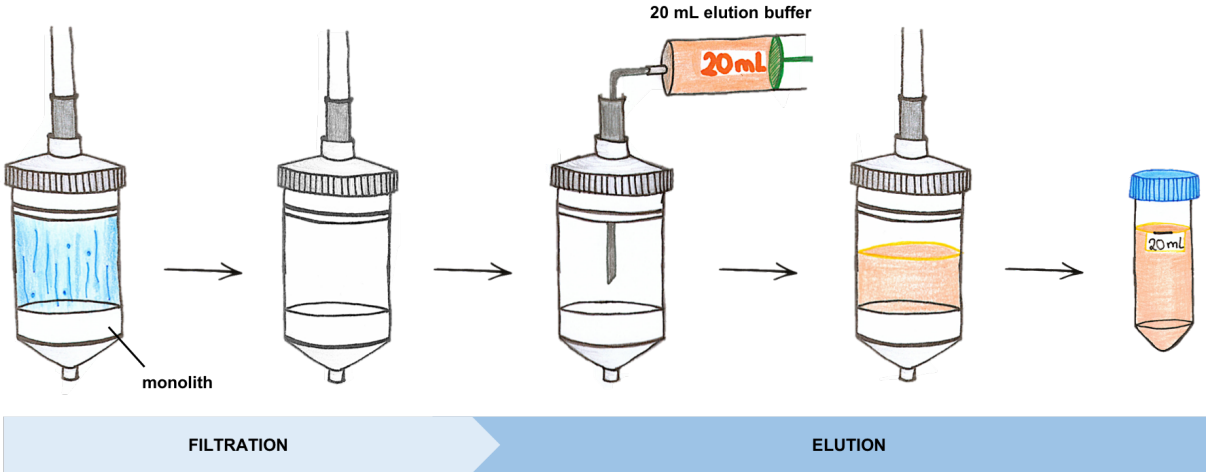


Figure 8: Schematic illustration of the monolithic adsorption filtration (MAF) workflow. Monoliths are inserted in a housing and connected to the water sample, which is then processed through the monolith. When the total sample volume has passed, 20 mL of elution buffer is added directly to the monolith and adsorbed microbes are eluted.

The turnaround time of MAF is primarily determined by the time needed for filtration, which depends on the total processing volume and the turbidity of the water sample. It was shown, that more than 100 L of high-quality water, such as drinking and ground water, can be processed at a flow rate of up to 1 L/min (Elsaesser, 2017). However, for water of lower quality, total volume as well as flow rate may have to be adjusted due to an increased back pressure and potential clogging of the monolith (Elsaesser, 2017; Wunderlich et al., 2016).

2.1.2. Virus concentration by size exclusion

In contrast to adsorption-elution approaches, viruses can also be enriched by size exclusion. This is achieved by ultrafiltration using microporous membranes with molecular weight cutoff (MWCO) levels of 30 - 100 kDa, which are usually composed of a polymer and constructed in a flat sheet, hollow-fiber or capillary format (Hill et al., 2009). UF is conducted by passing the water sample through the membrane, which retains viruses and other components larger than

the pore size on the side of the process stream, while water and low molecular weight substances are allowed to pass. UF can be performed in two modes: dead-end or cross flow. Dead-end flow is used for water of high quality with low turbidity. Here, the process stream is applied vertically to the membrane's surface and the sample is passed through the ultrafilter in a single run. In contrast, water of high turbidity is often processed in cross flow to prevent the formation of a boundary layer at the membrane's surface and thus clogging of the ultrafilter. Therefore, the process stream is applied tangentially to the membrane, which creates turbulences at its surface. A portion of the concentrate stream is recirculated to the system inlet and mixed with the incoming process stream. The concept of cross flow UF has already been applied successfully in combination with MAF for the concentration of viruses from large water sample of up to 100 L using MAF (Kunze et al., 2015; Pei et al., 2012; Peskoller et al., 2009).

The physical separation by size has the advantage that viruses are not exposed to any thermal, chemical or biological changes, which may have an influence on their stability. Furthermore, UF allows for the simultaneous concentration of multiple microbes and recovery efficiencies are often higher than those obtained with adsorption-elution techniques (Gibson and Schwab, 2011a; Hill et al., 2009; Morales-Morales et al., 2003; Polaczyk et al., 2008). However, efficiencies vary according to sample composition and operation conditions (Divizia et al., 1989; Patti et al., 1996; Soule et al., 2000). In addition, major drawbacks of UF are slow filtration rates, difficulties of field implementation and the tendency to clog when performed in dead-end flow (Olszewski et al., 2005). While cross flow UF seems to be an appropriate alternative, its experimental setup is complex and related to high capital costs of up to 1500 EUR/filtration (Lambertini et al., 2008). Therefore, adsorption-elution methods remain the preferred application as the primary concentration step for viruses from water (Ikner et al., 2011; Pei et al., 2012; Rutjes et al., 2005). Table 5 gives an overview of the concentration of adenoviruses and noroviruses using cross flow UF as primary concentration step.

Table 5: Overview of recoveries for HAdV and NoV using cross flow ultrafiltration.

Method	Type	Water matrix	Sample conditioning	Virus concentration	Sample volume (L)	MWCO/ Treatment	Time	Recovery (%)	Reference
Adenovirus									
	41	lake	none	5.9×10^4 GU/L	10.0	29 kDa none	> 30 min	1.0	Francy et al., 2013
	41	tap	0.01% NaPP	2.5×10^3 GU/L	100.0	30 kDa none	> 30 min	69.0	Rhodes et al., 2016
		river		2.6×10^3 GU/L	50.0			56.0	
Norovirus									
	MNV	surface	0.01% NaPP	10 – 100 PFU	100.0	70 kDa 0.1% NaPP	> 60 min	74.0	Gibson and Schwab, 2011a
	NoV (GII)	lake	none	4.6×10^3 GU/L	10.0	29 kDa none	> 30 min	2.0	Francy et al., 2013

NaPP: sodium polyphosphate; GU: genomic unit; PFU: plaque forming unit; NoV: human norovirus, MNV: murine norovirus; MWCO: molecular weight cutoff; kDa: kilodalton

2.1.3. Further methods: Aqueous polymer two-phase extraction

Aqueous polymer two-phase extraction is a liquid-liquid fractionation technique, which was first discovered by Albertsson in 1960 (Albertsson, 1960). The most commonly applied aqueous polymer two-phase extraction technique for virus concentration from water is polyethylene glycol precipitation (PEG). It consists of PEG as polymer phase, combined with another polymer, e.g. dextran or an aqueous salt-phase. PEG has been applied to concentrate viruses from sewage and different water (Lewis et al., 1985; Lund and Hedstrom, 1966; Nupen, 1970; Philipson et al., 1960; Shortridge et al., 1980; Shuval et al., 1967). Main advantages of PEG are that the method is rapid, inexpensive, nondestructive for viruses and allows for an application at neutral pH (Philipson et al., 1960). Although the underlying mechanism is still not clear, it is known that PEG achieves separation by precipitating proteins, which is affected by protein size, concentration, charge and initial ionic strength. Thus, viruses with their capsid composed of proteins are a major target for PEG. However, the main drawback of PEG is the lack of specificity, which leads to the co-concentration of other components, such as enzymatic inhibitors for downstream qPCR (Masclaux et al., 2013). Based on the complex partition mechanism, optimization of PEG is laborious as many trials have to be performed based on partitioning behavior screening to enhance and achieve higher specificities. This can drastically increase overall costs on one hand and limits the application of PEG at an industrial or commercial scale (Nestola et al., 2015). In addition, time for phase settling during precipitation can take several hours. Applying centrifugation decreases separation time to a few minutes, however, it limits the sample volume. Thus PEG is best applied as second concentration step (Grilo et al., 2016).

2.2. Virus detection by quantitative polymerase chain reaction (qPCR)

Traditional culture-based techniques have long been the gold standard in water research and their application is still promoted by international water quality standards. While these standards were developed with an explicit focus on the detection of health-related bacteria, application of culture-based methods reached their limit when viruses gained more attention as a cause of waterborne diseases. This is due to the reason that, in contrast to bacteria, some viruses propagate very slowly or not at all in culture, leading to long detection times and potential false-negative results. To overcome these limitations, quantitative polymerase chain reaction (qPCR) became an excellent alternative with a high acceptance. qPCR is a genome-based approach which is able to quantify viruses in less than two hours, while the method allows for the detection of variations down to the single-nucleotide level per reaction volume. Thus, it is a powerful tool to reliably quantify all virions present in a sample independently of their replication and can furthermore be applied to differentiate between virus species. However, its application is limited with regards to virus viability and infectivity (Francy et al., 2013; Heerden et al., 2005; van Heerden et al., 2003; van Heerden et al., 2004; Vergara et al.,

2016). While particularly DNA of degraded virions can remain intact in water for a long period of time, the sheer presence of a viral genome does not automatically implicate a related health risk. Therefore, qPCR results should be correlated with phenotypic and biochemical tests to avoid misleading interpretations (Levin, 2012; Osei Sekyere et al., 2015).

2.2.1. Sample preparation: Extraction and purification of nucleic acids

The first step of the qPCR workflow is the isolation of the viral genome, which is critical to allow for a downstream detection of viruses by qPCR. Isolation includes extraction of the viral genome and its purification from compounds interfering with its detection in qPCR. To prevent the loss of genetic material due to an insufficient genome extraction or degradation, commercial extraction kits are commonly applied. In contrast to in-house extraction methods these kits are highly reproducible while achieving a high yield and quality of purified nucleic acids and preventing the co-concentration of potential qPCR inhibitors, such as proteins, lipids, polysaccharides and extraction reagents (Vogelstein and Gillespie, 1979). Most commonly applied for viruses are kits based on spin columns or magnetic beads, both using a silica coated surface for nucleic acid binding. Independently of the used approach, lysis of viral particles is performed in a first step, leading to free nucleic acids, proteins and further impurities. The lysate is either transferred to a spin column or magnetic beads are directly added. In a next step, nucleic acids are bound to the silica coated surface of the spin column membrane or magnetic beads in the presence of salt. Proteins and other impurities are removed by washing and nucleic acids are eluted. An overview of both extraction workflows is displayed in Figure 9.

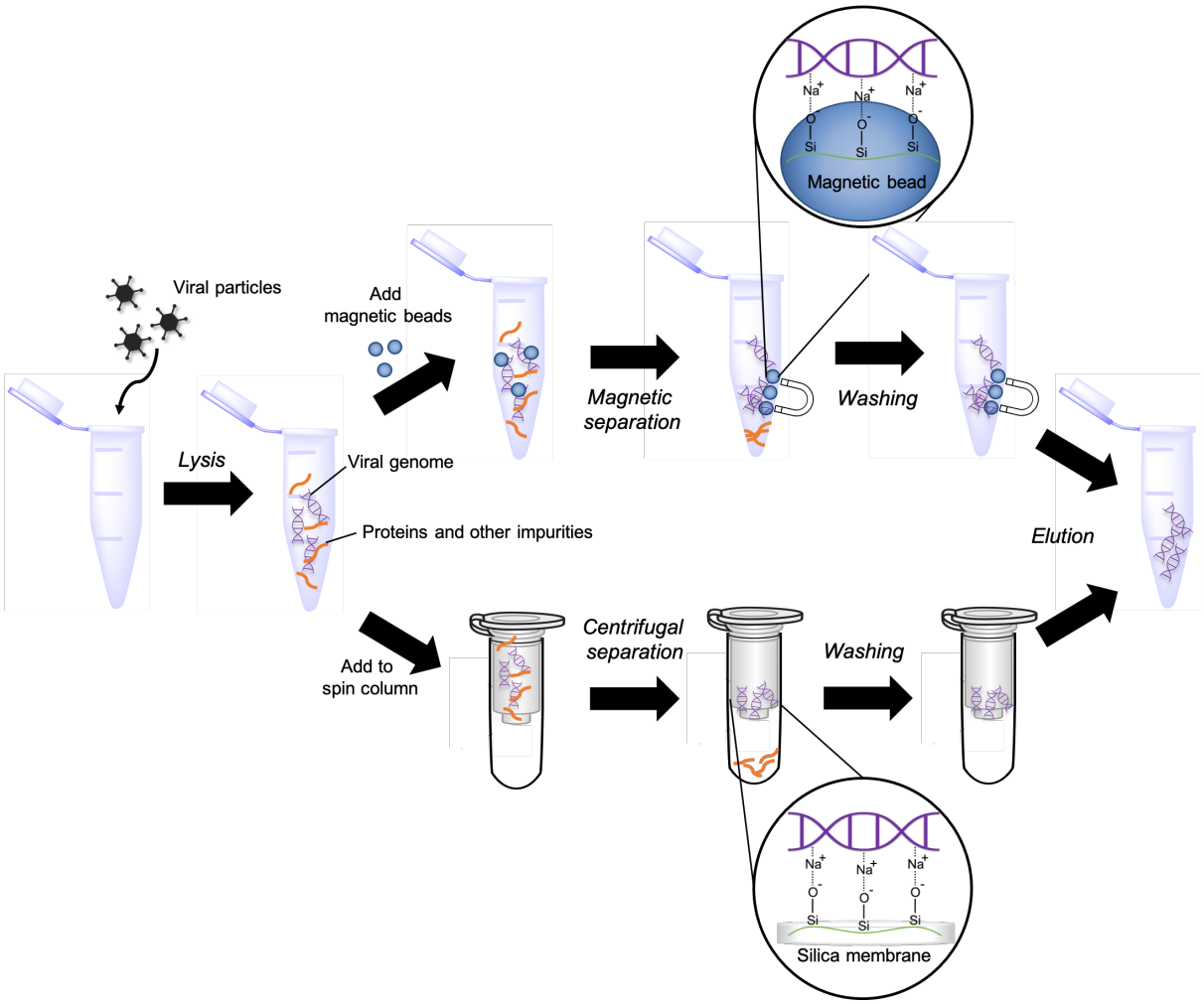


Figure 9: Schematic illustration of the extraction for viruses using either magnetic beads or spin columns. First, lysis of viral particles is performed, leading to free nucleic acids, proteins and further impurities. When using an extraction kit based on magnetic separation (upper workflow), magnetic beads are added, and free nucleic acids are bound to the silica coated bead surface in the presence of salt. Proteins and impurities are removed by further steps of washing before nucleic acids are eluted. When using spin columns (lower workflow), the lysate is transferred to a spin column containing a silica coated membrane. Proteins and other impurities are removed by centrifugation, while free nucleic acids bind to the silica coated membrane surface in the presence of salt. Further washing steps are perform before nucleic acids are eluted.

Which kit to choose depends mainly on the sample type (e.g. environmental, blood, cell culture), target (e.g. virus, bacteria, cells) and throughput. While the workflow of magnetic beads is performed in a single tube, these kits are often implemented in automation platforms and are the best choice for high throughput. However, with costs of around 5,33 EUR/sample (MagMAX™-96 Viral RNA Isolation Kit, AM1836, Thermo Fisher Scientific) they are more expensive than spin column kits carrying only around 3,94 EUR/sample (QIAamp Viral RNA Mini Kit, 52906, Qiagen). There are specific kits available for extraction of viruses from environmental samples, which have been shown to achieve better results in the presence of high levels of qPCR inhibitors than other conventional kits (Iker et al., 2013). Furthermore, it

has been reported that extraction with magnetic beads performed less effective than spin columns when comparing viral richness (Hjelmso et al., 2016). To avoid bias in results based on different extraction efficiencies it is therefore recommended to choose a kit specialized for the sample type and target and to use the same type of kit within a project as well as when comparing data between laboratories.

2.2.2. qPCR workflow for DNA and RNA

Depending on the virus type, purified nucleic acids can either be DNA or RNA. While DNA can be directly applied to qPCR, RNA needs to be transcribed into cDNA (complementary DNA) first, a step also known as reverse transcription (RT). RT can be combined with qPCR (one step RT qPCR) or performed as a separate step prior to qPCR (two step RT qPCR). Choosing one-step or two-step RT qPCR depends on convenience, budget and time. Two-step approaches are commonly less expensive as they allow for a flexible selection and combination of reagents applied in qPCR and RT. However, the total workflow time is longer and the additional pipetting step to transfer cDNA into qPCR involves an added risk of contamination. Here, one step approaches are more convenient and due to optimized buffer conditions, faster. Nevertheless, they are often at least two times more expensive.
A graphical overview of the qPCR workflow for DNA and RNA viruses is given in Figure 10.

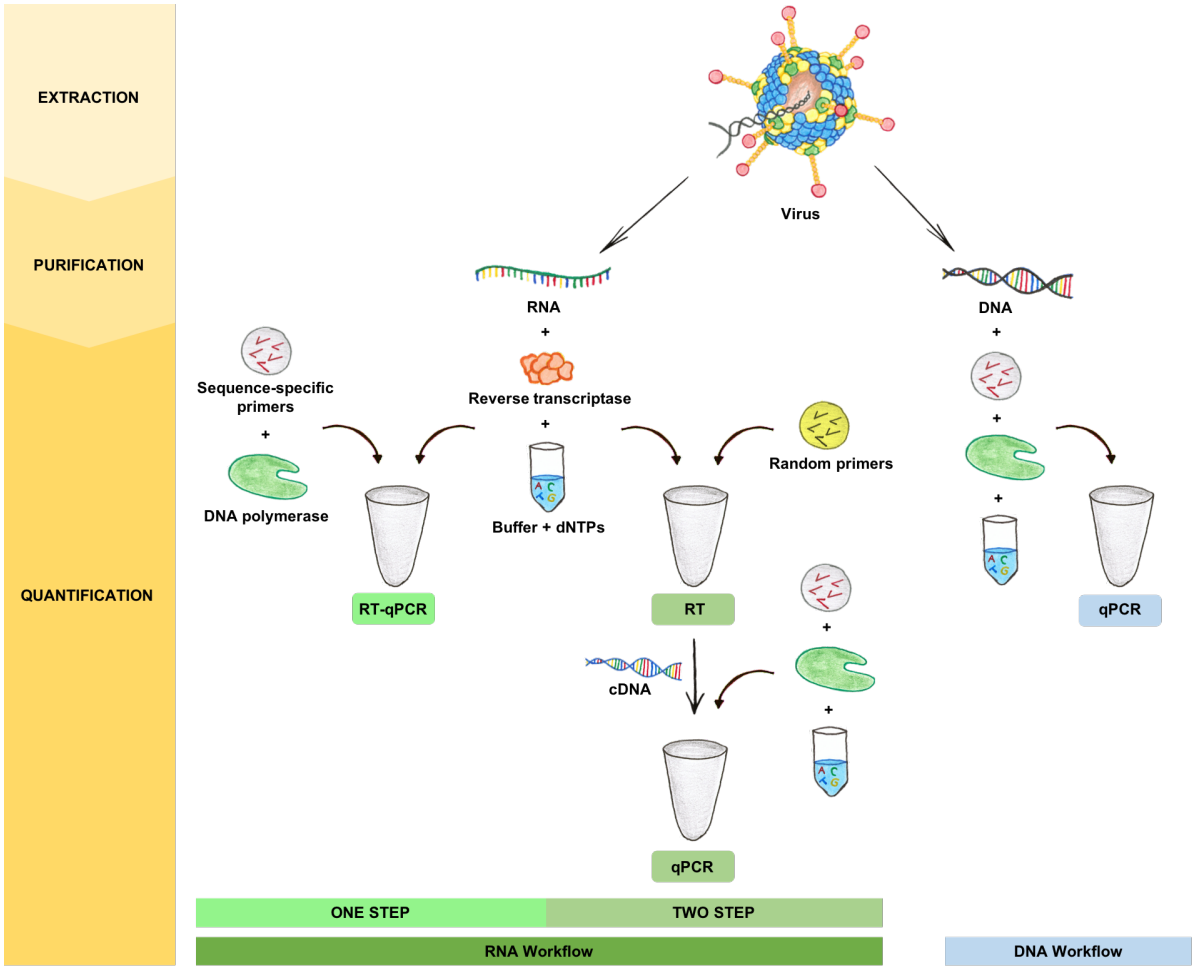


Figure 10: Graphical illustration of the qPCR workflow for DNA or RNA viruses. The virus genome (RNA or DNA) is extracted and purified prior to quantification. Purified DNA is mixed with all components needed for qPCR, including sequence-specific primers, DNA polymerase, buffer and dNTPs and is directly applied to qPCR. Purified RNA is transcribed in cDNA first, also known as reverse transcription (RT). RT can be combined with qPCR (one step RT-qPCR) or performed as a separate step (RT) prior to qPCR (two step RT qPCR).

2.2.3. Basic principle of qPCR

qPCR is based on PCR – a biochemical approach used to amplify a specific DNA sequence into millions of copies in a short time. One amplification cycle includes three steps: (I) denaturation, in which high temperature is applied to separate double stranded DNA; (II) annealing, where short oligonucleotides known as primers bind to flanking regions of the target DNA; and (III) extension, in which DNA polymerase extends the 3' end of each primer along the template strands. Commonly, one PCR run includes 25 to 35 of these cycles to exponentially increase the total amount of initial target DNA.
While the amount of accumulated PCR product is detected after a fixed number of cycles in traditional PCR, qPCR enables its monitoring and quantification in real time. This is achieved by the detection of a fluorescent signal at the end of each cycle, whose intensity is proportional to the amount of amplified DNA (Higuchi et al., 1992; Holland et al., 1991). Therefore, qPCR is also often referred to as real-time PCR.

Different qPCR chemistries exist; however, the one most commonly applied for virus detection is the fluorogenic 5' nuclease chemistry better known as TaqMan assay (Bustin, 2000; Kubista et al., 2006). TaqMan assays are highly sensitive and specific, which is achieved by the addition of a third oligonucleotide called probe. This probe is tagged with a reporter fluorophore on its 5' and a quencher on its 3' end. Based on the principle of FRET (fluorescence resonance energy transfer), the quencher absorbs the emitted fluorescent signal of the reporter fluorophore when in close proximity. During extension the probe is cleaved by the 5' nuclease activity of the DNA polymerase to allow target strand elongation. Thereby, the reporter fluorophore and quencher are separated resulting in a fluorescent signal, which is detected by a camera. A graphical illustration of an amplification cycle of a TaqMan assay is displayed in Figure 11.

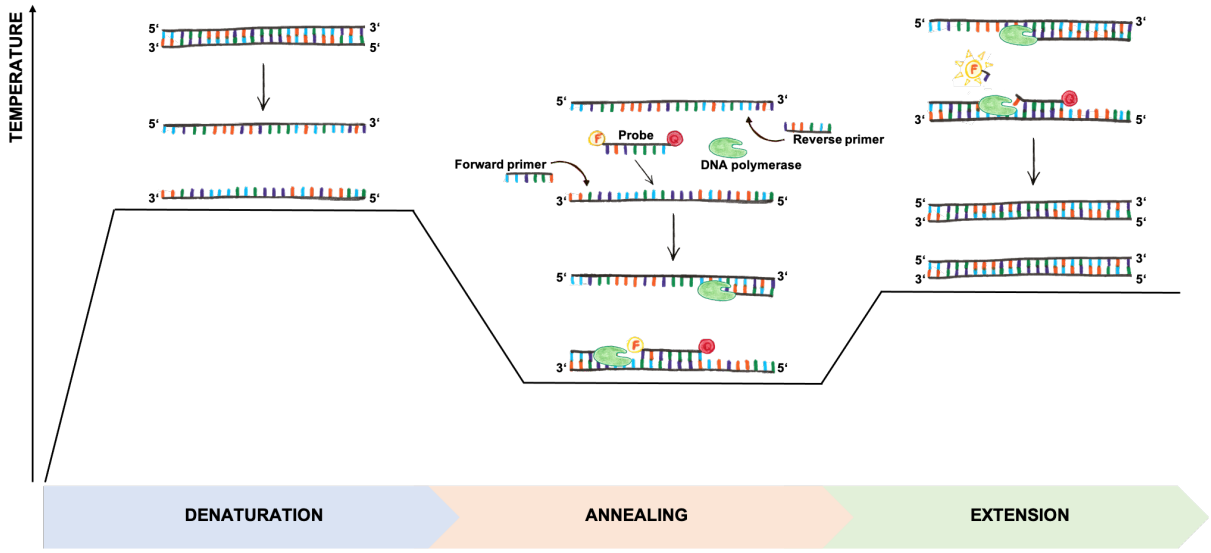


Figure 11: Graphical illustration of an amplification cycle of a TaqMan assay. Denaturation: High temperature is applied to separate double stranded DNA. Annealing: The temperature is decreased to allow hybridization of primers and probe to their complementary sequences on the target DNA stands. The DNA polymerase binds to the primers. Extension: DNA polymerase starts synthesizing the new strand. The probe is cleaved by the DNA polymerase's 5' nuclease activity to allow target strand elongation. Thereby, reporter fluorophore (F) and quencher (Q) are separated, and the resulting fluorescent signal is detected. The initial number of target DNA has doubled at the end of the extension step and the next amplification cycle begins.

2.2.4. Quantification and performance parameters of qPCR

The amount of target DNA doubles with each amplification cycle, while the fluorescent intensity increases proportionally to the progress of the reaction. The cycle (C_q), in which the fluorescent signal exceeds the background noise and enters the exponential phase is used for target quantification. At C_q the efficiency of the reaction is constant among cycles attributable to an excess of reagents and a highly active and efficient DNA polymerase, which allows for the collection of accurate data. Thus, a threshold is set at the fluorescent signal where all amplification plots of one qPCR experiment have entered the exponential phase. This threshold determines the C_q value of each sample, which is used to calculate the corresponding concentration. A non-template control (NTC) serves as a reference to avoid misleading interpretations. The change of fluorescent signal over the number of cycles is displayed by an amplification plot, which represents the accumulation of qPCR product and gives a graphical overview of the progression of the reaction. An illustration of an example amplification plot is displayed in Figure 12.

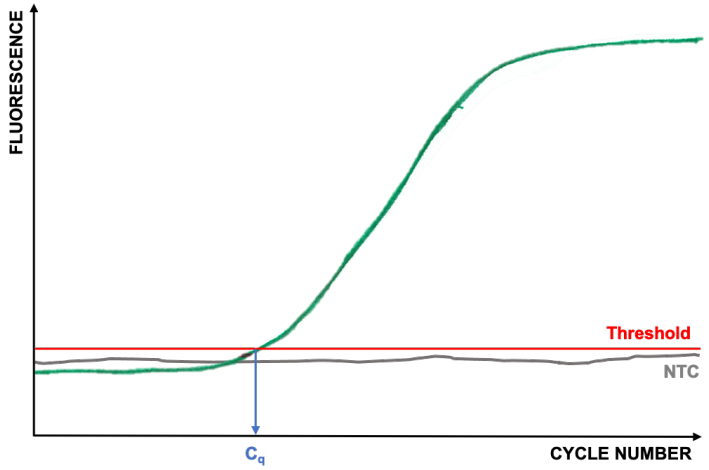


Figure 12: Graphical illustration of an amplification plot. The fluorescent signal increases over the number of cycles, representing the accumulation of qPCR product. Quantification is based on the cycle (C_q), in which the fluorescent signal exceeds the background noise. A threshold is set at the fluorescent signal where all amplification plots of one qPCR experiment have entered the exponential phase. This threshold is used to determine the C_q value of each sample. A non-template control (NTC) serves as reference to avoid misleading interpretations.

C_q values are instrumental readings, which depend on qPCR assay efficiency, baseline correction methodology and instrument calibration. A standard curve is used to transform these arbitrary signals into values with specific units (copies of organism, ng of DNA, concentration). This is crucial to allow for data comparison and interpretation between different qPCR experiments and instruments (Bustin et al., 2009; Johnson et al., 2013). The standard curve encompasses serial dilutions with known concentration in the expected range of the target DNA. Concentration is plotted against C_q values, which assigns cycle numbers to defined amounts of target DNA. This facilitates the transformation of sample C_q values into a corresponding concentration (Bustin et al., 2009; Kubista et al., 2006; Yang and Rothman, 2004). An example of a standard curve is given in Figure 13.

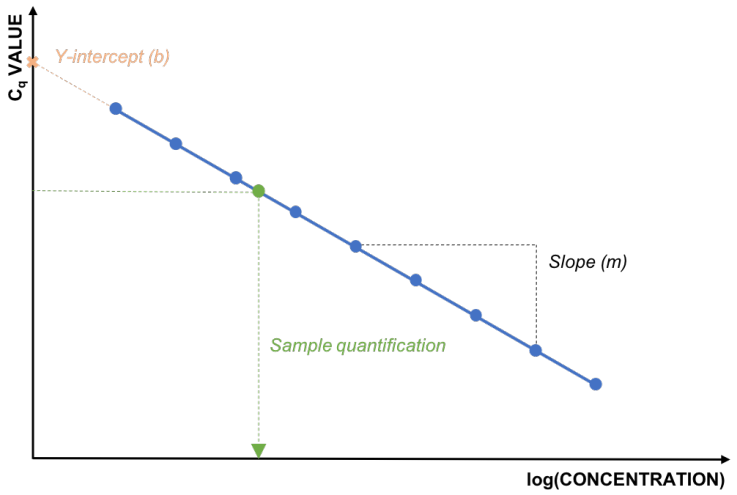


Figure 13: Graphical illustration of a standard curve. A standard curve is generated by plotting known concentrations of target DNA against C_q values, allowing for an easy quantification of samples. Parameters such as y-intercept (b) and slope (m) are used for analysis of qPCR performance.

The standard curve follows equation:

$$y = mx + b \qquad \textbf{Equation 1}$$

where y is the C_q value, m the slope, x the log(concentration), and b the y-intercept. Aside from sample quantification, the standard curve also contains information about qPCR performance. The linear area of the curve displays the working range of the qPCR assay, in which results show the highest level of precision, accuracy and linearity. This area is furthermore used to assess the curve's slope and determine the amplification efficiency, also known as qPCR efficiency. qPCR efficiency is directly related to the amplification factor, which describes the ratio of the number of DNA molecules at the end of a qPCR cycle divided by the number of initial target DNA at the beginning of the same cycle. With two strands as a template, the initial DNA molecules can at most double per cycle, resulting in an amplification factor of 2. The amplification factor can be assessed from the slope by:

$$AF = 10^{(-\frac{1}{m})} \leq 2 \qquad \textbf{Equation 2}$$

where AF is the amplification factor during the exponential phase of the qPCR reaction and m the slope of the standard curve. Equation 2 demonstrates that the slope tops out at -3.32, which is related to a qPCR efficiency of 100% as described by:

$$E = (AF - 1) * 100 \qquad \textbf{Equation 3}$$

where AF is the amplification factor and E the qPCR efficiency given as percentage. While errors in standard curve slopes are common, an efficiency between 90% and 110% is considered acceptable. These errors are usually caused by qPCR inhibitors, contamination, pipet precision or calibration errors, dilution point mixing problems as well as the assay itself, qPCR reagent performance and sample quality (Johnson et al., 2013).

Inhibitory effects in qPCR can be determined by the number of cycles between two amplification plots (ΔC_q) and the dilution factor of the corresponding samples. The relation between these two parameters under ideal qPCR conditions is given by:

$$2^n = dilution\ factor \qquad \textbf{Equation 4}$$

where n is the theoretical number of cycles between two amplification plots at a qPCR efficiency of 100%. Inhibition is detected when $\Delta C_q < n$. However, while qPCR efficiency rarely results in 100%, $\Delta C_q > 2.0$ is considered acceptable.

The y-intercept of the standard curve corresponds to the theoretical limit of detection (LOD). It displays the expected C_q value if the lowest copy number of target DNA marked on the x-axis gave rise to statistically significant amplification. There is still no standardized approach for determining the actual LOD in qPCR as conventional definitions do not fit for qPCR data (Klymus et al., 2020). The reason for this is that these definitions require a linear correlation between the analyte and the signal of response, which qPCR does not show. Furthermore, they assume a level of background noise in blank samples, from which the analyte must be distinguished (Armbruster and Pry, 2008). However, in qPCR NTC samples do not produce a signal that can be differentiated from the background signal of the instrument (Forootan et al., 2017; Hunter et al., 2017). Therefore, the LOD in qPCR is commonly defined as the lowest concentration of target DNA that can be detected with a certain level of confidence, usually 95% (Burd, 2010; Burns and Valdivia, 2007; Bustin et al., 2009; Forootan et al., 2017). Determination of LOD is achieved by running a large number (≥10) of replicate standard curves including low concentration standards and identifying the lowest standard concentration at which 95% of replicates produce an amplification signal. However, the incidence of nonspecific signals increases with the number of cycles, especially when using qPCR chemistries based on double stranded intercalating dyes, such as SYBR Green. Here, low concentration standards might produce a false-positive amplification signal leading to misinterpretation of LOD (Ruiz-Villalba et al., 2017). Furthermore, the instrument's sensitivity may also influence the detection of low concentrations. Consequently, a standardized approach to define LOD in qPCR, independently of the used instrument, is crucial to prevent analysis and reporting of inconsistent data leading to false discrepancies between laboratories (Stewart et al., 2013).

2.2.5. Standards in qPCR

Purified viral genomes, linear PCR amplicons, or cloned target sequences are most commonly used as a standard for quantification of viruses by qPCR. Typically the standard is produced by the laboratory itself, which requires certain technical and molecular biological equipment in addition to trained staff. Several steps are involved in obtaining a highly purified and robust standard with an acceptable qPCR efficiency, which apart from production, include validation, optimization, quantification and quality control. Each of these steps comprises a potential risk of contamination to the laboratory and to the standard itself (Cimino et al., 1990; Whelan et al., 2003). Thus, standard development and reproduction can be challenging, time-consuming and expensive. An excellent alternative to this is provided by synthetically designed dsDNA fragments, such as gBlocks®, which have several benefits over traditional standards. First, they are commercially produced, validated and quantified. Thus, no specialized equipment and training or cumbersome and expensive validation is required. Moreover, due to the commercial availability, gBlocks® also enable an easy transfer and establishment of the assay in another laboratory or on a different qPCR instrument (Conte et al. 2018). Second, they can be easily

customized and are highly specific for their target by comprising only the genomic region of interest. This is especially beneficial for the quantification of viruses, which in contrast to other microbes, only contain their genomic information. Thus, gBlocks® allow for a direct assessment of the total number of virions present in a sample by the count of fragments. Thereby, these synthetically designed standards represent an efficient tool for the development of a standardized, cost- and time-efficient process for the detection of viruses by qPCR.

2.2.6. Costs in qPCR

Apart from standard production and validation, reagents represent the main cost drivers of qPCR. Expenses depend primarily on the type of reagent used and the number of qPCR reactions needed. Reaction volumes in qPCR are very small and fall between 5 to 25 µL. To avoid, that small pipetting errors can lead to misinterpretation of qPCR data, standard dilutions and samples are commonly analyzed at least in duplicate, but most often in triplicate qPCR reactions. Consequently, the number of qPCR replicates needed can drastically increase costs, which vary between just a few cents up to a couple of Euros per reaction. In addition to this, costs increase even further when specific reagents are required to successfully perform qPCR. Demands on qPCR reagents are primarily determined by the type of sample and involve exceptional specificity, reproducibility, accuracy, robustness and stability among others. Particularly environmental water contains commonly a high level of qPCR inhibitors, such as toxic metals as well as humic and fulvic acids (Rock et al., 2010). Consequently, qPCR reagents validated for environmental application are typically needed to allow for an accurate and reliable quantification of viruses in the presence of such inhibitors.
While costs for the quantification of DNA viruses are often still manageable, analysis of RNA viruses is particularly expensive due to the additional step of reverse transcription needed prior to qPCR. Depending on the requirements of the sample type, costs per RT qPCR reaction start at around two Euros when applying a two step approach and are commonly at least two times higher for one step approaches.

2.3. Workflow efficiency: Costs, time and ecological footprint

The total costs for concentration and detection of viruses in water are hard to estimated. These depend on the applied methods on one hand, but also on the time needed, which might vary based on the experience level of the experimental operator on the other hand. Furthermore, it has to be distinguished between initial one-time costs of equipment needed to perform a method, operational and labor costs. To determine the workflow efficiency also the ecological footprint should be taken into consideration in addition to costs and time. This includes amount of disposable material and energy needed for performance.

The most accurate way to compare and estimate costs is to look at the main cost-drivers of the workflow and classify methods accordingly. Initial one-time costs of equipment are a

general cost-driver. Thus, alternative methods or partnering with other laboratories should be considered in case equipment will only be needed for a single project. Further cost-drivers for each step of the workflow are:

Virus concentration

While upfront material costs for e.g. filters are easy to calculate by the number of samples, sample volume is the main cost-driver of a concentration method. It directly determines the processing time needed, which is proportional to labor costs defined as hands on time. Furthermore, longer processing times can also have an influence on expenses for energy and lifetime of equipment (such as pumps). Thus, the most efficient concentration method needs only small sample volumes to achieve high recoveries. The second cost-driver represent disposables, which, together with the use of energy also influence the ecological footprint of a method.

Extraction and purification

Complexity of a sample is the main cost-driver for extraction and purification. To allow for an accurate and highly efficient downstream detection, specified extraction kits are needed, particularly when high levels of qPCR inhibitors are present as for example in sewage. The second cost-driver is the number of samples, which material as well as labor costs. In case of medium to high throughput (> 40 samples/day) investment in an automation platform should be considered, which increases efficiency while at the same time having a positive impact on the ecological footprint due to less disposables.

qPCR detection

Similar to extraction and purification, complexity of a sample represents also the main cost-driver in qPCR. Particularly when working with environmental samples specific qPCR reagents might be required to allow for an accurate detection in the presence of remaining qPCR inhibitors. Applying an extraction kit which is able to eliminate all qPCR inhibitors might be the most efficient solution to minimize qPCR costs. The second cost-driver is the number of samples. Although a sample needs to be analyzed at least in duplicates to achieve reliable results, operational costs can be minimized by working in smaller reaction volumes. While standard reaction volumes are between 15 and 25 µL, they can be reduced to 5 µL when working in 384 well formats. This would also optimize the ecological footprint of qPCR as more samples can be analyzed in a single run leading to less disposables.

3. Conducting interlaboratory studies

Interlaboratory studies are those, in which several laboratories analyze the same material or materials. They are carried out to examine the performance characteristics of a specific method (collaborative trials), the proficiency of a laboratory (proficiency studies) or to provide certified reference material (material-certification studies). This work focuses on collaborative trials, which are often part of developing standard operating procedures for the concentration of viruses from water. Further information about the two other interlaboratory study types can be found in Hund et al., 2000.

3.1. Definition

Collaborative trials also known as method-performance studies focus on the performance characteristics of a specific method. Based on ISO guideline 5725, these studies are also known as accuracy experiments (ISO 5725). Here, the accuracy of a test method is evaluated in terms of its trueness and precision from the interlaboratory trial. Trueness describes the bias of the measurement method in an interlaboratory context, defined in ISO 5725-4, and expresses the closeness of test results to the “true” value or the accepted reference value. Precision, on the other hand, follows ISO 5725-2 and determines the repeatability and reproducibility of a test method.

3.2. Method validation

Before performing a collaborative trial, non-standard and in-house-developed methods require method validation. Validation is performed to ensure that the method fits for the intended purpose in terms of quality, reliability and consistency of results. Thus, when being applied in a different laboratory under the same conditions and control parameters, a validated method should lead to comparable results. If a method is part of a workflow, also the complete workflow can be validated. This is particularly recommended in case the workflow should be applied as a SOP or at an interlaboratory scale. In a first step, the purpose and scope of a method/workflow are defined before its validation is performed according to the following parameters:

- Specificity and selectivity
- Linearity
- Range
- Accuracy
- Precision
- Limit of detection
- Robustness

The workflow of validation and evaluation of a method is displayed in Figure 14.

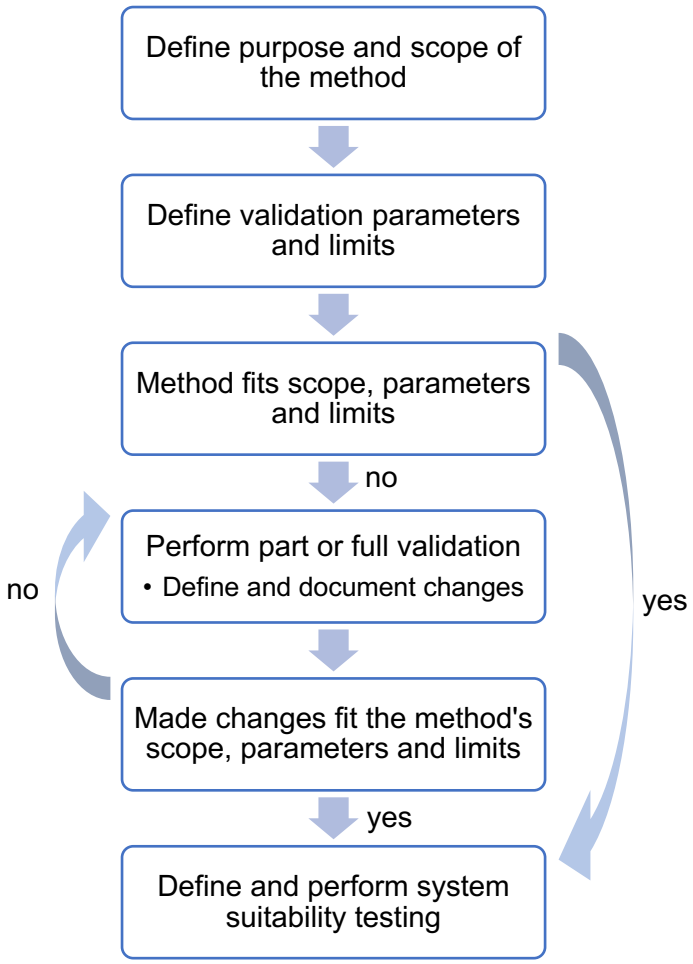


Figure 14: Brief overview of the workflow of evaluation and validation of a method (Gajra et al., 2011 (modified)).

For the detection of HAdV in surface water the complete workflow including concentration with MAF, extraction and purification of viruses and detection by qPCR needs to be validated.

One of the most important criteria for method validation is specificity, which describes the unequivocal response of a method to a specific analyte present in a sample, here HAdV. It needs to be ensured, that the response of the analytical measurement is particularly induced by the target of analysis and not by other interfering components leading to false positive or negative results. In the here presented workflow, specificity is directly determined by qPCR detection. The application of qPCR assays based on TaqMan chemistry makes qPCR highly specific for its target. Specificity of the assay is commonly checked upon assay design by screening genomic databases, such as FASTA, for the binding behavior of the qPCR assay to other targets. Although, TaqMan assays are unlikely to result in false positive signals, their specificity can also be double checked by analyzing the lengths of qPCR products using gel electrophoresis. Furthermore, specificity should be confirmed by running qPCR on blank samples of the water matrix in the absence of the target virus. In addition to the assay itself, also the used reagents can influence specificity. Depending on the amount of present qPCR

inhibitors, specific reagents may be required to allow for a reliable detection of target. qPCR inhibition can be checked by running 1:10 dilutions of samples.

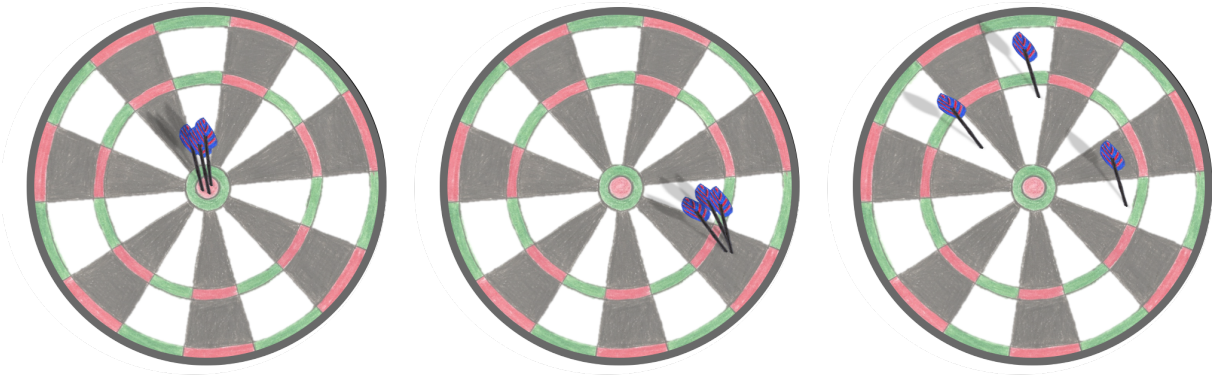
To allow for an accurate and precise detection, also the linearity and range of the applied qPCR assay need to be determined. Linearity describes the ability of an analytical procedure to produce results in direct proportion to the concentration range of the analyte in samples within the required concentration levels. It should be determined by a minimum of 6 standards and needs to include the slope, linear range and the correlation coefficient, which should be greater than or equal to 0.99 in the working range. The range is determined by the calibration plot, which is the interval between the upper and lower concentration of analyte falling in the linear range. Results within the range demonstrate acceptable levels of precision, accuracy and linearity. Linearity and range are commonly determined by a reference standard. However, as previously described in section 2.2.5., the quality of commonly reference standards in qPCR may highly vary due to their in-house production. Thus, for validation of the here presented workflow these standards are not ideal. An excellent alternative of high and reproducible quality represents synthetically designed and commercially produced DNA fragments. However, these fragments have not been validated for the MAF workflow yet.

In contrast to specificity, selectivity can be a collective response to a group of analytes sharing similar chemical and physical characteristics, for example all viruses present in a sample. For the here presented workflow, MAF defines the selectivity based on the surface modification of the applied monolith as well as the processing conditions (type of water, pH of water, flow rate, elution buffer). Also the applied kit for nucleic acid extraction can have an influence on selectivity, as there are extraction kits available, which purify DNA only, while RNA is degraded. Thus, it is crucial to choose a kit specified for the target of interest.

Accuracy describes the degree to which the determined value of analyte corresponds to the true value. It can vary over the expected concentration range and should be determined using a working or reference standard. Working or reference standards for virus analysis when concentrating water are tricky. Most commercially available reference standards are based on viral vectors, while the most commonly used working standard is bacteriophage MS2. However, vectors as well as MS2 might show different adsorption/elution behaviors than the target virus, or even compete for adsorption sites one the monolith’s surface – both factors, which could affect results and consequently, are not ideal for determination of accuracy. Thus, a spiking approach is the best way to analyze accuracy. Here, a water sample is spiked with a defined concentration of target virus, which has previously been determined by qPCR. Samples are taken of the spike in, and the processed water sample after MAF and concentrations are compared. The closer the value of the virus in the processed water is to the spike in control, the higher the accuracy of the workflow.

Precision on the other hand describes the closeness of a series of measurements of the same sample under identical conditions, which is expressed as variance, standard deviation or as coefficient of variation of a series of measurements. Here, a minimum of five replicate samples should be carried out to determine precision.

Figure 15 displays graphically the difference between accuracy and precision.



A. Accurate and precise **B. Precise, but not accurate** **C. Poor accuracy and precision**

Figure 15: Graphical illustration of accuracy and precision. The bull's eye of the dart board represents the center of the target, which is aimed to be hit by the darts. A. The darts are tightly clustered with an average position in the center of the bull's eye, representing an accurate and precise pattern. B. The darts are clustered together but did not hit the intended mark. The result is precise, but not accurate. C. The darts are neither clustered together nor near the center of the target, referring to a poor accuracy as well as precision.

The limit of detection (LOD) defines the lowest amount of an analyte that can be detected but not necessarily quantified. The limit of detection is calculated based on the average response of a blank sample containing no analyte plus three times its standard deviation. The blank sample should be the water matrix of validation as complexity of the matrix may have influences on its signal and thus affect the LOD. For the here presented workflow, the LOD has to be determined by qPCR. As previously described in section 2.2.4, there are still no standardized approaches available to determine the LOD according to the given definition as a blank sample in qPCR does not result in a response. Here, research is required to establish an approach based on qPCR to determine the LOD for the concentration workflow of viruses from water.

Robustness examines the impact of changes in operational parameters on the analytical results. These parameters are pH, temperature and operational conditions, such as flow rate or processing volumes.

3.3. Organizational structure of collaborative trials

Standard procedures of how to conduct a collaborative trial are specified by different organizations, such as the International Organization for Standardization (ISO), the "Deutsche Industrienorm" (DIN), the Association of Official Analytical Chemists (AOAC), the American

Society of Testing and Materials (ASTM) and the Analytical Methods Committee of the British Chemical Society (AMC), among others. They all share a common structure of how to organize and plan collaborative trials. This involves a panel of experts, who are familiar with the method of interest. The panel is responsible for the overall organization, planning and coordination of the study as well as the supervision of participants (ISO 5725-2, 1994; DIN, 1984). It decides on the experimental design, which includes the number of laboratories and test items for analysis as well as their levels of concentration. Furthermore, the panel appoints an executive officer from one of the participating laboratories and an internal or external statistical expert. The executive officer is responsible for the actual execution of the study including supervision of sample preparation, test item distribution and collection of results and they keep in close contact with the statistical expert, who is involved in the analysis of data. Participating laboratories are required to have a certain level of competence and should be representative for laboratories which will apply the method in the future. Within each laboratory, one supervisor is selected, who is responsible for the internal organization and the final reporting of results to the executive officer. Furthermore, the supervisor chooses a proficient operator, who executes all experiments. An overview of the structure is displayed in Figure 16.

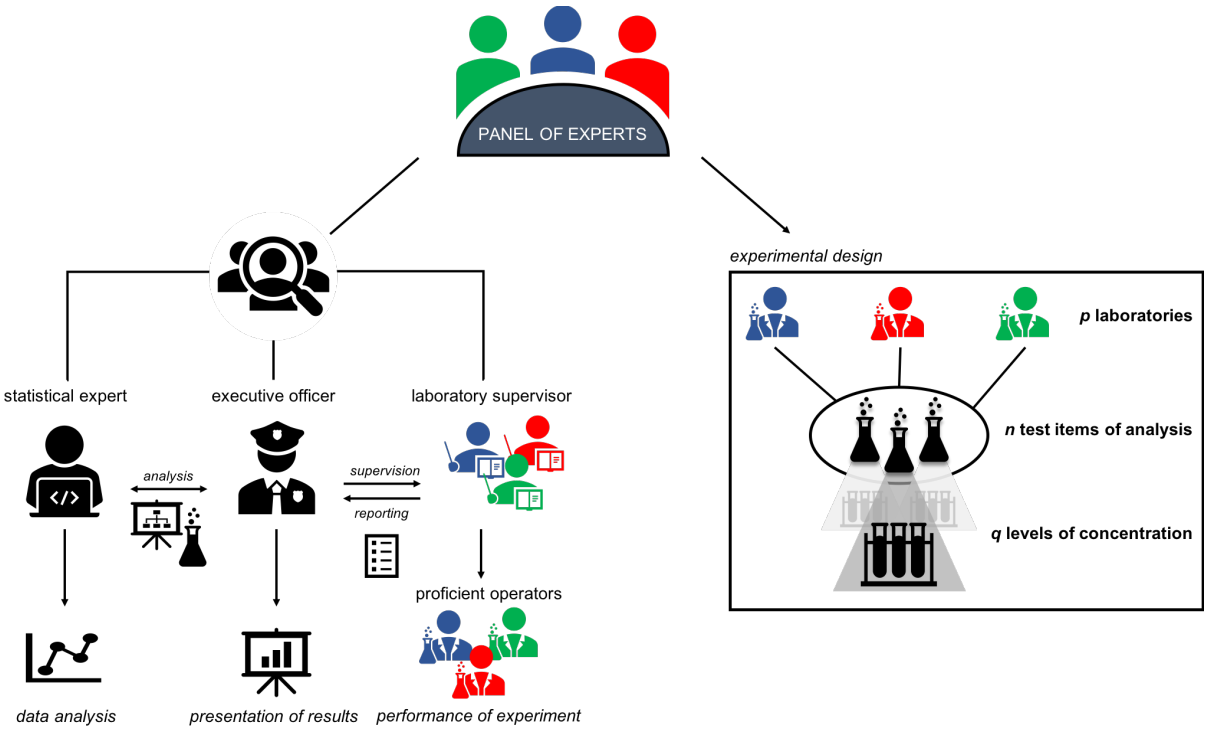


Figure 16: Planning a collaborative trial – organizational structure. A panel of experts, who are familiar with the method of interest plans and coordinates the collaborative study. The panel decides on the experimental design of the study, which defines the number *p* of laboratories, the number *n* of test items of analysis as well as the levels *q* of concentration per test item. Furthermore, the panel assigns an executive officer, who belongs to one of the participating laboratories and an internal or external statistical expert. The executive officer is responsible for the actual organization of the study, which includes supervision of sample preparation, test item distribution and collection of results. In addition, the executive officer is in close contact with the statistical expert, who is involved in the analysis of data. Within each

laboratory a supervisor is chosen, who is responsible for the internal organization and the final reporting of results to the executive officer. Furthermore, the supervisor selects a proficient operator who conducts all experiments.

III. Results and Discussion

1. Establishment of a standard operating procedure (SOP) for the detection of enteric viruses in water by molecular biological methods

Although it is known that the presence of enteric viruses in water poses a high risk to human health and can further lead to epidemics causing severe illness or even death, fast and affordable workflows are still missing to allow for routine virus assessment. To take a first step towards the development of such an approach, three expert European laboratories in the area of water research, IWC-TUM (Institute of Hydrochemistry, Technical University Munich, Germany), UB (University of Barcelona, Spain) and DTU (Technical University Denmark, Denmark) established a standard operating procedure (SOP) for the molecular biological quantification of adenovirus and norovirus in water. The goal of this collaboration was to provide a state-of-the-art workflow, where protocols for concentration, nucleic acid extraction and qPCR detection currently performed in each laboratory for virus detection in water were harmonized. Two concentration approaches were included in the SOP: (I) monolithic adsorption filtration using monoliths with a negatively charged hydroxyl surface (MAF-OH), a method developed at IWC-TUM by Peskoller et al. in 2009 and Kunze et al. 2015, and (II) skimmed milk flocculation (SMF), a process developed at UB. The harmonized SOP was established in each partner laboratory first before a pilot trial was conducted to evaluate its application and the performance of both concentration methods at an interlaboratory scale. Therefore, HAdV serotype 35 (HAdV35) and murine norovirus (MNV), referred to as adenovirus and norovirus in the following sections, were used in spiking experiments representing one DNA and one RNA enteric virus with a low biosafety level (Jiang, 2006).

1.1. Harmonization of qPCR detection

Before establishing the complete workflow including MAF-OH and SMF in each laboratory, qPCR assays for adenovirus and norovirus detection were evaluated regarding their performance to avoid misleading interpretation of data when conducting the pilot trial, particularly as laboratories used qPCR instruments of different manufacturers.

1.1.1. Establishment of qPCR assays

To facilitate the comparison of qPCR data among laboratories, synthetically designed dsDNA fragments (gBlocks®) were implemented as a standard in the qPCR workflow. In comparison to traditional standards, gBlocks® have the advantage that they are commercially available, highly specific and the concentration can be determined by UV-VIS spectrometry. Thus, using gBlocks® assured that each laboratory applied a standard of similar quality in qPCR. To validate their performance in an interlaboratory scale, each laboratory performed a standard curve from a dilution series of gBlocks® ranging from 1 to 10^8 genomic units/µL (GU/µL) and

assessed working range, qPCR efficiency and the lowest measured concentration. Table 6 displays the average mean of each performance parameter among the three laboratories.

Table 6: Average mean of performance parameters of qPCR assays for adenovirus and norovirus detection among the three laboratories.

Virus	Adenovirus	Norovirus
WR (GU/µL)	10^2 - 10^8	10^2 - 10^8
Efficiency (%)	94.6 ± 3.2	95.5 ± 4.0
Lowest measured concentration (GU/µL)	1	1

WR: working range; GU: genomic unit

All laboratories achieved a comparable working range and limit of detection, only qPCR efficiencies varied slightly. Contributing reasons for variations in efficiency may have been caused by differences in pipet precision and calibration or due to mixing errors of gBlocks® dilutions among laboratories. Nevertheless, efficiency values were still in the acceptable range of 90% and 110% (Johnson et al., 2013).

Precise and accurate data could still be achieved after multiple cycles of freezing and thawing of serial gBlocks® dilutions, which highlights their reproducibility and robustness. Consequently, applying gBlocks® accelerated the establishment of robust and reliable qPCR assays for adenovirus and norovirus detection, which effectively saved time and limited costs commonly associated with qPCR standard production and validation (Conte et al., 2018). Furthermore, gBlocks® showed a similar performance among laboratories and allowed for an easy comparison of data, independently of the used instrument. Taken together, these results demonstrate that gBlocks® represent an ideal tool for interlaboratory applications and a convenient alternative to traditional standards.

1.1.2. Evaluation of qPCR reagents regarding costs

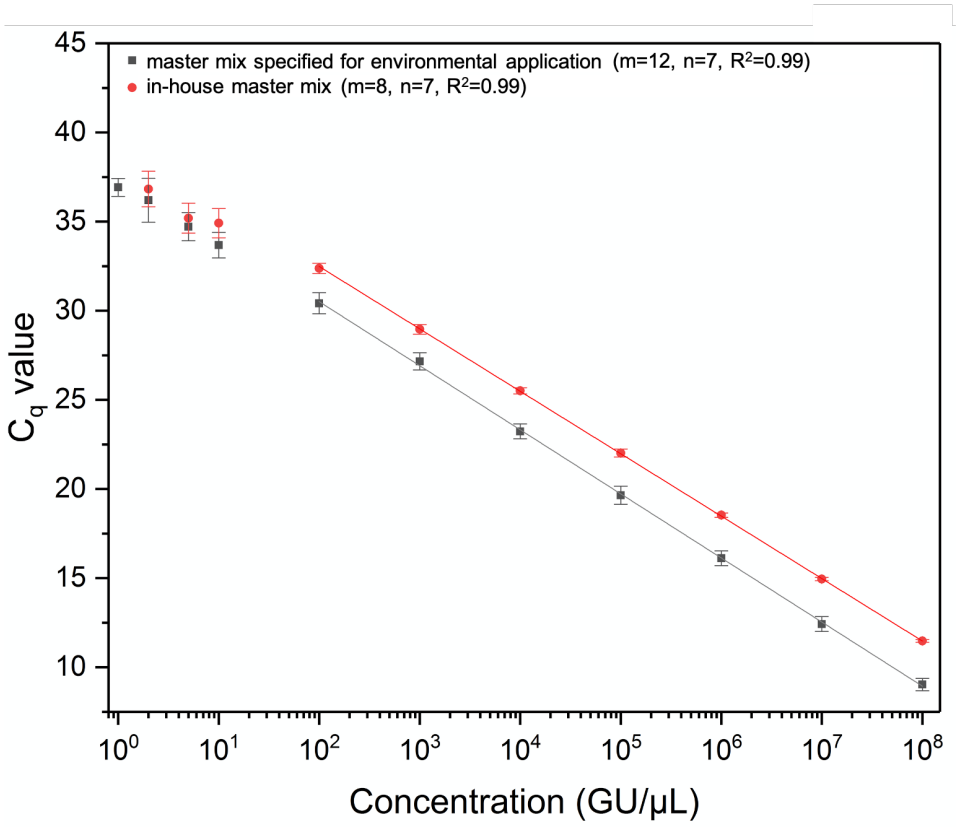
Although gBlocks® effectively reduced upfront expenses for establishment of qPCR assays, qPCR represented still the major cost driver of the SOP. The reason for this is attributed to the qPCR reagents chosen for adenovirus and norovirus detection. To allow for an accurate quantification of both viruses in water qPCR reagents validated for environmental applications were included in the SOP. These reagents are resistant to high levels of qPCR inhibitors, which may be present in environmental water. Although the robustness of qPCR to inhibitors is crucial for an application of the SOP in routine virus surveillance, costs of these specified reagents were extremely high. The applied master mix for adenovirus detection carried costs of around 2,40 EUR/reaction, while the used one step RT qPCR kit for norovirus was related to expenses of 5,00 EUR/reaction.

To find a more economical solution and verify, if reagents specified for environmental application are truly required, IWC-TUM tested two alternative reagents (one per virus), which

were commonly used at the institute for less complex samples. These reagents were up to five times less expensive, but not validated for environmental applications.

To compare the performance of specified and in-house used master mixes and one step RT qPCR kits directly to each other, standard curves were generated from the same dilution series of gBlocks® for each virus ranging from 1 to 10^8 genomic units/µL (GU/µL). Results are displayed in Figure 17.

A. Adenovirus



B. Norovirus

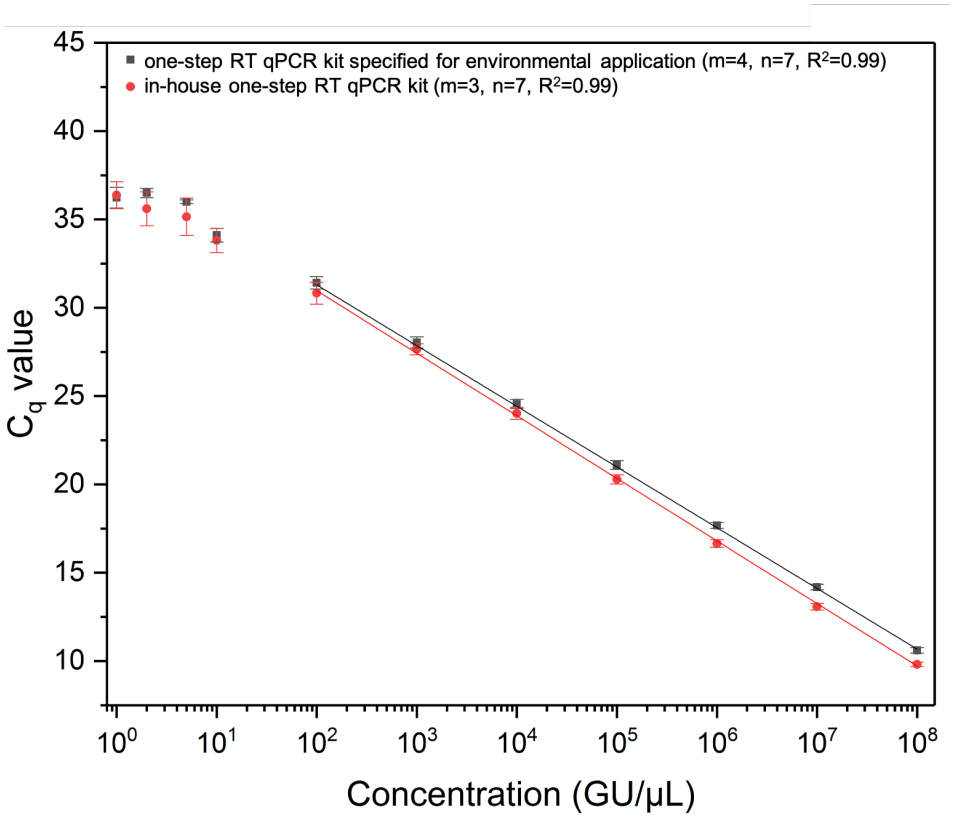


Figure 17: Overview of standard curves generated by a dilution series of gBlocks® for adenovirus and norovirus detection displayed by C_q values against concentration (GU/µL). A. Direct comparison of TaqMan® Environmental Master Mix 2.0 (qPCR master mix specified for environmental application) and Takyon™ No Rox Probe MasterMix dTTP Blue (in-house used master mix) for adenovirus detection. B. Direct comparison of RNA UltraSense™ One-Step Quantitative RT-PCR System (one step RT qPCR kit specified for environmental application) and Takyon™ One-Step Kit Converter (in-house used one step RT qPCR kit) for norovirus detection. *m* stands for number of replicates, *n* for number of measurement points.

Direct comparison of standard curves did not reveal any peculiarities between the reagents. Results showed a high linearity (Pearson R > 0.99) while the detected differences in C_q values were expected as C_q values are arbitrary units of the instrument, which may vary in relation to the master mix composition. When looking at the performance parameters in more detail it was found that specified reagents achieved slightly better efficiencies of 95%, whereas efficiencies of in-house used reagents were lower, but still in the acceptable range with 93% for adenovirus and 91% for norovirus (Johnson et al., 2013). Apart from this, working range and limit of detection were comparable. A detailed overview including costs is given in Table 7.

Table 7: Overview of performance parameters for qPCR reagents for adenovirus and norovirus detection.

A. Adenovirus

Master mix	**Specified for environmental application**	**In-house used**
Name	TaqMan® Environmental Master Mix 2.0	Takyon™ No Rox Probe MasterMix dTTP Blue
Costs/rct (EUR)	2,40	0,52
WR (GU/µL)	10^2 - 10^8	
Efficiency (%)	95	93
Lowest measured concentration (GU/µL)	1	
Replicates	12	8

WR: working range; GU: genomic unit; rct: reaction

B. Norovirus

One step RT qPCR kit	**Specified for environmental application**	**In-house used**
Name	RNA UltraSense™ One-Step Quantitative RT-PCR System	Takyon™ One-Step Kit Converter
Costs/rct (EUR)	4,97	1,04
WR (GU/µL)	10^2 - 10^8	
Efficiency (%)	95	91
Lowest measured concentration (GU/µL)	1	
Replicates	4	3

WR: working range; GU: genomic unit; rct: reaction

Although overall performance looked promising, tests with environmental samples led to a total inhibition of qPCR when using in-house reagents, whereas no impact was found with specified solutions. Thus, qPCR reagents specified for environmental application are required to allow for a reliable and reproducible detection of viruses in water and remain the best fit for the SOP.

1.2. Experimental setup of pilot trial between three European laboratories

After establishing the SOP in each laboratory, a pilot trial was conducted to validate its application and the performance of each concentration method at an interlaboratory scale. The structure of the pilot trial was organized as following: The three partner laboratories made up the panel of experts with IWC-TUM and UB as developers and professionals of MAF and SMF, and DTU as representative laboratory, being well experienced in concentration methods but new to MAF and SMF. IWC-TUM was appointed as the executive officer responsible for

sample distribution and final analysis of the results. For spiking experiments Evian mineral water was used as common matrix due to its well-defined, highly controlled and consistent water chemistry as well as availability to each partner site. For the purpose of generating a microbial environment and simulate conditions representative for a contamination of surface water by enteric viruses, Evian mineral water was spiked with artificially contaminated wastewater effluent containing a high or a low contamination load of adenovirus and norovirus. To achieve circumstances representative of a real case scenario in environmental virology, the pilot trial was conducted as a blind experiment. Therefore, UB and DTU provided IWC-TUM with stocks of adenovirus and norovirus. IWC-TUM spiked viruses at two different concentration levels in wastewater effluent samples, which were previously collected at the Kläranlage, Garching, Germany. Seeded wastewater samples were blindly coded and sent back to partner laboratories. An overview of sample distribution is displayed in Figure 18.

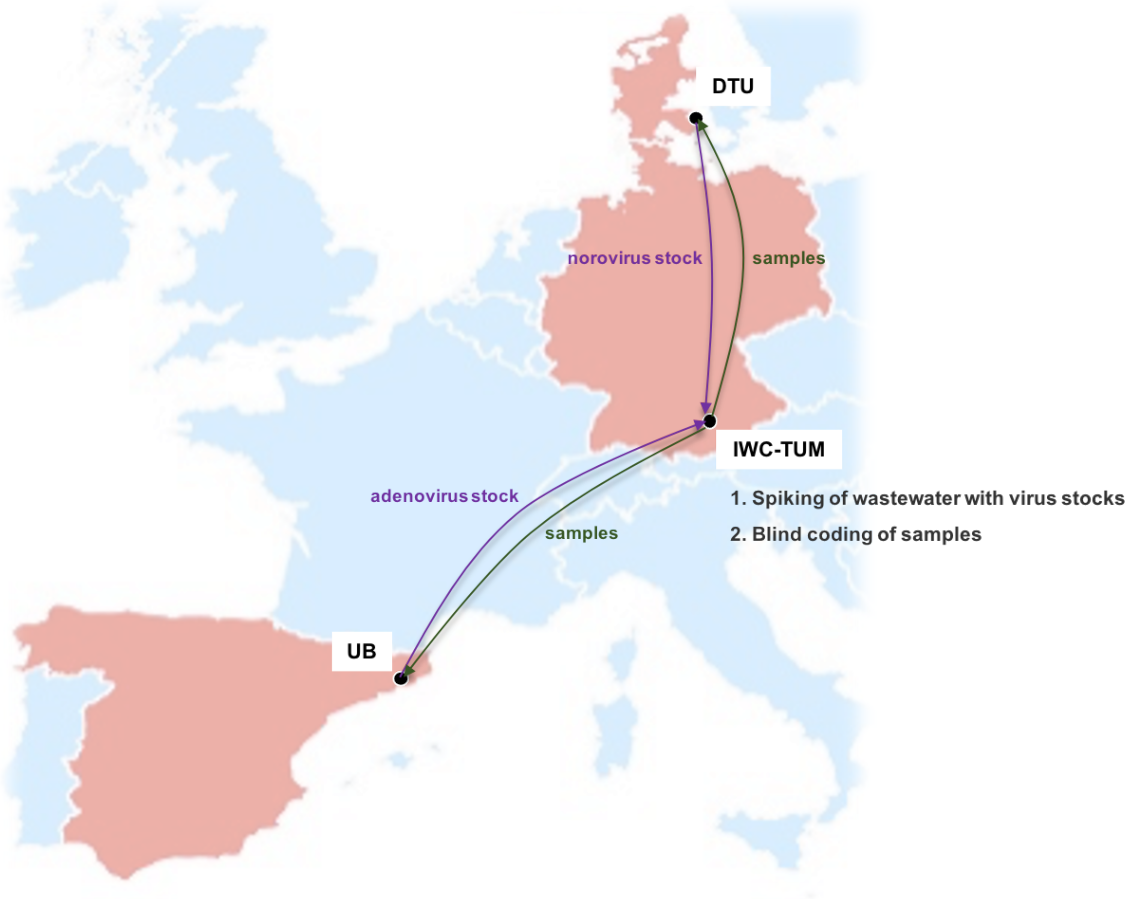


Figure 18: Overview of sample distribution for the pilot trial between the three laboratories. UB and DTU provided IWC-TUM with stocks of adenovirus and norovirus, respectively. IWC-TUM spiked viruses in wastewater previously collected at the Kläranlage Garching, Germany, blindly coded samples and sent aliquots back to partner laboratories.

Upon arrival at partner institutes spiking of Evian mineral water and concentration of viruses by MAF-OH and SMF were performed at capacity level of each laboratory. An illustrational overview of the workflow is given in Figure 19.

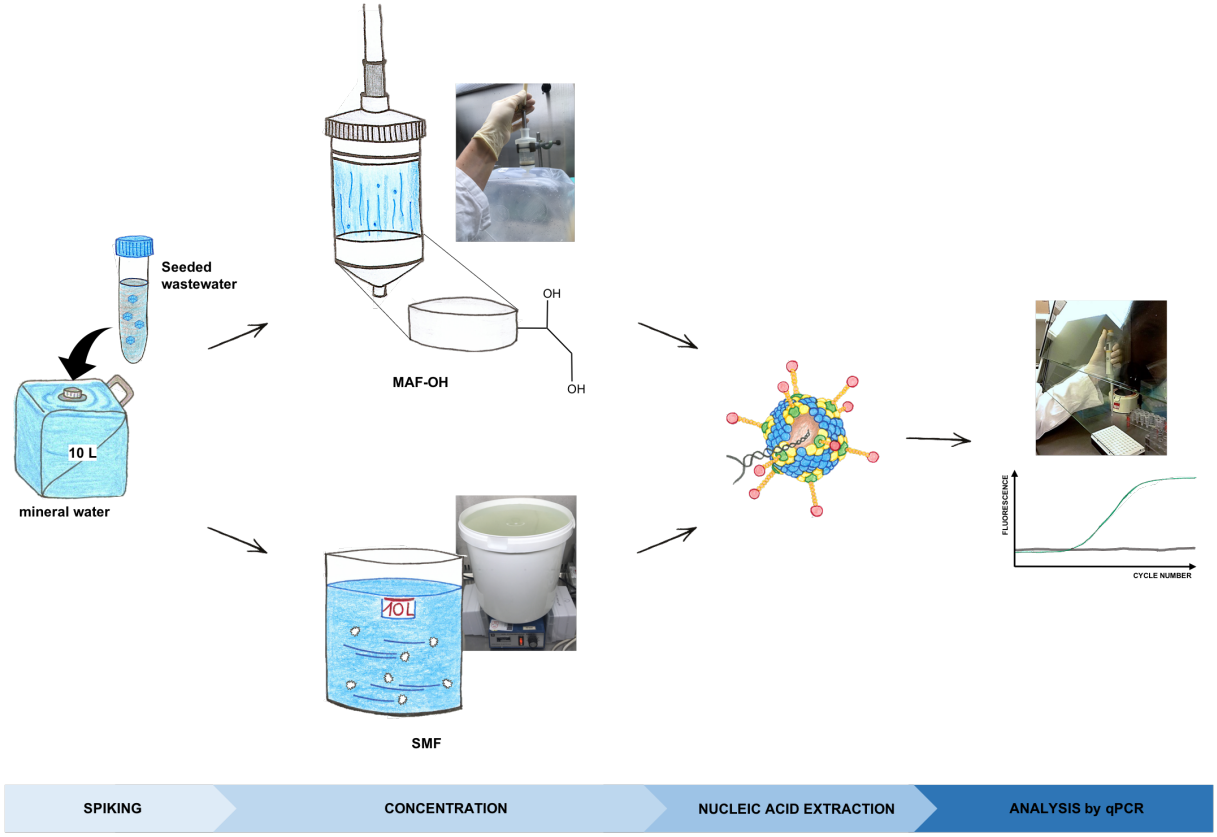


Figure 19: Illustrational overview of the workflow for concentration of viruses from water. Seeded wastewater is spiked into 10 L mineral water and viruses are concentrated by MAF-OH and SMF. Nucleic acids are extracted from viruses and quantified by qPCR. MAF-OH: monolithic adsorption filtration using monoliths with a negatively charged hydroxyl surface, SMF: skimmed milk flocculation.

1.3. Evaluation of MAF-OH and SMF for viral concentration

MAF-OH and SMF have already been used for the successful concentration of viruses from water in previous studies (Calgua et al., 2008; Pei et al., 2012). For the purpose of comparing the performance of both methods at an interlaboratory scale, 10 L of Evian mineral water was spiked with two different viral loads, either with 1.0×10^8 GU and 1.1×10^9 GU of adenovirus and norovirus, respectively representing a high viral level, or with 1.0×10^6 GU and 1.3×10^8 GU of adenovirus and norovirus, respectively representing a low viral level. Recoveries of both viruses obtained with each method were calculated and mean viral levels were compared between the laboratories (Figure 20).

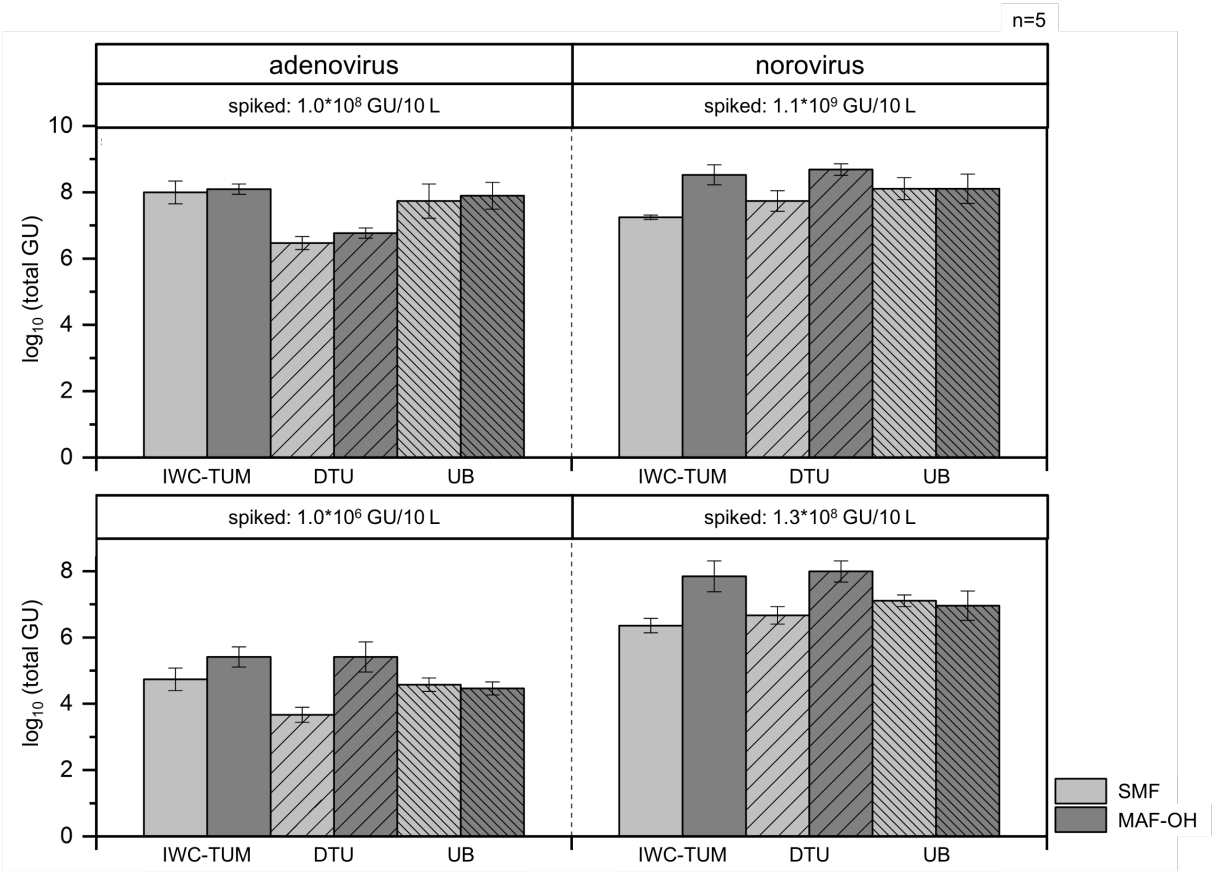


Figure 20: Overview of mean viral levels of adenovirus and norovirus recovered from 10 L seeded mineral water after MAF-OH (dark grey) or SMF (light grey) obtained in each laboratory. Spiked virus load is displayed on top of each graph. *GU* stands for genomic units and *n* for number of replicates.

Results demonstrate, that MAF-OH led to better recoveries with a higher reproducibility in most cases, independently of the spiked viral load or the executing laboratory. An exception represents the concentration of norovirus conducted by UB, where SMF achieved slightly better results. While this could not be reproduced by IWC-TUM or DTU, it can be assumed that the better performance of UB is attributed to its high level of expertise in SMF as the developer of the method. When correlating level of expertise with performance among laboratories in general, it was found that beginners could achieve comparable results to professionals with MAF-OH and SMF. However, independently of the experience level of the laboratory, results from SMF showed higher standard deviations in all cases, which illustrates an inconsistent reproducibility of the method - an observation that has already been described by various studies (Gonzales-Gustavson et al., 2017). It is likely that running more replicates may improve the reproducibility of SMF. However, a higher number of samples would drastically increase the total time needed to achieve comparable results to MAF-OH. This is based on the fact that SMF takes more than 16 hours to process one sample. In the same time, MAF-OH achieves a 48 times higher throughput for 10 L mineral water when conducted at a flow rate of 1 L/min. A detailed overview of the workflow in relation to the turnaround time of each method is displayed in Figure 21.

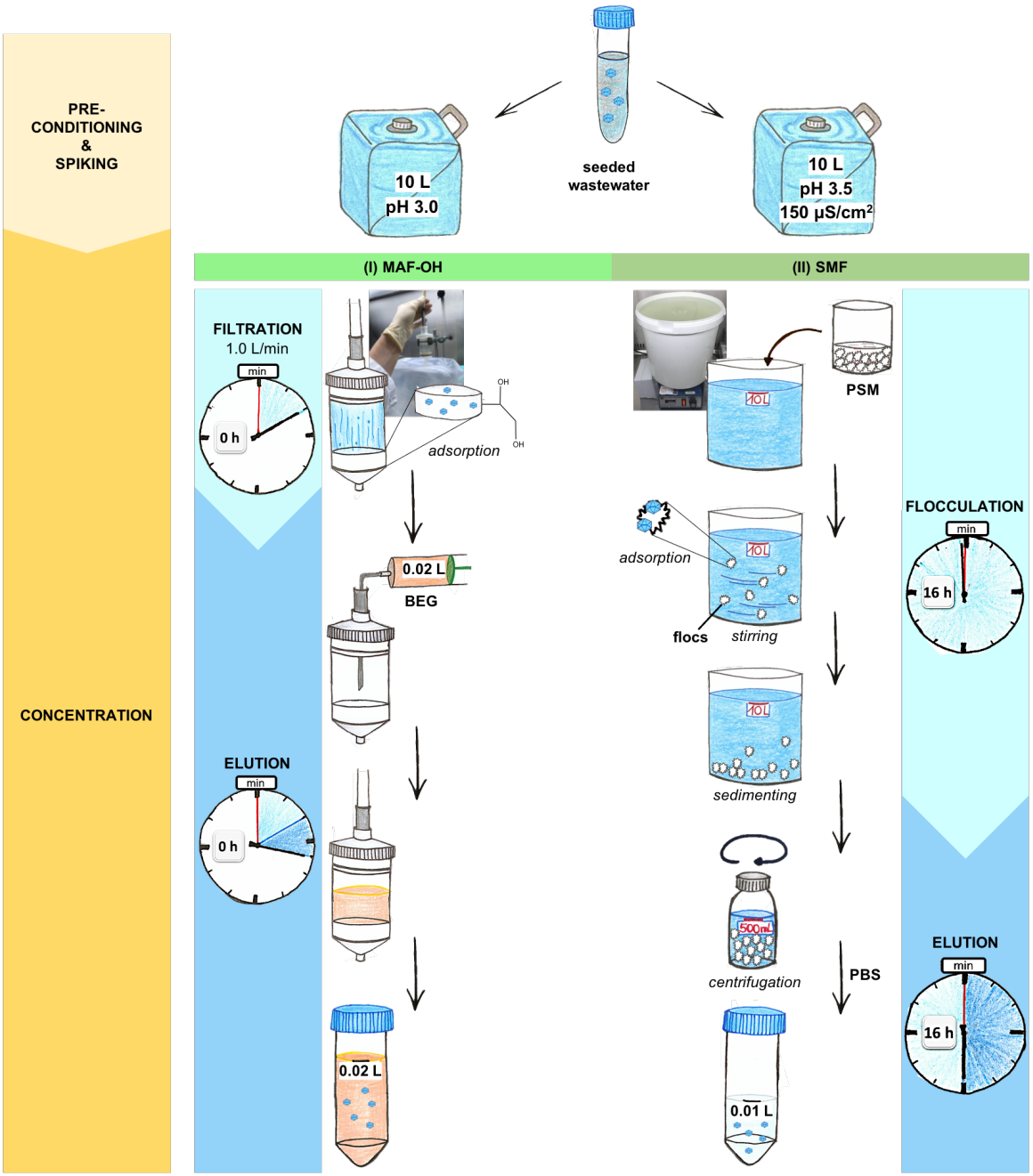


Figure 21: Workflow overview for (I) MAF-OH or (II) SMF in relation to turnaround times. (I) MAF-OH: 10 L water sample is pre-conditioned to pH of 3.0 and spiked with seeded wastewater. Filtration is performed at a flow rate of 1 L/min, resulting in a processing time of 10 min. Beef extract glycine buffer (BEG) is added to the monolith and elution is performed in a total time of 7 min. Taken filtration and elution together, MAF-OH takes 17 min for the whole workflow. (II) SMF: 10 L water sample is pre-conditioned to pH 3.5 and 150 µS/cm², spiked with seeded wastewater and mixed with pre-flocculated skimmed milk (PSM). Flocculation is performed by 8 hours of stirring followed by 8 hours of sedimentation. Elution is carried out by 30 min of centrifugation, leading to a total turnaround time of around 16.5 hours for SMF.

Taken together, results illustrate that both methods were easy to establish in a short period of time and did not require a certain level of expertise or additional training to achieve results comparable to professional operators. In addition, it could be shown that MAF-OH and SMF can be applied for the in-parallel concentration of adenovirus and norovirus from water.

However, in depth comparison of performance revealed, that MAF-OH stands out with a higher efficiency due to a better reproducibility and a significantly faster turnaround time. Based on these results both methods seem to fulfill some of the most important requirements for an application in routine virus surveillance. Nevertheless, their main disadvantage remains the pre-conditioning of water samples to promote virus adsorption. On one hand, pre-conditioning takes time, a factor particularly crucial in outbreak situations. On the other hand, it increases the risk of introducing contaminations and restricts field application.

1.4. Evaluation of the SOP

1.4.1. Workflow

Although the overall execution of the pilot trial worked well, challenges were experienced in verifying results due to a lack of reference material and missing pre-experimental data, particularly about the impact of transportation and storage time on viral stability.

One of the leading factors in determining virus stability, which involves virus inactivation and genome degradation, is temperature. Thus, virus strains are commonly kept at -80 °C to preserve viral stability. A potential increase of temperature and the time of viruses being exposed to such a change may have an impact on their stability, particularly on laboratory strains, which may not reflect the resistance of naturally occurring ones (Bertrand et al., 2012). When distributing virus samples, for example for interlaboratory studies, changes in temperature and variations in delivery times are expected, which may have for the aforementioned reasons a direct impact on virus stability. Even though this is well known, the effect of transportation on viral stability of viral stocks and spiked wastewater samples was inadvertently forgotten to be considered when planning and conducting the pilot trial and was neither checked in preliminary tests. Instead, distribution and quantification of samples were performed as following: Prior to distribution, virus stocks were quantified at providing laboratories UB and DTU respectively and sent to IWC-TUM. Rather than being re-quantified upon arrival at IWC-TUM, stocks were spiked directly into wastewater based on communicated concentrations by providing institutes and sent back to partner laboratories. Thus, the true viral load spiked into wastewater may have differed from the theoretically calculated one resulting in variances of recoveries among laboratories. Figure 22 gives an overview of the spiking procedure and highlights checkpoints where viral quantification would have been crucial.

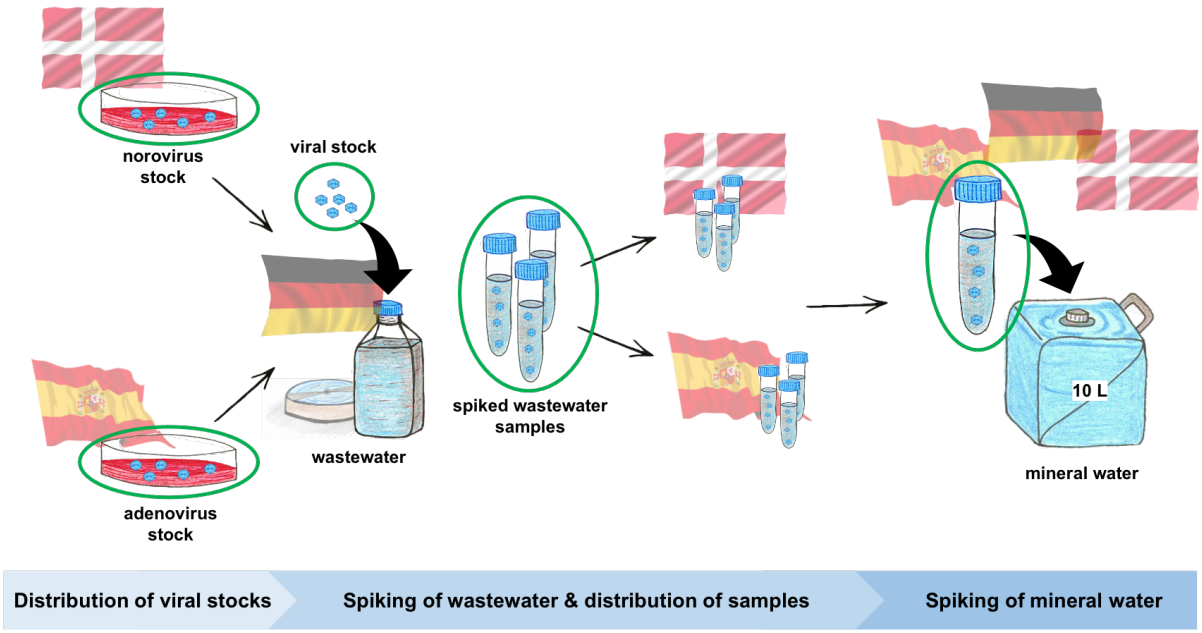


Figure 22: Overview of spiking procedure and checkpoints of viral quantification. Checkpoints at which viruses need to be quantified are circled in green.

In addition to changes in temperature, also sample storage time can have an impact on viral stability. During the pilot trial laboratories performed experiments at their material and working capacity levels, which were primarily determined by the equipment and number of professional operators available at the time. On one hand, this generated circumstances representative of a real case scenario in routine virus surveillance. On the other hand pre-experimental data was missing to verify if inconsistent storage times of spiked wastewater samples prior to concentration could have had an impact on viral stability. Here, also reference material could have been used to verify if variations in recovered viral loads between the laboratories were a direct result of differences in viral stability caused by transportation or storage of samples. However, no reference samples of stocks or spiked wastewater were kept or included in the study due to limited feed material.

1.4.2. Costs

Cost is a key parameter for the successful implementation of a SOP in routine viral surveillance. Main cost drivers can be attributed to the chosen concentration method on one hand as well as qPCR for detection on the other hand. MAF-OH and SMF are highly economical with costs of around 1 EUR/sample, which makes them very attractive from a pure cost-benefit standpoint. Therefore, they are the most economical concentration methods available to date and present an ideal cost-saving solution to commonly applied approaches. An overview is given in Table 8.

Table 8: Overview of costs of MAF-OH and SMF in relation to most commonly applied concentration methods for viruses in water.

Method		Costs/sample (EUR)
MAF-OH		~ 1
SMF		~ 1
Glass wool filtration		~ 1
VIRADEL	Filterite	18
	HA Membrane	18
	NanoCeram®	40
	1MDS filter	250
Ultrafiltration		250 - 1500

The QIAamp Viral RNA Mini Kit (Qiagen, Hilden) was used for extraction with expenses of 3,94 EUR/sample. To further reduce costs of sample preparation it would be recommended to compare and evaluate the applied kit to similar kits of other manufacturers. Expenses for detection by qPCR are primarily attributed to the qPCR reagents as well as the number of reactions needed. Although using gBlocks® as standard reduced the number of reactions and in turn the upfront costs needed for the establishment of qPCR assays, expenses for qPCR reagents still remain high. Verification experiments showed that qPCR reagents specified for an environmental application are required to successfully detect viruses in environmental water. Thus, related costs to qPCR and one step RT qPCR for adenovirus and norovirus quantification, respectively, could not be reduced further and amount to 2,50 EUR/qPCR reaction and 5,00 EUR/RT qPCR reaction.

Although specific reagents are required, it may be possible to improve costs at least for the detection of norovirus. The detection of RNA viruses, such as norovirus, is generally more expensive due to the additional RT step required prior to qPCR. Instead of performing one step RT qPCR as included in the SOP, another more cost-efficient alternative could be a two-step approach, in which RT is decoupled from qPCR and performed separately. This allows for the flexible choice of reagents for each step, which may reduce total costs per RT qPCR reaction and can also lead to an increased sensitivity and reproducibility as RT and qPCR can be performed under ideal experimental conditions. However, the flipside of two step RT qPCR approaches is, that they commonly need more time for establishment and optimization to evaluate the best fitting conditions for each step. Consequently, upfront costs can hardly be estimated, and total expenses might finally be higher compared to an initial application of a one step RT qPCR approach. In addition, the total turnaround time of two step RT qPCR is commonly longer. A summary of the most important decision-making criteria for choosing a one step or a two step RT qPCR approach is given in Table 9.

Table 9: Comparison of decision criteria for choosing a one step or two step RT qPCR approach.

Type of RT qPCR	One step	Two step
Costs/reaction	€€€	€€
Flexibility	low	high
Handling	fast	time consuming
Average run time (min)	120	235
Sensitivity	medium	high

1.5. Conclusion of pilot trial

The established SOP presents a promising framework for the development of a standardized workflow for routine virus testing in water. A key stepstone towards a harmonized approach was the implementation of gBlocks® for virus detection, which accelerated the establishment of qPCR assays, efficiently reduced upfront qPCR expenses and simplified the comparison of data between laboratories, independently of the qPCR instrument used. While the application of specific qPCR reagents validated for environment samples is required to allow for a reliable detection of viruses in water, further reduction in qPCR costs may be achieved by applying a two step RT qPCR approach for the detection of norovirus.

The conducted pilot trial further illustrated, that both concentration methods, MAF-OH and SMF, can be applied for virus concentration at an interlaboratory scale, although MAF-OH offered a faster solution with a higher reproducibility. However, it needs to be verified whether this also applies for other water matrices than the one tested in the pilot trial. It can be anticipated that SMF will perform better for water of high turbidity as monoliths of MAF tend to clog with increasing amounts of suspended particles present in water. Although both methods represent an excellent cost-saving solution to commonly applied concentration methods, pre-conditioning of water samples remains to be the main drawback of their application in routine virus surveillance.

In order to further improve the SOP, pre-experiments need to be included to study viral stability during transportation and storage of samples. Moreover, also the incorporation of reference samples in the SOP is crucial for determining and controlling viral integrity during the workflow. Furthermore, references samples may allow for the normalization of differences between laboratories. In addition to this, a defined time frame for experimental execution is recommended to avoid variations in results due to different time spans between single steps of the workflow. Nevertheless, an inherent variability of results will still remain and has to be expected when performing the SOP at an interlaboratory scale. This is attributed to factors, which cannot always be completely controlled involving the operator, the applied equipment, the calibration of the equipment on one hand and environmental conditions, such as temperature, humidity and pressure among others on the other hand.

2. Optimization of SOP based on MAF for the concentration of HAdV from surface water

Based on the promising results gained in the pilot trial, the SOP was further optimized by IWC-TUM for MAF and its application in surface water using adenovirus as only target due to its common presence in surface water, independently of the season.

2.1. Evaluation of monolith's type and elution buffer

To overcome the drawback of MAF-OH to condition water prior to concentration, monoliths with positively charged DEAE-groups exposed on their surface (MAF-DEAE) were tested, which have already been shown to successfully enrich bacteriophages at neutral pH (Elsaesser et al., 2018). To examine the suitability of MAF-DEAE for concentrating adenovirus, its performance was evaluated in direct comparison to MAF-OH. These proof-of-principle experiments were carried out in tap water as spiking matrix with a less defined and controlled water chemistry than mineral water. In contrast to previous experiments of the pilot trial, HAdV5 was used as target serotype due to limited availability of HAdV35. However, similar to HAdV35, also HAdV5 is one of the commonly applied laboratory adenovirus strains, because of its easy production and safe handling given its low biosafety level (Jiang, 2006).
1 L tap water was spiked with HAdV5 to a final concentration of 10^5 GU/mL and processed with MAF-OH or MAF-DEAE. The resulting recoveries are displayed in Table 10.

Table 10: Recoveries of HAdV5 from 1 L tap water after concentration with MAF-OH and MAF-DEAE. Recoveries are displayed in % as mean ± SD. n=3

Method	Elution buffer	Recovery (%)	pH of water sample
MAF-OH	BEG	32.4 ± 0.6	3.0
MAF-DEAE	BEG	39.8 ± 16.1	7.5

MAF-OH: monolithic surface functionalized with OH-groups; MAF-DEAE: monolithic surface functionalized with DEAE-groups; BEG (beef extract glycine buffer): 3% (w/v) beef extract, 0.5 M glycine at pH 9.5. *n* for number of replicates.

Comparison of MAF-DEAE to MAF-OH showed that MAF-DEAE successfully enriched HAdV5 at neutral pH from tap water, and, with a recovery of almost 40%, performed slightly better than MAF-OH achieving 32% only. However, MAF-DEAE resulted in a higher degree of standard deviation. Different studies reported that concentration of HAdV in particular exhibits significant variations in recoveries in contrast to other viruses. It was hypothesized that these variations are attributed to a hindered elution of HAdV from the filter matrix in general, whereas the level of impact was found to be filter matrix type specific (Albinana-Gimenez et al., 2009; Gibbons et al., 2010; Ikner et al., 2011; McMinn, 2013; Schaudies and Robinson, 2007). Based on these observations, it is likely to suppose that such a correlation between elution efficiency and filter matrix type also applies for MAF. If so, it can be assumed that MAF-DEAE with its

positively charged surface adsorbs HAdV stronger than MAF-OH, resulting in a weaker elution when applying BEG buffer for HAdV elution from MAF-OH and MAF-DEAE. Consequently, using a different elution buffer for MAF-DEAE may enhance its efficiency. It was shown, that particularly high salt buffers achieved increased elution efficiencies compared to BEG buffers when concentrating bacteriophages with MAF-DEAE (Elsaesser, 2017). To evaluate if a high salt buffer can also improve results for HAdV, the experiment was repeated by spiking 1 L tap water with HAdV5 to a final concentration of 10^5 GU/mL using MAF-DEAE for concentration and a high salt buffer for elution. Thereby, a recovery of 34.4 ± 2.9% was achieved, which shows, that the high salt buffer could not increase elution efficiency of MAF-DEAE and also decreased recovery.

In addition to a correlation between filter matrix type and elution efficiency, it is furthermore reported that elution also depends on the water matrix and is subject to multiple interferences such as changes in pH, concentration and type of salts as well as the composition and loading of dissolved organics present in a sample (Melnick et al., 1984; Sobsey and Glass, 1984; Victoria et al., 2009; Straub and Chandler, 2003). Thus, it can be assumed, that elution efficiency of HAdV5 from MAF-DEAE may be different between water matrices. While BEG buffer offered the highest recovery for HAdV5 in tap water, it was further chosen as elution buffer in following experiments, in which MAF-DEAE should be evaluated for river water.

However, independently of the elution efficiencies, MAF-OH and MAF-DEAE achieved comparable and, in some cases, even better results than those published for the concentration of HAdV from tap water using positively charged NanoCeram® filters (39%) or negatively charged Filterite (36%) and HA membranes (2.4 - 2.8%) (Ahmed et al., 2015; Enriquez and Gerba, 1995; Ikner et al., 2011).

2.2. Evaluation of MAF-DEAE for river water

River water was chosen as representative matrix for surface water illustrating a matrix of high complexity due to an undefined water chemistry. Prior to spiking experiments, the maximal processing volume and flow rate of MAF-DEAE for river water was assessed. Water samples were collected from the river “Würm” in Gräfelfing, Germany, which showed an average turbidity of 0.18 ± 0.02 FNU (m=30) independently of the sampling season. In contrast to previous experiments with tap water, the turbidity of river water required an adjustment of the processing conditions. When applying river water, it was observed that monoliths were strongly compressed during filtration (Figure 23) and clogged at a maximum sample volume of 10 L, before MAF columns started leaking.

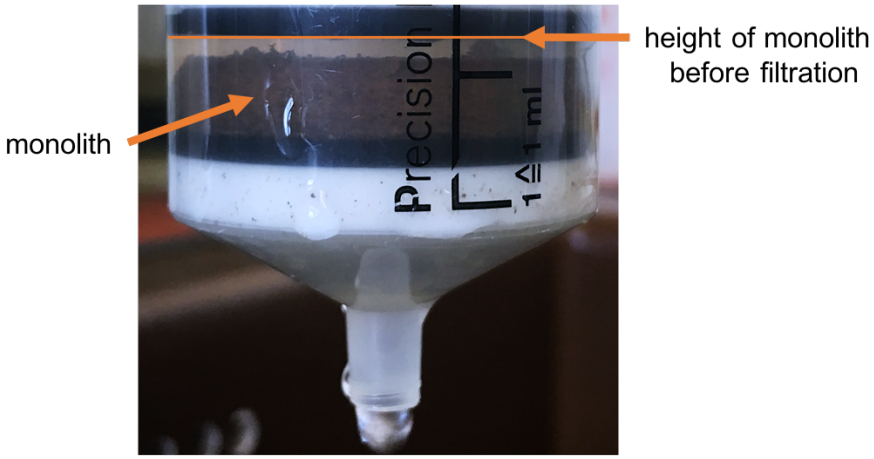


Figure 23: Compression of monolith during filtration of river water. Orange line displays the height of the monolith before filtration.

Furthermore, it was found that suspended particles present in river water samples accumulated primarily on border areas of the monolith (Figure 24).

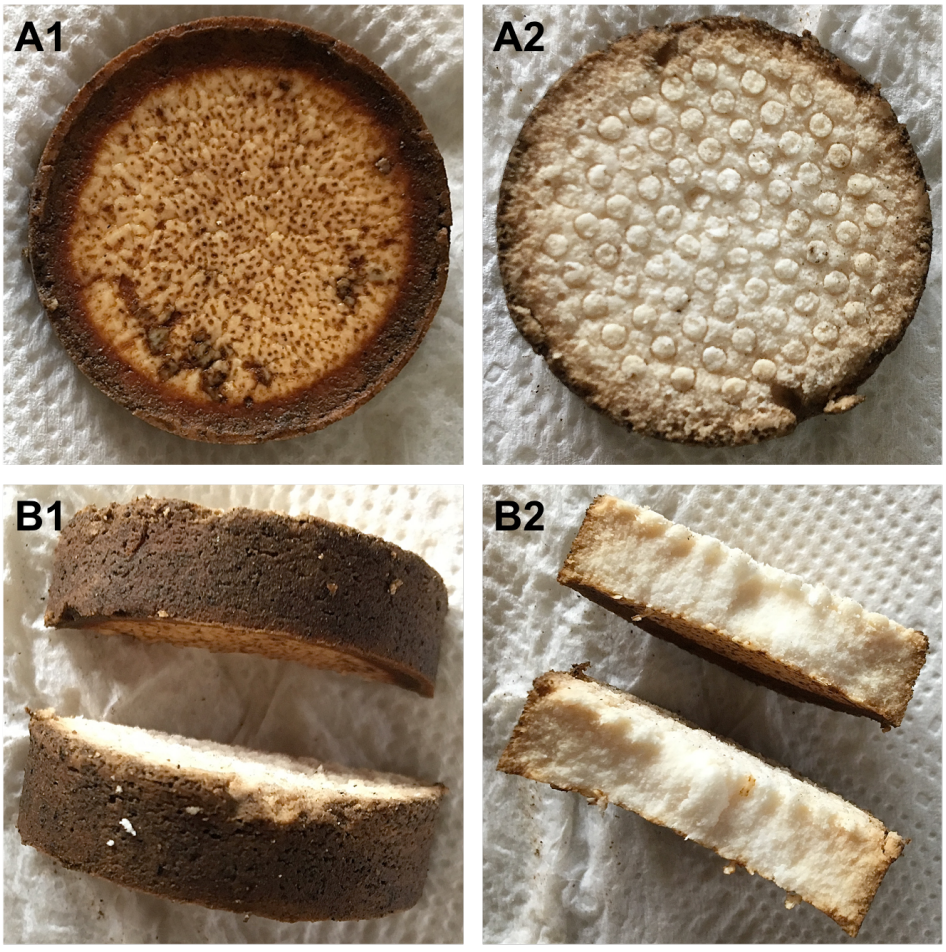


Figure 24: Overview of monolith after filtration of ten liters "Würm" river water. Displayed are the entire monolith from the top (A1) and the bottom (A2) as well as the outside border (B1) and inner (B2) cross-sectional area.

Thus, it was assumed that the compressed monolith allows water to pass through on its sides, which would lead to a loss of viruses when applying spiked water samples. Elsaesser, 2017

described similar observations for monoliths when processing water samples of different turbidity. He found that the compression of monoliths is induced by an increasing back pressure during filtration, which is directly related to the load of suspended particles present in the water sample. To control the back pressure and avoid that water may pass the monolith without being filtered, he reduced the flow rate and demonstrated that a lower flow rate had no effect on recovery rates (Elsaesser, 2017). To find a compromise solution between processing time and back pressure during filtration, the flow rate was restricted to 0.57 L/min. This relieved compression from the monolith and resulted in a turnaround time of 24 min including 17 min filtration and 7 min elution for MAF-DEAE using 10 L of river water.

A solution to further decrease processing times could be the operation of MAF-DEAE in a cross flow instead of a dead-end mode, which may also potentially increase the total processing volume for river water. Here, more research is required to set up such a device. However, with a total time of 24 min, MAF-DEAE achieves a competitive turnaround time to other concentration methods for surface water, such as VIRADEL, and can still process 10 L water samples up to 40 times faster than SMF (Francy et al., 2013).

2.3. Optimized structure of SOP

Evaluated processing conditions including flow rate, maximum sample volume and best elution buffer were implemented in the previously developed SOP to adjust the workflow to MAF-DEAE and river water. In addition, reference samples and a schedule for experimental execution were defined and also included in the SOP. A graphical illustration of the optimized experimental setup is displayed in Figure 25.

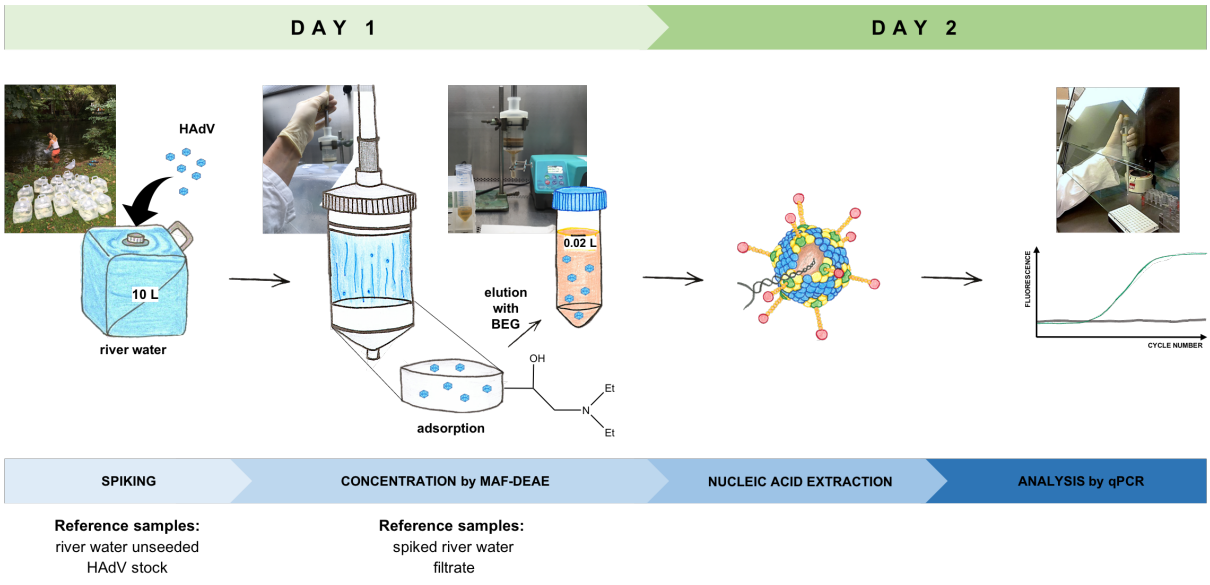


Figure 25: Experimental setup for the concentration and detection of HAdV from river water. Day 1: 10 L of river water is spiked with HAdV, reference samples are taken of unseeded river water and HAdV stock. The virus is concentrated by MAF-DEAE and eluted in a final volume of 0.02 L using BEG (beef extract glycine

buffer: 3% (w/v) beef extract, 0.5 M glycine at pH 9.5). Reference samples are taken of the spiked river water and the filtrate. Day 2: Nucleic acid extraction is performed and HAdV load is quantified by qPCR.

In more detail, the optimized SOP included the following steps, which were performed in the order mentioned below:

Day 1:

All steps of the MAF-DEAE workflow are performed at room temperature under a flow hood. Reference samples taken during the workflow are kept at room temperature under the hood to avoid impacts of temperature changes on viral stability. When the final step of the workflow is completed, eluate and reference samples are stored at -80°C until the next day.

1. Reference samples of HAdV stock and unseeded river water are taken (1 mL each)
2. 10 L river water is spiked with HAdV stock at defined concentration and mixed by inverting
3. Reference sample of spiked river water is taken (1 mL)
4. Filtration of spiked river water by MAF-DEAE (Figure 26) at a flow rate of 0.57 L/min – total processing time: 17 min

Figure 26: Filtration of river water by MAF-DEAE under flow hood.

5. Reference sample of filtrate is taken (1 mL)
6. Elution of viruses with BEG buffer (Figure 27) – total processing time: 7 min

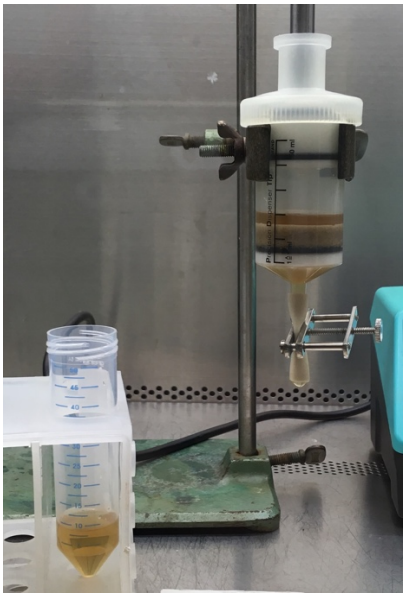


Figure 27: Last step of elution using BEG buffer.

7. Sample of eluate is taken (1 mL)
8. Eluate and reference samples are stored at -80°C until next day

Day 2:

1. Thawing of 1 mL samples
2. Nucleic acid extraction
3. Quantification of viral load by qPCR – qPCR run time: 90 min
4. Data analysis

2.4. Additional performance parameter for quantitative evaluation of MAF-DEAE: Limit of detection

The SOP was further improved by adding the limit of detection (LOD) as a quantitative performance parameter of MAF-DEAE, which should be evaluated by assessing the impact of MAF-DEAE to detect HAdV spiked in river water. To do so, the LOD for HAdV before and after MAF-DEAE needed to be determined by qPCR. As previously described in *Fundamentals - 2.2.3.* the determination of LOD in qPCR is challenging as conventional definitions are not directly applicable (Klymus et al., 2020). According to these definitions, which vary slightly among regulatory bodies and standards organizations, the LOD is determined by the lowest concentration that can be distinguished from the background signal of the blank sample while analyte and signal of response follow a linear correlation (Armbruster and Pry, 2008; Shrivastava and Gupta, 2011). However, in qPCR neither such a linear correlation is observed nor does NTC, which represents the blank sample in qPCR, return a positive signal (Burns and Valdivia, 2007; Forootan et al., 2017; Hunter et al., 2017). The reason for this is based on the fact, that qPCR reports C_q values, which represent an amplification of the target DNA by

displaying the point at which the fluorescent signal of a sample exceeds the background noise and enters exponential growth. In turn, no C_q value is reported for NTC, which does not contain a target DNA that can be amplified. Thus, the conventional analysis of qPCR data by assigning C_q values to sample concentrations does not allow for the evaluation of the impact of MAF-DEAE on the LOD for HAdV. Elsaesser, 2017 introduced a solution by using raw fluorescence data instead of C_q values, which allowed for the assessment of signals for NTC and samples. By plotting raw fluorescent signals at a selected C_q against corresponding sample concentrations a sigmoidal dose-response curve was generated and the LOD could be assessed. The selected C_q value for signal plotting was defined by the cycle at which NTC did not yet show a positive signal (Elsaesser, 2017).

While this approach was applied for qPCR assays using the double stranded intercalating SYBR Green dye to detect amplification, it is not directly transferable to TaqMan assays. This is based on the fact that SYBR Green dyes are often associated with non-specific signals occurring in latter cycles due to an incorporation of the dye in double stranded artifacts, such as primer dimer formations (Ruiz-Villalba et al., 2017). In contrast, TaqMan assays are highly specific as an amplification signal is only generated when the included probe is cleaved by the DNA polymerase during strand elongation (as described in detail under *Fundamentals - 2.2.2.*). Consequently, the raw fluorescent signal of NTC is stable over the total number of cycles when using TaqMan chemistry.

To find a definition that is easily applicable to every qPCR assay and instrument and thus can also be used as a standardized approach in routine virus surveillance, the original approach by Elsaesser, 2017 was modified as following: To plot raw fluorescent signals of samples against concentration the first C_q outside of the linear area of the standard curve was defined as cutoff cycle. On one hand, this cycle can be assumed to represent still accurate data compared to latter cycles, which prevents the inclusion of false-positive signals into LOD determination potentially occurring when using SYBR Green dyes. On the other hand, using the working range of the standard curve as a reference point for cutoff cycle determination offers a guided approach, which can easily be applied to every qPCR assay, while assay specific parameters associated with the standard curve are incorporated and used to tailor the approach not only to the assay itself, but also to its performance on the qPCR instrument used. Based on the defined cutoff cycle, corresponding fluorescent signals of samples are plotted against concentrations and the Levenberg Marquardt curve-fitting algorithm is applied to determine the LOD. A detailed illustration of how the cutoff cycle is determined and applied is displayed in figure 28.

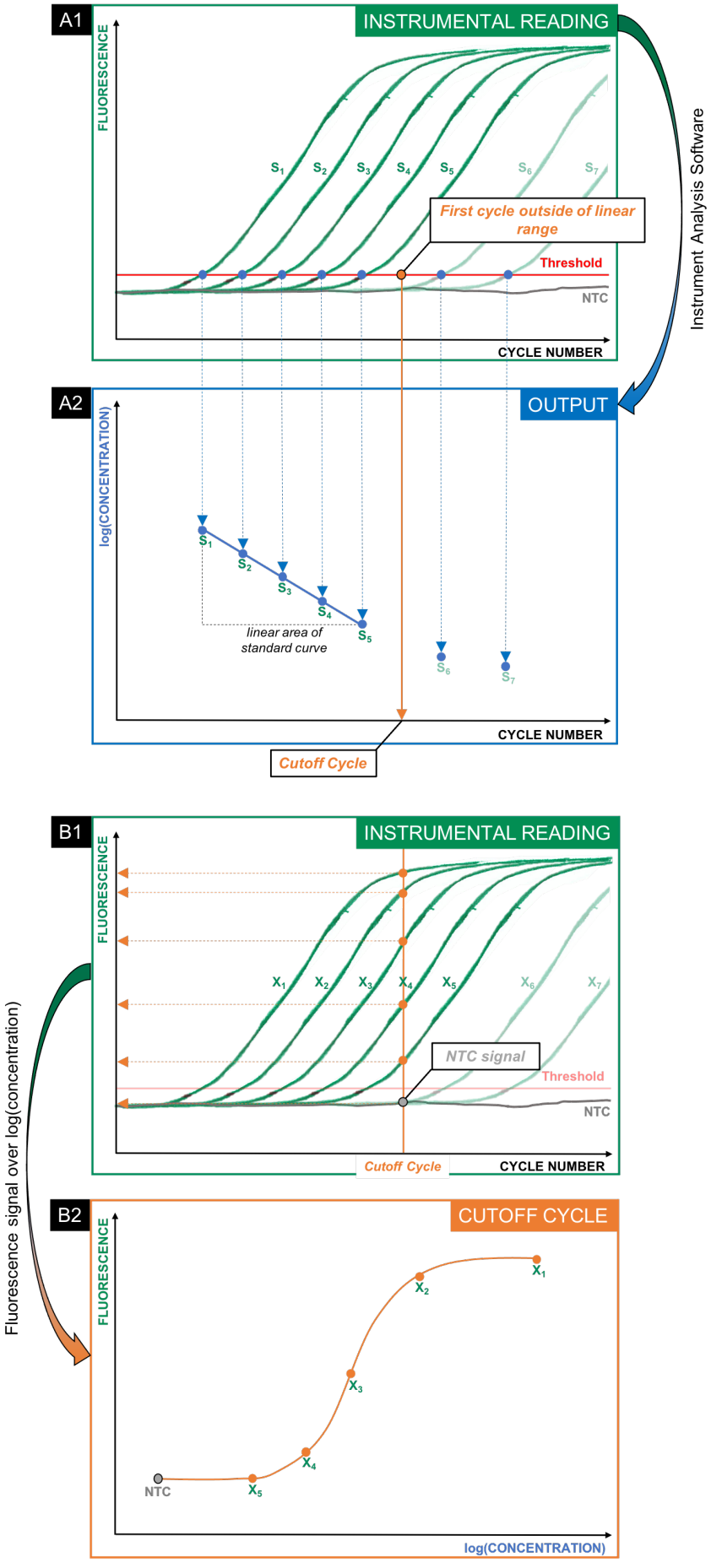


Figure 28: Definition of cutoff cycle in qPCR to determine the impact of MAF on the limit of detection (LOD).

A: Analysis of dilution series of standard (S_1 - S_7, concentration S_1>S_7) to determine cutoff cycle of the qPCR assay. A1: Instrumental readings of amplification plots for standard dilutions S_1 to S_7 and NTC given by fluorescent signal over cycle number. A threshold is set at the fluorescent signal at which all plots exceeded the background signal and entered exponential growth. The corresponding cycle number of each dilution is used by the instrument analysis software to generate a standard curve of cycle number plotted against log(concentration), here displayed inversely by log(concentration) against cycle number for an easier graphical presentation of cutoff cycle assessment (A2). C_q values inside of the linear area of the standard curve are used for sample analysis. To set a cutoff cycle in qPCR for the determination of the impact of MAF-DEAE on the LOD, the first cycle outside of the linear area of the standard curve is chosen, which can be assumed to still represent accurate data compared to latter cycles. B: Analysis of samples X_1 to X_7 (concentration X_1>X_7). B1: Amplification plots of samples X_1 to X_7 and NTC given by fluorescent signal plotted against cycle number. The cutoff cycle previously determined by the standard of the same qPCR assay is applied to the amplification plots. B2: Corresponding fluorescent signals of samples and NTC are plotted against concentrations (log(concentration)), which are determined by the standard curve beforehand. A sigmoidal dose-response curve is generated by applying the Levenberg Marquardt curve-fitting algorithm to assess the corresponding LOD.

According to the described approach, the cutoff cycle for the HAdV assay was determined at a C_q value of 32 corresponding to an LOD of 3.7×10^1 GU/µL by a standard curve of gBlocks® ranging from 1 to 10^8 GU/µL (Figure 29).

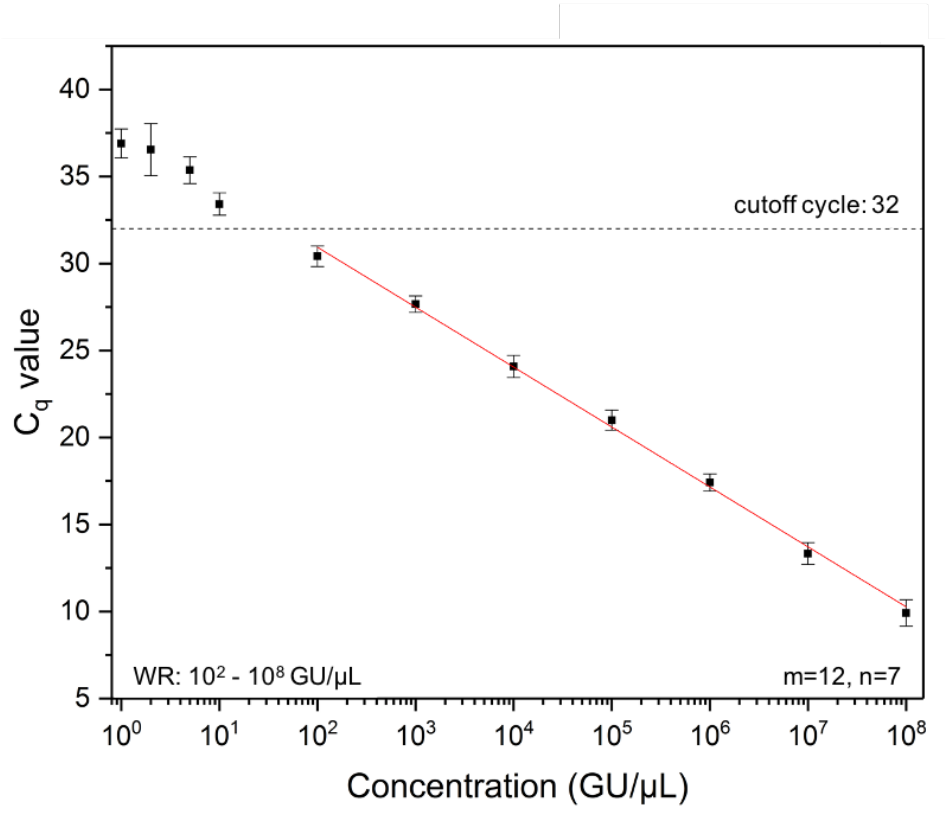


Figure 29: Standard curve of dilution series of gBlocks® for adenovirus detection displayed by C_q values over concentration (GU/µL) including working range (WR) and cutoff cycle at a C_q value of 32 corresponding to an LOD of 3.7×10^1 GU/µL. Used master mix: TaqMan® Environmental Master Mix 2.0. *m* stands for number of replicates, *n* for number of measurement points.

3. Concentration of HAdV from river water

The optimized SOP was applied to evaluate the performance of MAF-DEAE for the concentration of HAdV spiked in river water. Experiments were performed first with HAdV5 for general evaluation of MAF-DEAE and then repeated with HAdV41, the HAdV serotype most commonly present in environmental water and a representative target for an application of the SOP in routine virus surveillance (Haramoto et al., 2007). Results were directly compared to each other.

3.1. Influence on limit of detection

To validate the impact of MAF-DEAE on the LOD of qPCR for HAdV detection, 10 L river water samples were spiked to final concentrations ranging from 10^1 to 10^5 GU/mL. The determined cutoff cycle of 32 was applied to plot raw fluorescence data against sample concentrations (Figure 30).

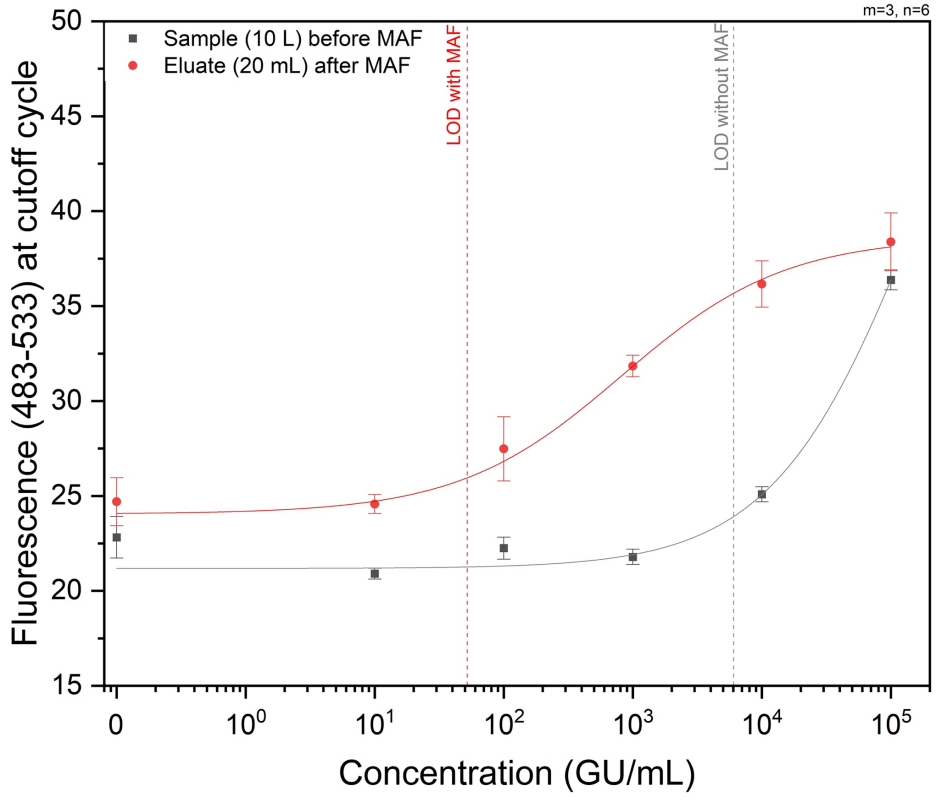


Figure 30: Influence of MAF-DEAE on LOD of qPCR for HAdV5. Grey graph displays spiked water before MAF, red graph eluates after MAF. Concentration is given in genomic units (GU)/mL river water. *m* stands for number of replicates, *n* for number of measurement points. The curve-fitting algorithm of Levenberg-Marquardt was used.

The LOD of qPCR for HAdV5 before MAF-DEAE was determined to be 6.0×10^3 GU/mL, which could be decreased to 5.2×10^1 GU/mL after MAF-DEAE. These results show, that MAF-DEAE significantly reduced the LOD by more than two orders of magnitude, which enhanced the detection of HAdV5 in river water by 115 times. Thereby, MAF-DEAE allows for

the quantification of HAdV5 by qPCR at concentration levels commonly found in the environment, which would otherwise not be detectable (Seidel et al., 2016).

When repeating the experiment with HAdV41, it was found, that MAF-DEAE had a lower impact. The LOD of qPCR for HAdV41 in 10 L river water before MAF was determined to be 5.9×10^3 GU/mL, which could only be enhanced by 15 times to 3.9×10^2 GU/mL after MAF (Figure 31).

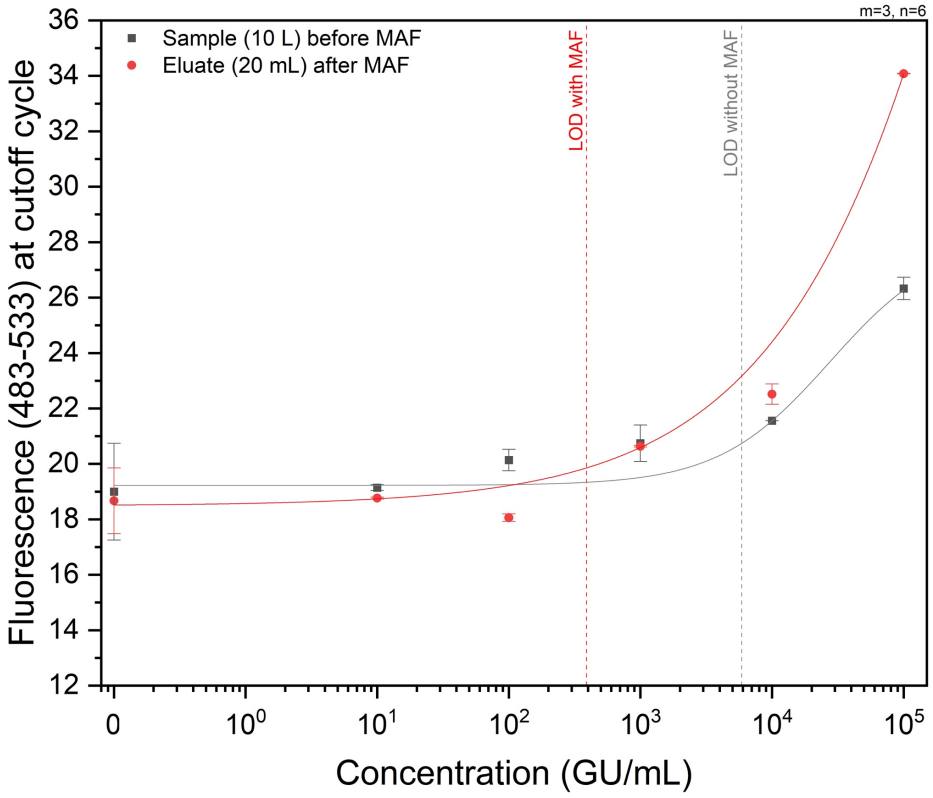


Figure 31: Influence of MAF-DEAE on LOD of qPCR for HAdV41. Grey graph displays spiked water before MAF, red graph eluates after MAF. Concentration is given in genomic units (GU)/mL river water. *m* stands for number of replicates, *n* for number of measurement points. The curve-fitting algorithm of Levenberg-Marquardt was used.

A reason for the difference in performance of MAF-DEAE observed for HAdV5 and HAdV41 could be due to a variation in physicochemical properties between the two serotypes, such as alterations of the isoelectric point or small structural differences. It can be assumed, that such variations may have an effect on the interaction of virions with the monolith, an observation already described for HAdV when applying other membrane-based approaches (Favier et al., 2004; Gerba, 1984; Gibbons et al., 2010; Kidd et al., 1993; Michen and Graule, 2010; Sobsey and Glass, 1984; Yeh et al., 1994). Consequently, the physical entrapment of HAdV by MAF-DEAE may vary in relation to the serotype. This would also explain the small differences observed between the two serotypes when looking at average recoveries of HAdV5 and HAdV41 determined for each concentration level (Figure 32).

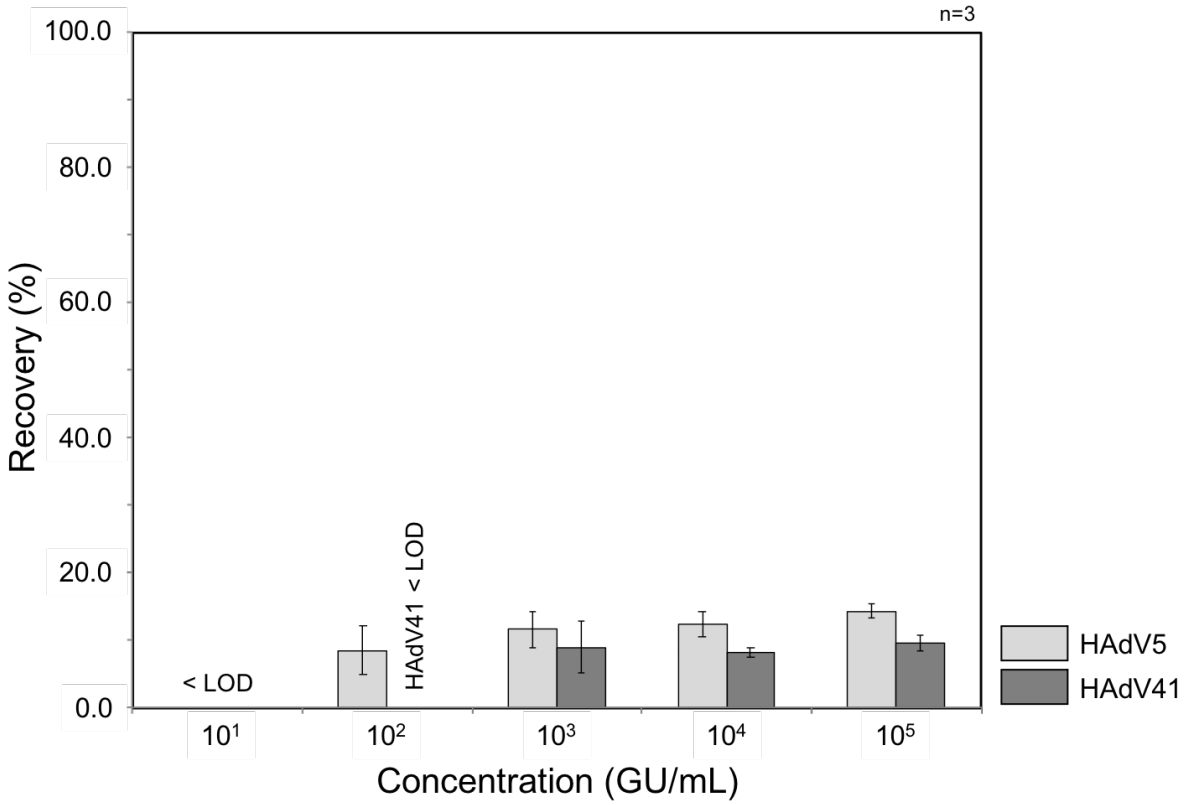


Figure 32: Recovery of HAdV5 (light grey) and HAdV41 (dark grey) spiked in different concentrations in "Würm" river water after MAF-DEAE. Recoveries are displayed in % as mean ± SD, *n* stands for number of replicates.

Results demonstrate, that MAF-DEAE showed a consistent performance and low standard deviation independently of the spiked concentration level and HAdV serotype. However, it achieved a slightly higher mean recovery of 11.7 ± 2.1% over four orders of magnitude of concentration levels for HAdV5 than for HAdV41 with a mean recovery of 8.8 ± 0.6% over three orders of magnitude of concentration levels only. In comparison to earlier experiments when evaluating elution buffers for MAF-DEAE using tap water and HAdV5, these results prove that elution efficiency does not only rely on the elution buffer but rather depends on a combination of elution conditions, HAdV serotype and water matrix. Thus, it can be assumed, that the difference in recovery between the two serotypes may be caused by a less efficient elution of HAdV41 from the monoliths, which has been reported to be particularly challenging compared to other HAdV serotypes when using positively charged filter surfaces (Enriquez and Gerba, 1995; Gibbons et al., 2010; Lemiale et al., 2007). A higher elution efficiency of HAdV41 may be achieved by adjusting elution conditions. Instead of performing extensive buffer screening and replacing the currently used BEG buffer by another elution fluid, a faster and often successful alternative represents the adjustment of pH conditions of the BEG buffer in a first step (Elsaesser, 2017).

3.2. Influence of pH on HAdV41 elution

To evaluate if recovery of HAdV41 can be enhanced by pH, different pH conditions of the BEG elution buffer were tested. To do so, HAdV41 was spiked in 10 L river water to a final concentration of 10^5 GU/mL, concentrated by MAF-DEAE and eluted with BEG adjusted to pH levels between 9.0 and 10.5 (Figure 33).

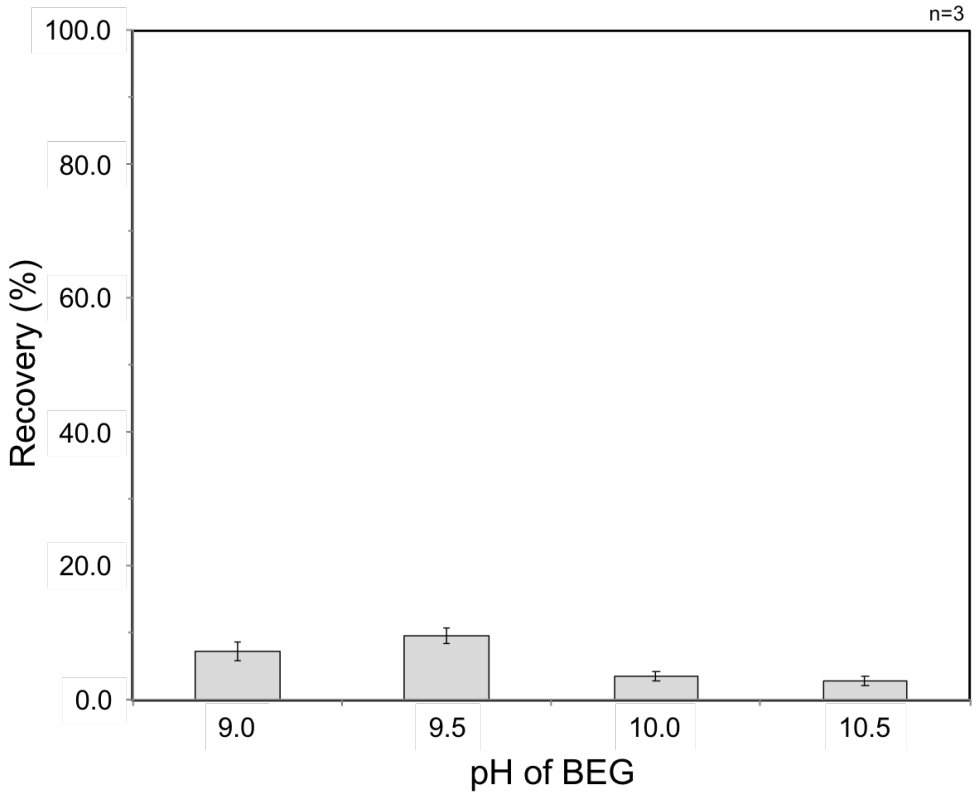


Figure 33: Effect of pH on HAdV41 elution. HAdV41 was spiked in 10 L river water to a final concentration of 10^5 GU/mL, concentrated with MAF-DEAE and eluted by applying BEG of different pH levels. Recoveries are displayed in % as mean ± SD, *n* stands for number of replicates.

The highest recovery of HAdV41 was found at pH 9.5 with 9.5 ± 1.2%, followed by 7.3 ± 1.4% at pH 9.0, 3.4 ± 0.7% at pH 10.0 and 2.8 ± 0.6% at pH 10.5, which illustrates that changes of pH conditions of the BEG buffer trigger the elution of HAdV41 in general. Nevertheless, results demonstrate that pH levels higher or lower than 9.5 seemed to stabilize the adsorption of HAdV41 to the monolith's surface and could not enhance elution. Thus, further buffer screening is required to improve elution efficiency and recoveries. Examples for an alternative to BEG are glycine or high salt buffers, which were shown to successfully enhance the elution of bacteriophages from MAF-DEAE by a factor of up to 66 times compared to BEG when applied at acidic pH (Elsaesser, 2017). Further experiments are required to verify if these buffers also improve the elution of HAdV41. Unfortunately, verification could not be included within the scope of this study due to limited availability of HAdV41 stock.

Nevertheless, with recoveries between 9% and 14% for HAdV41 and HAdV5, respectively, MAF-DEAE operated remarkably better than other adsorption-elution methods used for fresh water, such as NanoCeram® Filters (0.02% for lake water), glass wool filtration (4.7% in lake water and 1.3 – 3.0% for river water) and HA membranes (1.3 – 5.3%, river water) (Ahmed et al., 2015; Ahmed et al., 2016; Fong et al., 2010; Francy et al., 2013).

In contrast to above mentioned methods, relatively high recovery between 25% and 95% were reported for SMF when concentrating HAdV serotype 2 (HAdV2) from 5 L river water (Calgua et al., 2013). To verify if SMF can achieve similar results for HAdV41 in river water and to compare its performance directly to MAF-DEAE, 10 L river water was spiked with HAdV41 to a final concentration of 10^5 GU/mL and processed by SMF. Achieved recovery of 28.6 ± 12.0% for HAdV41 were lower than those reported for HAdV2, but still three times higher than results previously observed with MAF-DEAE. Nevertheless, similar to the findings of the pilot trial, SMF showed a high standard deviation. Consequently, more replicates are needed to achieve reliable and reproducible results with SMF, while MAF-DEAE stands out with its high precision and the direct processing of water samples without pre-conditioning.

3.3. Influence of volume on HAdV recovery

It was reported for different adsorption-elution methods that the accuracy of virus detection can be affected strongly by the sample volume (Bofill-Mas and Rusinol, 2020; Haramoto et al., 2018; Hryniszyn et al., 2013; Sidhu et al., 2013). Furthermore, various studies showed that large sample volumes often result in lower recoveries than smaller volumes, which was found to be mainly attributed to a co-concentration of qPCR inhibitors (Albinana-Gimenez et al., 2009; Qiu et al., 2016). To assess the influence of the sample volume on HAdV recovery, different volumes of river water ranging from 0.1 L to 10 L were spiked with HAdV5 to a final concentration of 10^5 GU/mL and processed by MAF-DEAE. Mean percentage recoveries were calculated for each volume, which are displayed in Figure 34.

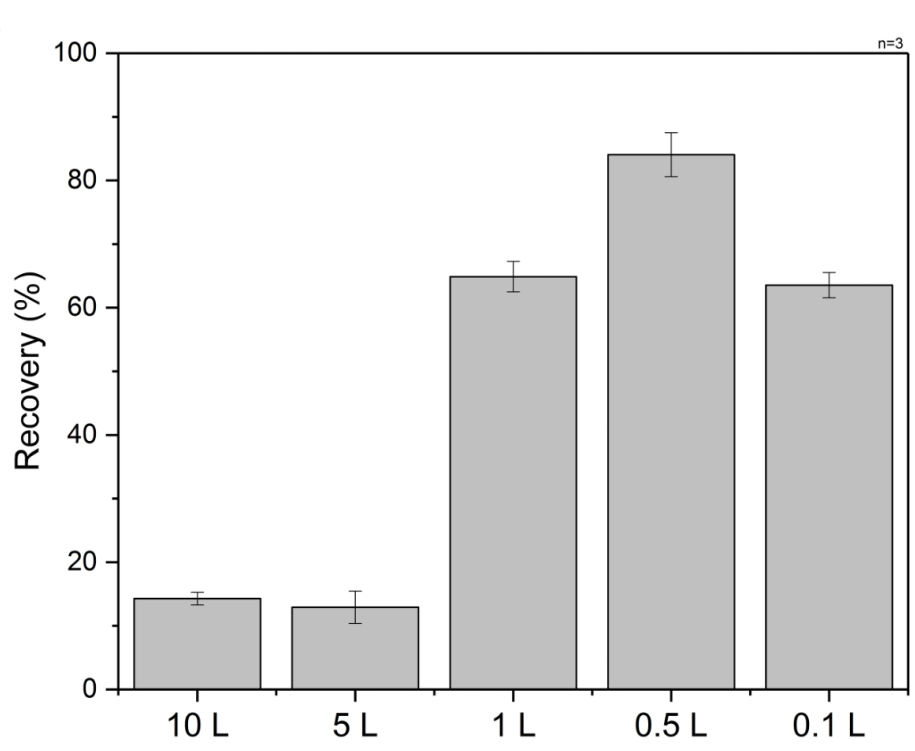


Figure 34: Recoveries of HAdV5 spiked in different volumes of "Würm" river water after MAF-DEAE. Recoveries are displayed in % as mean ± SD. *n* stands for number of replicates.

Results demonstrate that recoveries of HAdV5 for smaller volumes of 1 L or less were significantly higher than for large volumes. The highest recovery of 84.0 ± 3.5% was found in 0.5 L, followed by 64.9 ± 2.4% for 1 L and 63.5 ± 2.0% for 0.1 L. The lowest recovery was detected in 5 L with 13.0 ± 2.5%, which did not differ considerably from the one found in 10 L with 14.3 ± 1.0%. Consequently, no correlation between the accuracy of detection and the sample volumes could be observed. However, results revealed a connection between sample volume and recovery. While comparable recovery levels were found in 10 L and 5 L water samples, recoveries increased significantly by more than 50% for smaller volumes. Interestingly, the highest level of 84% was observed in 0.5 L, whereas smaller sample volumes of 0.1 L showed recoveries of only 64%. A reason for the drop in recovery observed for 0.1 L samples may be the small processing volume itself, which might directly affect the adsorption-elution efficiency of MAF-DEAE. It is likely to assume that viruses in such small processing volumes rather retain on top of the monolith instead of adsorbing to its surface, which in turn may result in an inefficient elution. A second elution step or a concentration dependent analysis of HAdV recoveries in small sample volumes may give a better insight into the binding behavior of HAdV to MAF-DEAE.

In comparison to experiments in 1 L tap water, it was found that MAF-DEAE achieved a 1.6 times higher recovery of HAdV5 from river water of the same volume. These results prove that recovery efficiency of MAF-DEAE does not only rely on the sample volume but rather depends on a combination of water matrix and sample volume. Thus, it can be assumed that differences

in recoveries between the two water matrices may be caused by variances in their water chemistries affecting the adsorption-elution behavior of HAdV5 to MAF-DEAE - an observation reported by different studies for the concentration of viruses from water using other adsorption-elution methods (Melnick et al., 1984; Sobsey and Glass, 1984; Victoria et al., 2009). Unfortunately, chemical data were not generated, and therefore emphasis cannot be placed on any single characteristic of the water samples used. However, it can be concluded that the adequate sample volume for a certain water matrix has to be verified and adapted if needed to ensure a high viral recovery using MAF-DEAE.

Decreasing the sample volume to 0.5 L for HAdV5 concentration from river water, does not only significantly enhance viral recoveries, but also simplifies handling as well as shortening the total processing time of MAF-DEAE to less than 10 min. Further experiments are needed to validate if a similar correlation between sample volume and recovery also exists for HAdV41. Unfortunately, these experiments could not be conducted within the scope of this study due to previously mentioned availability reasons of HAdV41 stock. However, based on the gained results for HAdV5, it is likely that an adapted sample volume may also enhance HAdV41 recoveries.

3.4. Conclusion of the optimized SOP to quantitatively detect HAdV from river water by MAF-DEAE and qPCR

The SOP for the enrichment of HAdV with MAF and its detection by qPCR could be successfully optimized. Pre-experiments demonstrated that not only monoliths with a negatively charged OH-surface, but also those with a positively charged DEAE-surface can be applied to concentrate HAdV from water, which allowed for the processing of water samples without pre-conditioning. Thereby, sample handling could be simplified leading to a reduction in preparation time as well as the risk of contamination. The processing volume of MAF for river water was restricted to 10 L with a limited flow rate of 0.57 L/min due to an increasing back pressure caused by the water's turbidity which resulted in a total processing time of 24 min for 10 L. In addition to processing conditions for MAF-DEAE reference samples were defined, and a timeline of sample execution was added to the SOP. Furthermore, an optimized approach for analyzing the impact of MAF on LOD in qPCR was established, which allows for a standardized application to every qPCR assay, independently of the qPCR instrument used.

Applying the optimized SOP for the concentration of HAdV5 and HAdV41 from river water showed that MAF-DEAE reduced the LOD in qPCR and enabled the quantification of HAdV at concentration levels, which would otherwise not be detectable. While MAF-DEAE stood out with its overall performance for HAdV concentration compared to other adsorption-elution methods in general, it was found that its efficiency depends on HAdV serotype, water matrix, elution conditions and drastically increases for smaller sample volume. The highest recovery of 85% was achieved in 0.5 L water samples when concentrating HAdV5, which reduced the

turnaround time of MAF-DEAE to less than 10 min. Although further experiments are needed to verify if such a correlation between sample volume and recovery also applies for HAdV41, results already demonstrate the potential of the optimized SOP based on MAF-DEAE and qPCR, which represents one of the most efficient, easy-to-implement and cost-effective workflows available for the concentration of HAdV from river water providing reliable and reproducible results in less than two hours.

4. Remaining challenge of the SOP

4.1. Loss of material during the workflow

Previous experiments highlight the huge potential of the resulting SOP and its application in routine virus surveillance. Apart from costs, efficiency and sensitivity of concentration and detection, the simple loss of virions during the workflow represents an additional challenge, which can lead to an inconsistency between the analyzed viral load and the true viral load present in the water of investigation. Such a discrepancy can have a big impact, especially under circumstances in which the true viral load is crucial for assessing the infectious risk of a certain water body. Often the loss of virions during the workflow cannot be completely avoided, even if every step is conducted with the highest level of performance possible. The reason for this is due to the inherent loss related to the workflow itself. Sources are displayed in Figure 35 and include a possible adsorption of viruses due to their physicochemical properties to any plastic disposable used, such as sampling bags, tubing systems and pipetting tips among others, but also more complex causes like an insufficient elution during filtration as well as during nucleic acid extraction (Gerba, 1984).

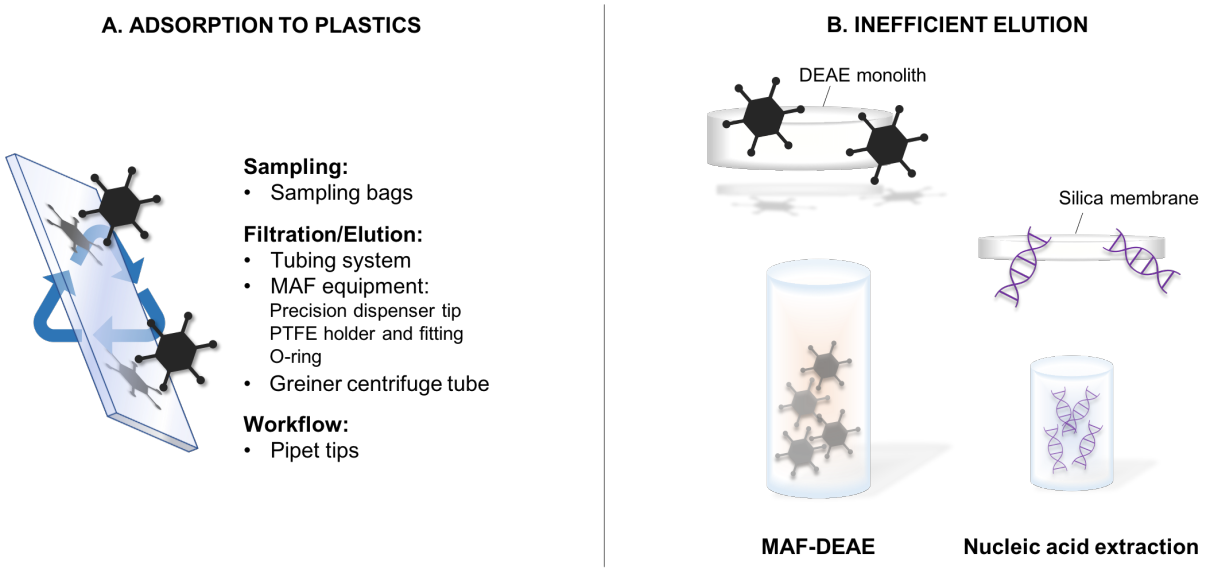


Figure 35: Sources of inherent loss of virions during the workflow of the SOP. A. Adsorption of HAdV to surfaces of plastic equipment used during the workflow. B. Inefficient elution of HAdV from DEAE monoliths during filtration or of purified viral genomes during nucleic acid extraction.

The utilization of specific plastic equipment, where possible, as well as highly efficient nucleic acid extraction kits should minimize the loss of viral material based on the aforementioned reasons when performing the SOP. Although reference samples for MAF-DEAE taken during the workflow gave information about the efficiency of concentration, the level of loss associated with sampling and nucleic acid extraction has not yet been determined. The easiest and most cost-effective solution for analysis is associated with process controls (Blanco Fernández et al., 2017; Haramoto et al., 2018). In comparison to reference samples, which are taken during

the workflow, process controls of known concentration are inoculated into the workflow. Thereby, they are exposed to similar experimental conditions and treatment as the target virus. The ratio of target virus concentration to process control concentration determines the level and cause of virion loss. The loss is inherent to the workflow itself in case the concentration of target virus and process control are equally affected, resulting in a ratio of 1. When the ratio is smaller than 1, the loss is target virus specific and the workflow needs to be optimized. To allow for a reliable correlation of workflow impacts on target virus and process control, an appropriate process control should be a model virus, which is genetically closely related to and, at the best, shares similar properties as the target virus, but does not occur naturally in the water under investigation.

Depending on the point of inoculation, process controls provide information about the efficiency of the entire workflow or of just a single step. Three types of process controls exist: (I) whole workflow process controls, which are inoculated into the water sample before virus concentration, (II) molecular process controls, which are inoculated into the viral concentrate before nucleic acid extraction and give information about the efficiency of nucleic acid extraction, (III) qPCR/RT qPCR controls, which are inoculated prior to qPCR/RT qPCR and are commonly used as an external positive control to assess false-negative qPCR/RT qPCR results (Kitajima et al., 2020).

Whole workflow controls can be applied to analyze the loss of target due to adsorption to plastic equipment used for sampling and MAF-DEAE. To do so, sampling and filtration should be performed as described by the SOP, but without the utilization of a monolith for MAF. Thereby, the target virus and process control are only exposed to the used plastic equipment itself. Samples should be taken prior to and directly after filtration to determine the impact of sampling bags on viral loss separately to the one of further plastics used for MAF.

Target virus and process control need to be quantified simultaneously, which can be achieved by a duplex approach in qPCR or, alternatively, by digital PCR (dPCR) (Blaise-Boisseau et al., 2010; Farkas et al., 2020; Pasquale et al., 2010; Racki et al., 2014a). A duplex approach describes the in-parallel detection of two targets present in a single sample using two qPCR assays with probes of different fluorescent labeling in one reaction (Haramoto et al., 2018; Newby et al., 2009). While qPCR quantifies both targets relative to their corresponding standard curve, dPCR provides an absolute quantification without the need of a standard as a reference. This is achieved by dividing the PCR reaction into thousands of individual sub-reactions each containing only one or no copies of the target. Then, endpoint PCR is performed, and the fluorescent signal of each sub-reaction is detected after amplification. While only sub-reactions containing a copy of the target give a positive signal, the ratio of these reactions to the total number of all sub-reactions promotes the absolute quantification of target in the original sample by applying binomial Poisson statistics (Dube et al., 2008; Pinheiro et

al., 2012). Particularly when determining the loss of target virus, the application of dPCR over qPCR has the advantage that the total number of positive signals of the process control can be used to normalize results of the target virus and assess directly the level of loss. In addition, dPCR does not require any replicates, which reduces the number of total reactions needed compared to qPCR and, in turn, limits expenses. Furthermore, dPCR has a greater precision and is less sensitive to PCR inhibitors (Coudray-Meunier et al., 2015; Monteiro and Santos, 2017; Racki et al., 2014b).

Based on the differing elution profiles of HAdV5 and HAdV41 found for MAF-DEAE it can be assumed that the impact of loss is serotype-specific. Thus, it would be recommended to always implement a process control. Different process controls for enteric viruses have been validated; however, an appropriate one for HAdV has not yet been found (Blanco Fernández et al., 2017). Here, more research is needed to find an appropriate process control and verify that the analyzed viral load of HAdV represents the actual one present in the water under investigation

4.2. Viral viability

The application of qPCR to detect HAdV and enteric viruses generally stands out with an improved sensitivity and reduced time as well as the capability to quantify non-culturable viruses compared to traditional culture-based technologies. While these are powerful features, especially under circumstances when the viral load is low and time is critical, it has to be pointed out that particularly adenoviral genomes can persist in water for a long period of time and are more stable than genomes of other viruses (El-Senousy et al., 2013). Thus, it may be possible that qPCR detects not only DNA of viable HAdV, but also free adenoviral DNA present in a water sample. For this reason, the sole detection of adenoviral DNA may be misleading to determine the true human health risk of a water body, which is related to viable, infectious HAdV only. Expanding detection by implementing culture-based methods such as plaque assays in addition to qPCR may be a solution to assess the number of infectious particles; however, it does not take into account that the viability of HAdV may also be affected by the concentration procedure (Ogorzaly et al., 2010). When performing MAF-DEAE, virions are exposed to high sheer forces during filtration and changing pH conditions during elution. Both may cause a degradation of the viral capsid leading to free adenoviral DNA. In case this occurs during filtration, free adenoviral DNA may be lost within the filtrate, whereas when occurring during elution, free adenoviral DNA will be found in the concentrate, which will undergo nucleic acid extraction in the next step and can still be detected by qPCR. While free DNA is commonly negatively charged, it is likely that free adenoviral DNA may adsorb to MAF-DEAE monoliths (Lipfert et al., 2014). This applies to both free adenovirus DNA present in the water sample prior to concentration and to potentially released adenovirus DNA due to degradation of HAdV

capsid during filtration. An overview of the described routes of how adenoviral DNA may enter qPCR when performing MAF-DEAE is displayed in Figure 36.

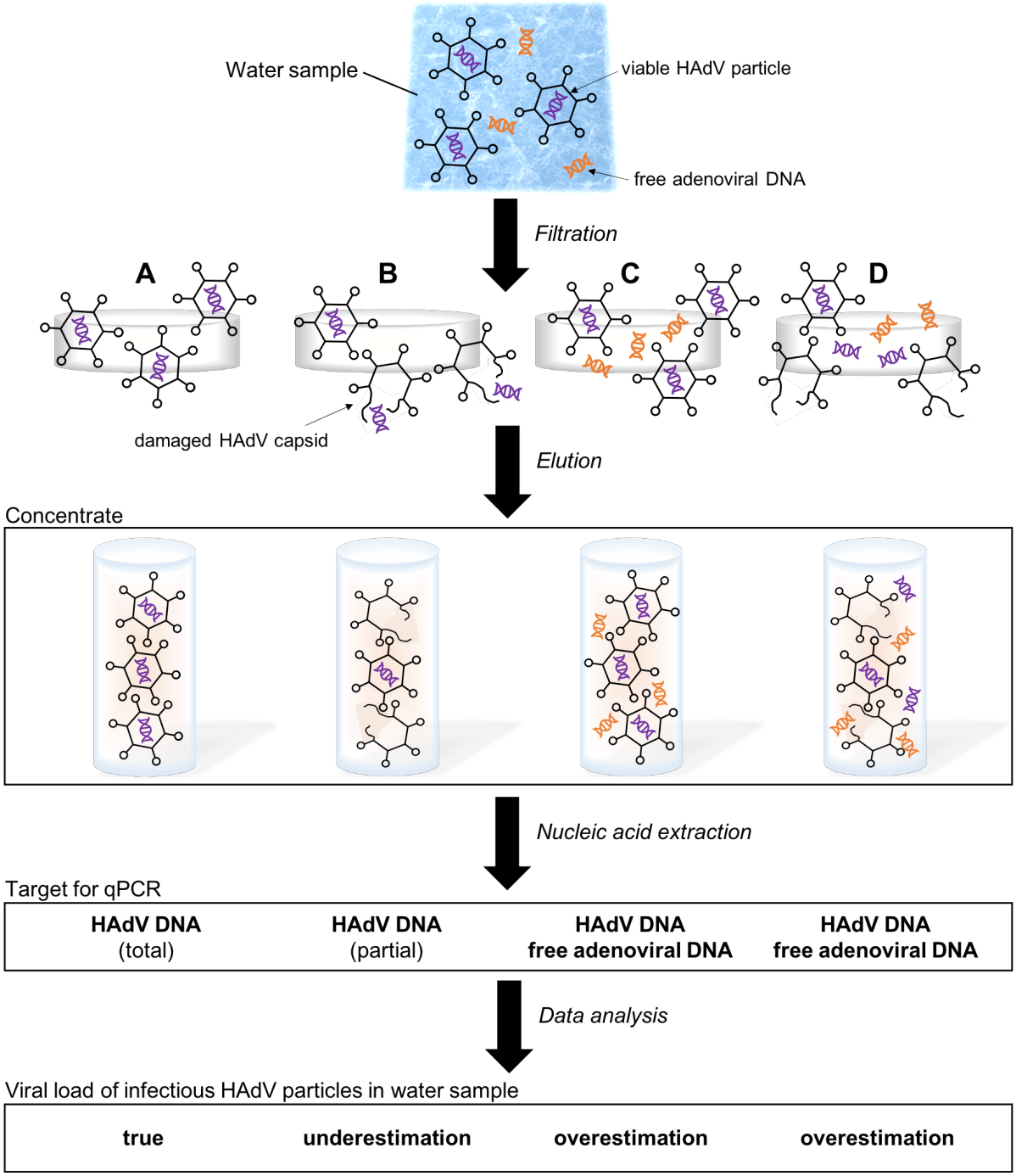


Figure 36: Varying routes of how adenoviral DNA may enter qPCR and influence results of the viral load of infectious HAdV particles in a water sample when performing MAF-DEAE.

Water sample contains viable, infectious HAdV particle and potentially free adenoviral DNA. When applying MAF-DEAE, four scenarios are possible: A. Adsorption of viable HAdV particles to monolith, free adenoviral DNA present in the water sample is lost during filtration and only DNA of viable HAdV particles enters qPCR leading to the assessment of the true viral load of infectious HAdV particles in the water sample. B. Adsorption of viable HAdV particles to monolith. Due to sheer forces during filtration some HAdV capsids are damaged leading to a loss of adenoviral DNA of viable particles during filtration. Consequently, only a partial amount of DNA from viable HAdV particles enters qPCR, resulting in an underestimation of the true

viral load of infectious HAdV particles in the water sample. C. Adsorption of viable HAdV particles and free adenoviral DNA to monolith. Both are entering qPCR leading to an overestimation of infectious HAdV particles present in the water sample. D. Adsorption of viable HAdV particles and free adenoviral DNA to monolith. Free adenoviral DNA includes the one present in water sample prior to filtration as well as released one from damaged capsids during filtration. Total DNA of all virions and free adenoviral DNA are entering qPCR resulting in an overestimation of infectious HAdV particles present in water sample.

More research is needed to analyze the potential of DEAE-monolith to adsorb free adenoviral DNA and, in case DNA adsorption occurs, also the efficiency of the BEG buffer to elute adsorbed DNA from the monolith.

A solution for preventing free adenoviral DNA present in the water sample from affecting qPCR detection can be achieved by removing free DNA which is not contained within an intact viral capsid. The most common approach is sample digestion with DNase. Although its application is fairly easy, the flipside of using DNase can be poor removal of particle-bound, non-viable DNA and the loss of DNase activity in some environmental matrices (Bibby et al., 2019). An alternative to this can be achieved using intercalating dyes, such as propidium monoazide (PMA) or ethidium monoazide (EMA). After activation by light these dyes integrate into free DNA, which prevents its amplification by qPCR (Fittipaldi et al., 2011). This azo-dye based and culture independent approach is also known as capsid integrity qPCR ((ci)-qPCR) and has already been successfully applied for the detection of infectious adenovirus in the aquatic environment (Leifels et al., 2019). Further experiments are needed to evaluate if (ci)-qPCR or a similar approach can also be implemented in the SOP to gain more information about which impact MAF-DEAE has on viral viability and to provide an ideal solution for the risk assessment of HAdV in water.

IV. Summary and Outlook

Enteric viruses are the most commonly found and highly persistent viruses in water and one of the major causes of waterborne diseases in the human population worldwide. Although the associated risk for human health is well known, their assessment has still not been implemented in routine microbial surveillance of water quality and is currently only performed during outbreak situations or when an outbreak is suspected. The reason for this is due to the challenging examination of viruses in aquatic environments, in which their concentration levels are commonly below the limit of detection of qPCR, the gold standard for virus analysis in the environment. Thus, qPCR is usually combined with upstream methods for the concentration of viruses from water, which are often time consuming and expensive. To implement virus assessment in routine water surveillance, a standardized workflow is needed which provides a time and cost-efficient solution to reliably detect viruses in water.

The aim of this work was the establishment of a standard operating procedure (SOP) for the analysis of enteric viruses in water based on monolithic adsorption filtration (MAF) for concentration and qPCR for detection. The SOP should allow for an easy and fast implementation of included methods, have a short turnaround time and lead to reliable and reproducible results at an interlaboratory scale with manageable costs. Furthermore, it should be compatible with field application.

To establish a state-of-the-art workflow applicable at an interlaboratory scale, IWC-TUM and two other European expert laboratories in the area of water research shared commonly performed protocols for concentration, nucleic acid extraction and qPCR detection of viruses in water and set up a harmonized SOP for the in-parallel concentration of two enteric viruses, human adenovirus 35 (HAdV35) and murine norovirus (MNV) from artificially contaminated mineral water. Two concentration methods were included in the SOP: (I) monolithic adsorption filtration (MAF) using monoliths with a negatively charged hydroxyl surface (MAF-OH) and (II) skimmed milk flocculation (SMF).

To provide the most cost-efficient solution, experiments for SOP establishment focused on qPCR detection as one of the main cost drivers of the workflow. Two qPCR and RT qPCR reagents were compared regarding costs and each approach was evaluated for its compatibility to detect adenovirus or norovirus, respectively. It was found that reagents particularly suited for environmental application provided a reliable detection of both viruses due to their resistance to qPCR inhibitors and, although they are related to higher costs, these reagents are required for a successful application of the SOP for surface water. Furthermore, it was shown that synthetically designed dsDNA fragments known as gBlocks® represent an ideal alternative to traditional standards. gBlocks® allowed for a fast establishment of qPCR

assays with highly reproducible results and comparable performance parameters between the three partner laboratories, independently of the used qPCR instrument. For both assays a similar working range of 10^2 – 10^8 GU/µL and lowest detectable concentration of 1 GU/µL could be achieved, while resulting qPCR efficiencies lay in an acceptable range between 94.6 ± 3.2% and 95.5 ± 4.0% for adenovirus and norovirus, respectively. Thus, implementing gBlocks® in qPCR effectively minimized the amount of time and expenses typically needed for standard production, validation and establishment due to their commercial availability. Moreover, it offered a qPCR standard of high and comparable performance at an interlaboratory scale.

For the purpose of evaluating MAF for the concentration of enteric viruses from water, a cascade of different spiking experiments was conducted. With each step the complexity of the spiking matrix was increased, and the SOP tailored to the application of MAF to concentrate HAdV from surface water.

In a first step, a pilot trial between the three laboratories was performed to compare MAF-OH and SMF regarding their performance and evaluate the workflow of the SOP for its application at an interlaboratory scale. Artificially contaminated Evian mineral water was used as spiking matrix due to its well-defined, highly controlled and consistent water chemistry as well as availability to each partner side.
Validation of the concentration methods showed that MAF-OH and SMF were easy to establish in a short period of time without the need of additional training to achieve results comparable to those of laboratories well experienced in each method. Both methods were able to successfully enrich HAdV35 and MNV spiked in 10 L Evian mineral water in parallel. While recoveries of SMF varied strongly, MAF-OH achieved reproducible, and in most, cases slightly better results in all three laboratories at an outstanding turnaround time of less than 10 min. Nevertheless, the main drawback of MAF-OH and SMF proved to be the required pre-conditioning of water samples to allow for virus concentration.
While the SOP offered an attractive solution from a time and cost perspective, most notably when performing MAF-OH, validation of the workflow highlighted the importance of including reference samples on one hand and a defined timeline for experimental execution on the other hand to allow for the normalization and verification of results, particularly when applying the SOP at an interlaboratory scale. In addition to this, it would be recommended to perform pre-experiments to assess the influence that the transportation and storage of samples has on viral degradation and inactivation.

In a next step, IWC-TUM further optimized the SOP for its application in concentrating adenovirus from surface water with MAF based on gained results of the pilot trial. Here, HAdV5 was chosen as target serotype due to a limited availability of HAdV35. To overcome the main drawback of pre-conditioning of water when applying negatively charged MAF-OH, positively charged monoliths exposing diethyl aminoethyl groups on their surface (MAF-DEAE) were

successfully validated for the concentration of HAdV5 at neutral pH. Experiments were conducted in 1 L tap water representing a more complex matrix than mineral water due to a less defined and controlled water chemistry. Comparing MAF-DEAE directly to MAF-OH showed that MAF-DEAE achieved a slightly better recovery of 39.8% than MAF-OH with 32.4% and, when comparing elution buffers that BEG at pH 9.5 led to better results than a high salt buffer.

Next, MAF-DEAE was evaluated under environmental conditions. River water was chosen as representative matrix for surface water illustrating a matrix of high complexity due to an undefined water chemistry. Water samples were collected at the river “Würm” in Gräfelfing, Germany and showed a turbidity of 0.18 ± 0.02 FNU. Compared to previous experiments with tap water, the turbidity of the river water required an adjustment of processing conditions. Thus, the applied flow rate was restricted to 0.57 L/min due to an increasing back pressure in relation to the processed volume of river water. Clogging of the monolith limited the sample volume to a maximum of 10 L. The turnaround time of MAF-DEAE was determined to a total of 24 min including 17 min filtration and 7 min elution for river water samples of 10 L. Thereby, MAF-DEAE achieves a competitive turnaround time to other concentration methods for surface water and can still process 10 L water samples up to 40 times faster than SMF.

Next, processing conditions, sampling of references during the MAF-DEAE workflow and a defined timeline for experimental conduction were implemented into the previously developed SOP. Furthermore, the impact of MAF-DEAE on the LOD in qPCR was added to the SOP as an additional parameter for the evaluation of MAF-DEAE performance. Therefore, a novel approach was established, which determines the impact of MAF-DEAE on the LOD in qPCR based on raw fluorescent signals plotted over sample concentrations at a predefined cutoff cycle of 32. The cutoff cycle was defined in relation to the standard curve of a qPCR assay by the first cycle outside of its linear area. In comparison to described approaches elsewhere, the approach presented here offers a standardized solution by applying the standard curve as a reference point. On the other hand, because of using the first cycle outside of the linear area as cutoff cycle, it furthermore avoids the incorporation of false-positive signals sometimes occurring in latter cycles when using SYBR green dyes. Taking these two advantages together, this approach can easily be applied at an interlaboratory scale as it is applicable to every assay, independently of the qPCR chemistry or instrument used.

After optimization of the SOP as described above, spiking experiments with HAdV5 and HAdV41, one of the most commonly found HAdV serotype in environmental water, were conducted. First, the impact of MAF-DEAE on the LOD in qPCR was assessed. Experiments with 10 L river water spiked with concentration levels ranging from 10^1 to 10^5 GU/mL of HAdV5 or HAdV41, respectively, showed that MAF-DEAE successfully improved the LOD; however, the level of improvement was found to be serotype-specific. A higher impact on the LOD was

detected for HAdV5 with an improvement of 115 times from 6.0 × 10^3 GU/mL before to 5.2 × 10^1 GU/mL after MAF-DEAE, while for HAdV41 the LOD could only be enhanced by 15 times from 5.9 × 10^3 GU/mL before to 3.9 × 10^2 GU/mL after MAF-DEAE. It was assumed that variations in physicochemical properties between the two serotypes may cause a weaker elution of HAdV41, an observation particularly reported for HAdV41 by different studies when applying other adsorption-elution methods. In turn, a weaker elution from the DEAE monoliths would result in a lower impact of MAF-DEAE on the LOD of HAdV41 and explain the differences between HAdV5 and HAdV41. To verify if such a correlation also exists for MAF-DEAE, the mean recovery over the total concentration range of 10^1 to 10^5 GU/mL were compared among serotypes. Thereby, it could be confirmed that slightly higher recoveries were achieved for HAdV5 with 11.7 ± 2.1%, than for HAdV41 with 8.8 ± 0.6% on one hand, while on the other hand, it could be shown that MAF-DEAE achieved a consistent performance with high precision, independently of the spiked concentration level or serotype. To enhance recoveries of HAdV41 in a next step, different pH conditions of the elution buffer ranging from 9.0 to 10.5 were tested in spiking experiments of 10 L river water seeded with 10^5 GU/mL HAdV41. Even though elution of HAdV41 could not be improved, it could at least be confirmed that the previously applied pH of 9.5 still achieved the highest recovery of 9.5 ± 1.2%. In comparison, a recovery of 14.3 ± 1.0% was reached for HAdV5 under similar conditions. Here, further research is needed to enhance the elution of HAdV41; however, with recoveries between 9% and 14% for HAdV5 and HAdV41, respectively, MAF-DEAE achieved better results than those reported for other adsorption-elution methods applied to river water.
An exception seemed to be represented by SMF, for which recoveries of up to 95% were reported when concentrating HAdV2 from river water. To compare the performance of SMF directly to MAF-DEAE and to validate if such high recoveries can also be achieved for HAdV41, SMF was performed for 10 L river water spiked with 10^5 GU/mL of HAdV41. Results showed that SMF led to a higher recovery of 28.6 ± 12.0% for HAdV41 than MAF-DEAE; but, similar to the comparison of SMF with MAF-OH in the pilot trial, SMF results varied strongly, highlighting the improved precision of MAF-DEAE over SMF. In addition, MAF-DEAE still stood out with a faster turnaround time and, most importantly, no need for the pre-conditioning of water samples.

Different studies have described a correlation between the sample volume and recovery of HAdV and reported furthermore, that smaller volumes often lead to better results. Such a link could also be confirmed for MAF-DEAE. Comparison of recoveries determined for river water samples of 0.1 L, 0.5 L, 1.0 L, 5.0 L and 10.0 L spiked with 10^5 GU/mL of HAdV5 showed that the highest recovery of 84% was found in 0.5 L, whereas recoveries of 0.1 L and 1.0 L volumes lay around 64% and those of 10.0 L and 5.0 L around 13.5%. In comparison to experiments in 1 L tap water, MAF-DEAE achieved a 1.6 times higher recovery of HAdV5 from river water of

the same volume. These results show that recovery efficiency of MAF-DEAE does not only rely on the sample volume but rather depends on a combination of water matrix and sample volume. Thus, it can be assumed that differences in recoveries between the two water matrices may be caused by variances in their water chemistries affecting the adsorption-elution behavior of HAdV to MAF-DEAE. Unfortunately, chemical data were not generated in the present study, and therefore emphasis cannot be placed on any single characteristic of the water samples used. However, it can be concluded that the adequate sample volume for a certain water matrix has to be verified and adapted if needed to ensure a high viral recovery using MAF-DEAE. Due to a limited availability of stock, experiments could not be repeated for HAdV41. Therefore, more research is needed to verify if a similar correlation between sample volume and recovery also applies for this serotype. However, at least for HAdV5, the reduced sample volume of 0.5 L drastically shortened the time needed for MAF-DEAE to less than 10 min, which reduced the total workflow time - from filtration to results - to under two hours. Hence, the SOP represents one of the fastest workflows for HAdV detection in river water.

To further improve the SOP, it would be recommended to add process controls to the workflow. On one hand, these controls would uncover points of sample loss during the workflow, which may lead to differences between the true viral load and the analyzed one. On the other hand, process controls can be used for the normalization of results and promote a targeted optimization of single steps and even the total workflow.
In order to offer a complete risk assessment tool, it would also be advised to add virus viability tests in addition to molecular detection of HAdV to the SOP, which would verify the infection risk for the human population related to the water under investigation.

The optimized SOP for MAF-DEAE overcomes most drawbacks of existing approaches and represents one of the most efficient and easy-to-implement workflows available for the detection of HAdV in river water due to its fast turnaround time of less than two hours, high recoveries, precision of results, manageable expenses and the direct processing of water samples. Even though processing controls and virus viability testing have to be added and more research is needed to further validate a serotype specific relation between MAF-DEAE performance and elution conditions as well as processing volumes, the SOP already represents a promising tool for future application in routine virus surveillance. Applying the SOP in ongoing research and field studies will promote a better understanding of the mechanisms and underlying conditions of how HAdV contaminates surface water, although its application may not be limited only to HAdV. Due to the easy adaption of MAF monoliths to the target of investigation, the workflow may also be applicable for other viruses. A current example of high importance could be an implementation in studying the surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) in waste and surface water (Giacobbo et al., 2021; Guerrero-Latorre et al., 2020; Haramoto et al., 2020; Rimoldi et al., 2020). Gained

information will help to uncover the route of viral transmission from source to target and increase awareness of the risk level associated to waterborne diffusion of viruses, which is greatly needed to develop water standards, enhance water safety and protect human health.

V. Experimental Part

1. Material and methods

1.1. Instruments and disposables

43 MµltiGuard®-tips, 0.1 – 10 µL (X595.1, Carl Roth, Karlsruhe, Germany)

49 MµltiGuard®-tips, 1 – 200 µL (X598.1, Carl Roth, Karlsruhe, Germany)

53 MµltiGuard®-tips, 100-1000 µL (X601.1, Carl Roth, Karlsruhe, Germany)

Autoclavable disposal bags (0381.1 Carl Roth, Karlsruhe, Germany)

Autoclave, Laboklav ECO (SHP Steriltechnik, Detzel Schloß, Germany)

Centrifuge Universal 320 R (Hettich Zentrifugen, Tuttlingen, Germany)

Disposable needles Sterican® (C630.1, Carl Roth, Karlsruhe, Germany)

Disposable pasteur pipettes (EA61.1, Carl Roth, Karlsruhe, Germany)

Disposable syringes Injekt®, 20 mL (0059.1, Carl Roth, Karlsruhe, Germany)

DNA LoBind tubes, 1.5 mL (0030108051, Eppendorf, Hamburg, Germany)

DNA LoBind tubes, 5.0 mL (0030108310, Eppendorf, Hamburg, Germany)

Greiner centrifuge tubes, 15 mL (T1943, Sigma-Aldrich, Taufkirchen, Germany)

Greiner centrifuge tubes, 50 mL (T2318, Sigma-Aldrich, Taufkirchen, Germany)

Laboratory scale Mettler PM 4600 Delta Range (Mettler-Toldeo, Giessen, Germany)

LightCycler® 480 Multiwell Plate 96 (04729692001, Roche Diagnostics, Mannheim, Germany)

Magnetic stirrer with heating, RCT basic (IKA Labortechnik, Staufen, Germany)

Marprene tubing, ID 6.4 mm (9020064016, Watson Marlow, Falmouth, England)

NanoPhotometer (IMPLEN, München, Germany)

Oven (Heraeus, Hanau, Germany)

Peristaltic pump SciQ 323 DU/D (Watson Marlow, Falmouth, England)

Portable Turbidimeter, Turb 430 IR (WTW GmbH, Weilheim, Germany)

Precision dispenser tips, 50 mL (EH38.1, Carl Roth, Karlsruhe, Germany)

qPCR platform LightCycler® 480 System (Roche Diagnostics, Mannheim, Germany)

Rotilabo®-specimen containers, 120 mL (CEP3.1, Carl Roth, Karlsruhe, Germany)

Rotilabo®-syringe filters, 0.22 µm (P668.1, Carl Roth, Karlsruhe, Germany)

Thermomixer C (Eppendorf, Hamburg, Germany)

Ultrapure Milli-Q plus 185 (Millipore, Bedford, MA, USA)

Vortexer Top Mix FB15024 (Fisher Scientific, Hampton, NH, USA)

Waterbags (N369.1, Carl Roth, Karlsruhe, Germany)

1.2. Software

ChemBio Office Professional 17.1 (Cambridge Soft, Waltham, MA, USA)

Microsoft Office 2016 (Microsoft, Redmond, WA, USA)

Origin 2018 (OriginLab, Northampton, MA, USA)

1.3. Chemicals

1,4-Dioxane (296309, Sigma-Aldrich, Taufkirchen, Germany)

Beef extract powder (B4888, Sigma-Aldrich, Taufkirchen, Germany)

Boron trifluoride diethyl etherate (175501, Sigma-Aldrich, Taufkirchen, Germany)

Diethylamine (KK00.2, Carl Roth, Karlsruhe, Germany)

di-Sodium hydrogen phosphate dihydrate (T877.2, Carl Roth, Karlsruhe, Germany)

Ethanol absolute, ≥ 99.8% (32205, Sigma-Aldrich, Taufkirchen, Germany)

Glycine, 99.7 – 101% (33226, Sigma-Aldrich, Taufkirchen, Germany)

HEPES (H3375, Sigma-Aldrich, Taufkirchen, Germany)

Hydrochloric acid, ≥ 37% (30721, Sigma-Aldrich, Taufkirchen, Germany)

Methanol, ≥ 99.9% (34860, Sigma-Aldrich, Taufkirchen, Germany)

Polyglycerol-3-glycidylether (CL9, ipox Chemicals, Laupheim, Germany)

Sea salt (S9883, Sigma-Aldrich, Taufkirchen, Germany)

Skim milk powder (70166, Sigma-Aldrich, Taufkirchen, Germany)

Sodium chloride, > 99.8% (9265.2, Carl Roth, Karlsruhe, Germany)

Sodium hypochlorite (9062.4, Carl Roth, Karlsruhe, Germany)

Sodium thiosulfate (1615107, Sigma-Aldrich, Taufkirchen, Germany)

Sulfuric acid, 95.0 – 97.0% (30743, Sigma-Aldrich, Taufkirchen, Germany)

tert-Butyl methyl ether, ≥ 99.8% (306975, Sigma-Aldrich, Taufkirchen, Germany)

Tris(hydroxymethyl)-aminomethan Sigma 7-9®, ≥ 99% (T1378, Sigma-Aldrich, Taufkirchen, Germany)

Toluene, ≥ 99.7% (244511, Sigma-Aldrich, Taufkirchen, Germany)

Water, PCR grade (03315932001, Roche Diagnostics, Mannheim, Germany)

1.4. Kits

TaqMan® Environmental Master Mix 2.0 (4396838, Applied Biosystems, Foster City, CA, USA)

QIAamp® DNA Mini Kit (51304, Qiagen, Hilden, Germany)

RNA Ultrasense™ One-Step Quantitative RT-PCR System (11732927, Applied Biosystems, Foster City, CA, USA)

Takyon™ No ROX Probe 2X MasterMix Blue dTTP, 1.5 mL (UF-NPMT-B0101, Eurogentec, Lüttich, Belgium)

Takyon™ One-Step Kit Converter, 1.5 mL (UF-RTAD-D0101, Eurogentec, Lüttich, Belgium)

1.5. Viruses

Murine Norovirus, strain CW3, extracted from cell culture (National Food Institute, Technical University of Denmark, Copenhagen, Denmark)

Human adenovirus, serotype 5, extracted from cell culture (Ruhr-University Bochum, Bochum, Germany)

Human adenovirus, serotype 35, extracted from cell culture (Laboratory of virus contaminants of water and food, University of Barcelona, Barcelona, Spain)

Human adenovirus, serotype 41, extracted from cell culture (Technische Universität München/Helmholtz Zentrum München, Munich, Germany)

1.6. Buffers and solutions

All buffers and solutions were prepared with ultrapure water. pH value was adjusted with NaOH or HCl, respectively.

1.6.1. Buffers

Beef extract glycine buffer (BEG)

0.5 M Glycine
3% (w/v) beef extract powder
BEG was adjusted to pH 9.0, pH 9.5, pH 10.0 or pH 10.5, respectively.

High salt buffer (pH 7.0)

1.5 M NaCl
0.05 M HEPES

Phosphate buffer (PBS) (pH 7.5)

1:2 (v/v) 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4

Pre-flocculated skimmed milk (PSM) (pH 3.5)

33.33 g artificial sea salt
1% (w/v) skimmed milk powder
add 1 L ultrapure ddH_2O

1.6.2. Cleaning solutions for MAF experiments

NaOCl (0.5%)

42 mL NaOCl
add 1 L ddH_2O

NaOCl (0.05%)

10 mL NaOCl (0.5%)
add 1 L ddH_2O

$Na_2S_2O_3$ (2%)

10 g $Na_2S_2O_3$
add 1 L ddH_2O and autoclave

$Na_2S_2O_3$ (0.005%)

2.5 mL $Na_2S_2O_3$ (0.05%)
add 1 L ddH_2O

2. Molecular biological techniques

2.1. Nucleic acid extraction

Nucleic acid extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sterile Eppendorf DNA LoBind tubes were used to enhance the final yield of purified nucleic acids. All steps were carried out at room temperature (RT). 20 µL of proteinase K was combined with 200 µL of sample. 200 µL of lysis buffer (AL) was added, the sample was pulse-vortexed for 15 sec and incubated for 10 min at 56 °C. Drops on the inside of the lid were spun down by brief centrifugation and 230 µL of Ethanol (absolute, 96 – 100%) was added. The sample was pulse-vortexed for 15 sec and centrifuged briefly. The total volume was transferred to a QIAamp® Mini spin column. Binding of nucleic acids to the column was carried out by centrifugation at 6000 × *g* for 1 min. The filtrate and collection tube were discarded. 500 µL of washing buffer 1 (AW1) was added and the spin column was centrifuged at 6000 × *g* for 1 min. The filtrate and collection tube were discarded. 500 µL of washing buffer 2 (AW2) was added and the spin column was centrifuged at 20000 × *g* for 3 min. The filtrate and collection tube were discarded, and the spin column

was placed in a new, non-sterile 1.5 mL tube. The spin column was centrifuged at full speed for 1 min to avoid carryover of AW2 buffer. The spin column was placed in a clean, 1.5 mL DNA LoBind tube and 60 µL of elution buffer (AE) was added. To increase the yield of purified nucleic acids the spin column was incubated at RT for 5 min before eluting nucleic acids by centrifugation at 6000 × *g* for 1 min. Purified nucleic acids were stored at -20 °C until further use.

2.2. Quantitative real-time PCR (qPCR)

All qPCR experiments were carried out in a 96-well standard format on the LightCycler® 480 System (Roche Diagnostics, Mannheim, Germany). 1.5 mL sterile Eppendorf DNA LoBind tubes were used for master mix and sample preparation. gBlocks® Gene Fragments (Integrated DNA Technologies, Leuven, Belgium) were applied as standard and PCR grade H_2O as negative control (ntc). Dilutions of samples and standards were performed with PCR grade H_2O. All preparation steps were carried out on ice. Loaded 96-well plates were covered with a sealing foil, centrifuged briefly to spin down contents and eliminate air bubble and qPCR was performed immediately. Depending on the master mix used, thermal cycling conditions were adjusted according to the manufacturer's instructions, which are displayed in Table 11 for TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) and in Table 12 for Takyon™ No ROX Probe 2X MasterMix Blue dTTP (Eurogentec, Lüttich, Belgium).

Table 11: Thermal cycling conditions for qPCR using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems).

Step	Cycles (#)	Temperature (°C)	Time (min)
Denaturation	1	95	10:00
Amplification	45	95	00:15
		60	01:00

Table 12: Thermal cycling conditions for qPCR using Takyon™ No ROX Probe 2X MasterMix Blue dTTP (Eurogentec).

Step	Cycles (#)	Temperature (°C)	Time (min)
UNG activation	1	50	02:00
Denaturation	1	95	03:00
Amplification	40	95	00:20
		60	01:00

2.2.1. Preparation of gBlocks® Gene Fragments as standard for qPCR experiments

Synthetically designed dsDNA fragments (gBlocks® Gene Fragments) were purchased from Integrated DNA Technologies, Leuven, Belgium and prepared according to the manufacturer's instructions as following: The tube containing dried gBlocks® Gene Fragments was centrifuged

for 5 sec at 3000 × g to ensure that DNA is located at the bottom. PCR grade H2O was added as indicated to reach a final concentration of 10 ng/µL. The tube was vortexed briefly and incubated for 20 min at 50 °C to resuspend the gBlocks® Gene Fragments. The final concentration of gBlocks® Gene Fragments in ng/µL was verified by a NanoPhotometer (IMPLEN, München, Germany). The following formula was used to calculate the copy number as described by the manufacturer:

$$c\left[\frac{ng}{\mu L}\right] * M\left[\frac{fmol}{ng}\right] * \left(10^{-15}\frac{mol}{fmol}\right) * N_A = copy\ number/\mu L \qquad \textbf{Equation 5}$$

where c is the measured concentration of gBlocks® Gene Fragment in ng/µL, M the molecular weight of gBlocks® Gene Fragments in fmol/ng as provided by the manufacturer and N_A the Avogadro's number.

gBlocks® Gene Fragments were stored at -20 °C until further use. For standard curve preparation a dilution series of gBlocks® Gene Fragments from 10^8 – 10^1, 5, 2 and 1 GU/µL in PCR grade H_2O was prepared immediately before each qPCR run.

Sequences of gBlocks® Gene Fragments used for viral quantification are given in Table 20.

Table 13: Sequences of gBlocks® Gene Fragments for detection of viruses by qPCR.

Target	Sequence of gBlock® (5'→3')	Gene	Size (bp)
HAdV	ATGATGCCGCAATGGTCTTACATGCACATCGCCGGGCAGGACGC CTCGGAGTATCTGAGCCCGGGCACACACACACACCTGGTGCAATT TGCCCGCGCCACCGATACGTACTTCAGCCTGGGGAACAAGTTCA GAAATCCCGCTGCGATTCGTGCCAGTCGACCGCGAGGACACCGC TTATTCTTACAAAGTGCGCTTTACGCTGGCCGTGGGCGACAACCG GGTGTTGGACATGGCCAGCACCTACTTTGACATCCGCGGCGTGC TGGATCG	Hexon protein	273
MNV	ACCAGTTTGGGTGGTACGGTCGTCTTGATCGTGCCAGCATCGACC GCCAGCTCCTCTGGACTAAAGGACCTACCCACCAGAACCCCTTTG AGACTCTCCCTGGACATGCTCAGAGACCCTCCCAACTAATGGCCC TGCTCGGTGAGGC	Poly protein	148

2.2.2. qPCR using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems)

qPCR using TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) was performed according to the manufacturer's instructions. Composition of the pre-mix solution is displayed in Table 14.

Table 14: Composition of pre-mix solution for qPCR using the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems).

Component	Volume/reaction (µL)
TaqMan® Environmental Master Mix 2X	12.5
Forward primer	1.0
Reverse primer	1.0
Probe	0.5
Total per reaction	15

The pre-mix solution was briefly vortexed, centrifuged and 15 µL of the pre-mix solution was transferred to the appropriate well. 10 µL of control or sample was added and qPCR was performed in a final reaction volume of 25 µL.

2.2.3. qPCR using the Takyon™ No ROX Probe 2X MasterMix Blue dTTP (Eurogentec)

qPCR using the Takyon™ No ROX Probe 2X MasterMix Blue dTTP (Eurogentec, Lüttich, Belgium) was performed according to the manufacturer's instructions. Composition of the pre-mix solution is displayed in Table 15.

Table 15: Composition of premix solution for qPCR using the Takyon™ No ROX Probe 2X MasterMix Blue dTTP (Eurogentec).

Component	Volume/reaction (µL)
Takyon™ MasterMix	10.0
Forward primer	0.5
Reverse primer	0.5
Probe	1.0
PCR grade H_2O	3.0
Total per reaction	15

The pre-mix solution was vortexed briefly, centrifuged and 15 µL of the pre-mix solution was transferred to the appropriate well. 5 µL of control or sample was added and qPCR was performed in a final reaction volume of 15 µL.

2.3. One Step quantitative Reverse Transcription PCR (RT qPCR)

All RT qPCR experiments were carried out in a 96-well format on the LightCycler® 480 System (Roche). 1.5 mL sterile Eppendorf DNA LoBind tubes were used for master mix and sample preparation. gBlocks® Gene Fragments (Integrated DNA Technologies, Leuven, Belgium) were applied as standard and PCR grade H_2O as negative control (ntc). Dilutions of samples and standards were performed with PCR grade H_2O. All preparation steps were carried out on ice. Loaded 96-well plates were covered with a sealing foil, centrifuged briefly to spin down contents and eliminate air bubble and RT qPCR was performed immediately.

2.3.1. RT qPCR using the RNA Ultrasense™ One-Step Quantitative RT-PCR System (Applied Biosystems)

RT qPCR using the RNA Ultrasense™ One-Step Quantitative RT-PCR System (Applied Biosystems, Foster City, CA, USA) was performed according to manufacturer's instructions. Composition of the master mix is displayed in Table 16.

Table 16: Composition of master mix for RT qPCR using the RNA Ultrasense™ One-Step Quantitative RT-PCR System (Applied Biosystems).

Component	Volume/reaction (µL)
RNA UltraSense™ 5X Reaction Mix	5.0
RNA UltraSense™ Enzyme Mix	1.25
20X Bovine Serum Albumin	1.25
Forward primer	1.0
Reverse primer	1.0
Probe	0.25
PCR grade H_2O	10.25
Total per reaction	20

The master mix was vortexed briefly, centrifuged and 20 µL of master mix was transferred to the appropriate well. 5 µL of control or sample was added and RT qPCR was performed in a final reaction volume of 25 µL. Thermal cycling conditions are displayed in Table 17.

Table 17: Thermal cycling conditions for RT qPCR using the RNA Ultrasense™ One-Step Quantitative RT-PCR System (Applied Biosystems).

Step	Cycles (#)	Temperature (°C)	Time (min)
Reverse Transcription	1	55	60:00
Denaturation	1	95	05:00
Amplification	45	95	00:15
		60	01:00
		65	01:00

2.3.2. RT qPCR using the Takyon™ One-Step Kit Converter (Eurogentec)

RT qPCR using the Takyon™ One-Step Kit Converter (Eurogentec, Lüttich, Belgium) was performed according to manufacturer's instructions. Composition of master mix is displayed in Table 18.

Table 18: Composition of master mix for RT qPCR using the Takyon™ One-Step Kit Converter (Eurogentec).

Component	Volume/reaction (µL)
2X Takyon™ MasterMix Blue dTTP	10.0
Forward primer	0.5
Reverse primer	0.5
Probe	1.0
Euroscript II RT	0.2

Component	Volume/reaction (µL)
Additive	0.2
PCR grade H_2O	2.6
Total per reaction	15

The master mix was vortexed briefly, centrifuged and 15 µL of master mix was transferred to the appropriate well. 5 µL of control or sample was added and RT qPCR was performed in a final reaction volume of 20 µL. Thermal cycling conditions are displayed in Table 19.

Table 19: Thermal cycling conditions for RT qPCR using the Takyon™ One-Step Kit Converter (Eurogentec).

Step	Cycles (#)	Temperature (°C)	Time (min)
Reverse Transcription	1	48	20:00
UNG activation	1	50	02:00
Denaturation	1	95	03:00
Amplification	45	95	00:20
		60	01:00

2.4. Sequences of oligonucleotides

Sequences of used primers and probes as well as their final concentration per qPCR reaction are given in Table 20.

Table 20: Sequences of primers and probes used for quantification of viruses by qPCR. FWD: forward primer, REV: reverse primer, P: probe.

Target	Sequences of primers and probe (5'→3')	Gene/Amplicon size (bp)	Concentration (µM/qPCR rct)	References
HAdV	**FWD:** CWTACATGCACATCKCSGG **REV:** CRCGGGCRAAYTGCACCAG **P:** 6-FAM-CCGGGCTCAGGTACTCCGAGGCGTCCT-BHQ1	Hexon protein/69	0.9 0.9 0.225	Hernroth et al., 2002; Bofill-Mas et al., 2006
MNV	**FWD:** TGATCGTGCCAGCATCGA **REV:** GTTGGGAGGGTCTCTGAGCAT **P:** 6-FAM-CTACCCACCAGAACCCCTTTGAGACTC-BHQ1	Polyprotein/101	0.5 0.9 0.25	Park et al., 2010; Rawsthorne et al., 2009

3. Concentration of viruses from water

3.1. Monolithic adsorption filtration (MAF)

Synthesis, surface functionalization of monoliths with DEAE- or OH- groups and assembling of MAF device was performed as initially established by Peskoller et al., 2009 including optimizations published by Kunze et al., 2015 and Elsaesser, 2017.

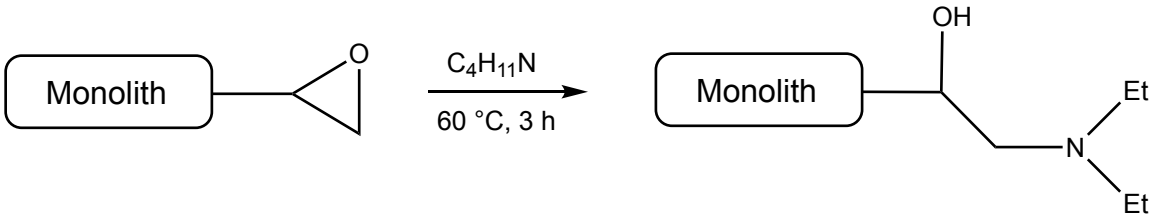
3.1.1. Synthesis of monoliths

A porogen was prepared by combining 5.65 mL of toluene and 3.77 mL of *tert*-Butyl methyl ether (60:40, v/v). Porogen mixture, PTFE molds and the monomer polyglycerol-3-glycidylether (CL9) were pre-heated at 28 °C for 1 h in a heating oven (Heraeus). The catalyst solution was prepared by diluting boron trifluoride diethyl etherate in 1,4-dioxane (1:10, v/v). 0.15 mL of catalyst was added to the pre-heated porogen and vortexed for 20 sec. 2.4 mL of monomer was immediately added (ratio monomer/porogen 20:80), the mixture was vortexed for 1 min and poured into pre-heated PTFE molds standing inside of the heating oven. The PTFE molds were covered with PTFE tops and self-polymerization reaction of monoliths was carried out at 28 °C for 45 min. Monoliths were carefully removed from the PTFE molds with a spatula, washed with methanol and stored in fresh methanol at RT until functionalization. Synthesized monoliths had a size of 38.6 mm in length and 9.0 mm in height.

3.1.2. Surface functionalization of monoliths

Epoxy surface groups of monoliths were functionalized either with $C_4H_{11}N$ for positively charged monoliths or with H_2SO_4 for negatively charged monoliths. Chemistry of monolithic surface functionalization is displayed in Figure 37.

A. MAF-DEAE



B. MAF-OH

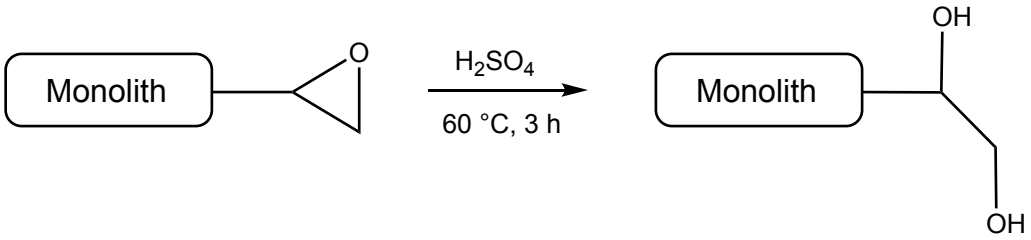


Figure 37: Overview of surface functionalization. Monoliths exposing epoxy groups on their surface are chemically modified to DEAE- (A) or OH-groups (B).

Surface functionalization was carried out for up to three monoliths at a time. Therefore, the end of a 50 mL precision dispenser tip was cut off and used as housing, in which the following components were inserted in the order as displayed: PTFE holding, an O-ring, up to three monoliths, a fitting and an adapter. This setup is referred to as *monolithic column* in the following section. The monolithic column was connected to a peristaltic pump and washed with 500 mL ultrapure ddH_2O. The reaction mixture used for functionalization was prepared, added to a beaker and heated up on a heating stirrer to 60 °C. The temperature was controlled by a

thermometer and functionalization was performed by circulating the reaction mixture through the monoliths using a peristaltic pump. Functionalization conditions and reaction mixture compositions are displayed in Table 21.

Table 21: Functionalization conditions and reaction mixture compositions for MAF-DEAE and MAF-OH.

Monolith type	Surface group	Reaction mixture	Temperature (°C)	Time (h)
MAF-DEAE	Diethyl aminoethyl-	10% Diethylamine 50% Ethanol	60	3
MAF-OH	Hydroxyl-	0.5 M Sulfuric acid	60	3

Functionalized monoliths were washed with 500 mL ultrapure ddH_2O, carefully removed from the housing and stored in sterile filtered, ultrapure ddH_2O at 4 °C until further use.

3.1.3. Performing MAF

To perform MAF a monolithic column was assembled as described under *3.1.2*. with the exception that only one monolith was applied at a time. When performing MAF-OH, pH of water sample and ultrapure ddH_2O for equilibration was adjusted beforehand to pH 3.0 with HCl. MAF was carried out at room temperature under a flow bench. The monolithic column was adapted to a peristaltic pump and equilibrated with 1 L ultrapure ddH_2O. The water sample was connected and its total volume was pumped through the monolith. Applied flow rates were adjusted to water matrix due to increasing back pressure during filtration when using water of higher turbidity. An overview of applied flow rates in relation to the water matrix is given in Table 22.

Table 22: Flow rates applied for equilibration and filtration in dependency of water sample type and volume.

Water matrix	Mineral water	Surface water	Tab water
Flow rate in L/min (rpm)	1.0 (372)	0.57 (320)	1.0 (372)

Next, 20 mL of elution buffer was directly injected onto the monolith by a syringe. The column was reconnected to the pump and elution was carried out at a flow rate of 160 mL/min (53 rpm) in three steps. One third of the elution buffer was pumped through the monolith, the end of the housing was closed, and the monolith soaked with elution buffer was incubated for three minutes. Then, the second third of the elution buffer was pumped through the monolith and the incubation step of three minutes was repeated. Last, the remaining elution buffer was pumped through the monolith while continuously increasing the flow rate to 1 L/min until all buffer passed through the monolith.

The used monolith and housing were discarded after each MAF experiment. The PTFE holding, O-ring, fitting and adapter were cleaned in 100% Ethanol and washed with ultrapure ddH_2O before reassembling. The tubing was cleaned by circulating 1 L of NaOCl (0.05%) for 10 min at RT followed by circulating 1 L of $Na_2S_2O_3$ (0.005%) for 10 min at room temperature.

Cleaning solutions were prepared before a MAF experiment, used up to three times per experimental day and discarded afterwards.

For quantification, samples of 1 mL were taken of the stock used for spiking, the spiked water sample and the eluate. All samples were kept at room temperature under the flow hood until the end of one MAF run. Samples as well as remaining eluate were stored at -80 °C until further use.

3.2. Skimmed milk flocculation (SMF)

Skimmed milk flocculation (SMF) was performed as described by Calgua et al., 2008. 10 L water sample was added to a bucket and pre-conditioned with artificial sea salt to a conductivity of 150 µS/cm^2 and adjusted to a pH of 3.5 with HCl. Pre-flocculated skimmed milk (PSM) and 100 mL was added to the spiked water sample. Flocculation was carried out by stirring the water sample for 8 h followed by sedimentation of flocs for an additional 8 h at RT under a flow bench. The supernatant was carefully discarded. Centrifugation of sedimented flocs in a remaining volume of 500 mL was performed at 8000 × *g* for 30 min at 4°C (Calgua et al., 2008). The pellet was dissolved in a total volume of 10 mL phosphate buffer.
Used buckets were autoclaved after each SMF experiment.

For quantification , samples of 1 mL were taken of the stock used for spiking, the spiked water sample and the concentrate. All samples were stored at -80 °C until further use.

4. Spiking procedure of water samples for pilot trial

Wastewater was collected at Kläranlage, Garching, Germany. Wastewater was spiked with two concentration levels of HAdV35 and MNV, representing a high and a low viral load. Spiked concentrations were calculated based on the levels communicated by laboratories providing viral stocks. Seeded wastewater was aliquoted in 10 mL samples, which were sent back blindly coded to partner laboratories and stored at -80 °C until further procedure. Final concentration levels and total number of distributed aliquots are displayed in Table 23.

Table 23: Overview of distributed wastewater samples for pilot trial including sample names, viral loads and number of aliquots per partner laboratory.

Viral level in GU	High	Low	Blank
Virus type			
HAdV	1.0×10^8	1.0×10^6	0
MNV	1.13×10^9	1.3×10^8	0
Number of aliquots	10	10	6

Spiking experiments were performed at capacity level of laboratories. 10 mL seeded wastewater was spiked into 9990 mL of Evian mineral water and processed with MAF-OH or SMF, respectively. Eluates were stored at -80 °C until further use.

Processing order of blindly coded aliquots corresponded to the following concentration levels:

Blank – low – low – low – high – high – high – Blank – high – low – high – low – Blank

5. Data analysis

One dataset included three independent repeats as well as one negative control sample. Parameter m describes the number of independent repeats, n describes the number of measurement points of an experiment. The number of virus was expressed as genomic units (GU).

5.1. Calculation of recovery

The recovery of viruses was calculated in percentage using the following equation:

$$recovery\ [\%] = \frac{\#\ recovered\ GU\ by\ qPCR}{\#\ seeded\ GU\ by\ qPCR} * 100 \qquad \textbf{Equation 6}$$

5.2. qPCR parameters

Serial dilutions of gBlocks® Gene Fragments including 10^8 – 10^1, 5, 2 and 1 GU/µL for HAdV or MNV were used to determine qPCR parameters for adenovirus and norovirus assays. Concentrations of dilutions were plotted against corresponding C_q values, creating a standard curve with the following equation:

$$y = mx + b \qquad \textbf{Equation 7}$$

where y is the C_q value, m the slope, x the log(quantity) and b the y-intercept.

The working range (WR) was determined by the linear area of the standard curve. QPCR efficiency was directly assessed from curve's slope and calculated in percentage based on the following equations:

$$AF = 10^{(-\frac{1}{m})} \qquad \textbf{Equation 8}$$

$$E = (AF - 1) * 100 \qquad \textbf{Equation 9}$$

where AF is the amplification factor of the exponential phase of the qPCR reaction, m the slope of the standard curve and E the qPCR efficiency in percentage. The limit of detection (LOD) of each assay was determined by the smallest concentration possible to detect.

5.2.1. Determination of inhibition

The distance between amplification plots of a diluted and an undiluted sample was calculated to assess inhibitory effects in qPCR by the following formula:

$$\Delta C_q = C_{q,n} - C_{q,n-1} \qquad \textbf{Equation 10}$$

where ΔC_q is the difference between two amplification plots and C_q the quantification cycle of a sample *n*.

The theoretical distance under ideal qPCR conditions was assessed from the dilution factor of the corresponding samples and calculated by:

$$2^n = dilution\ factor \qquad \textbf{Equation 11}$$

where n is the theoretical number of cycles between two amplification plots at a qPCR efficiency of 100%.

5.3. Impact of MAF-DEAE on limit of detection (LOD) in qPCR

To determine the impact of MAF-DEAE on the limit of detection for HAdV in 10 L river water, raw fluorescent signals of qPCR were plotted over template concentrations at a pre-determined cutoff cycle. The cutoff cycle was defined as the first C_q outside of the linear area of the standard curve. The limit of detection (LOD) of spiked water before and after MAF was calculated based on a generated calibration curve using the Levenberg Marquardt curve-fitting algorithm.

The following equation was used to determine the background fluorescent signal of the qPCR assay needed to apply curve-fitting:

$$background\ fluorescence\ signal = (mean\ of\ raw\ fluorescence\ signal\ of\ ntc) + 3 * SD(ntc)$$

VI. Abbreviation

AF	Amplification factor
AMC	Analytical Methods Committee of the British Chemical Society
AOAC	Association of Official Analytical Chemists
ASTM	American Society of Testing and Materials
AWWA	American Water Works Association
BEG	Beef extract glycine buffer
bp	Base pair
CCL	Contamination Candidate List
cDNA	Complementary DNA
cm	Centimeter
C_q	Quantification cycle
DEAE	Diethylaminoethan
DIN	Deutsche Institut für Normung e.V.
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DTU	Technical University Denmark
E	qPCR efficiency
E. coli	Escherichia coli
EUR (€)	Euro
fmol	Femtomole
FNU	Formazine nephelometric unit
FRET	Fluorescence resonance energy transfer

FWD	Forward primer
g	Gram
GU	Genomic unit
h	Hour
HAdV	Human adenovirus
HEPES	(4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
IEP	Isoelectric point
ISO	International Organization for Standardization
IWC	Institute of Hydrochemistry
kDa	Kilodalton
L	Liter
LOD	Limit of detection
MAF	Monolithic adsorption filtration
min	Minute
mL	Milliliter
MNV	Murine norovirus
MWCO	Molecular weight cutoff
N_A	Avogadro’s number
NaPP	Sodium polyphosphate
ng	Nanogram
nm	Nanometer
NMWL	Nominal molecular weight limit
NTC	No target control

NoV	Norovirus
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PSM	Pre-flocculated skimmed milk
PTFE	Polytetrafluorethylene
qPCR	Quantitative polymerase chain reaction
REV	Reverse primer
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
RT qPCR	Quantitative reverse transcription PCR
SD	Standard deviation
sec	Second
SMF	Skimmed milk flocculation
SOP	Standard operating procedure
TCID	Tissue culture infective dose
T_m	Melting temperature
TPB	Tryptose phosphate broth
TUM	Technical University Munich
UB	University of Barcelona
USEPA	United States Environmental Protection Agency

UV	Ultraviolet
VIRADEL	Virus adsorption-elution
WHO	World Health Organization
WR	Working range
µL	Microliter
µm	Micrometer
µS	Microsiemens

VII. References

Abbaszadegan, M., Huber, M.S., Gerba, C.P., Pepper, I.L., 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. Appl Environ Microbiol 59, 1318-1324.

Abbaszadegan, M., Lechevallier, M., Gerba, C., 2003. Occurrence of Viruses in US Groundwater. J Am Water Works Ass 95, 107-120.

Agrawal, S., Orschler, L., Lackner, S., 2021. Long-term monitoring of SARS-CoV-2 RNA in wastewater of the Frankfurt metropolitan area in Southern Germany. Sci Rep 11, 5372.

Ahmed, S.M., Hall, A.J., Robinson, A.E., Verhoef, L., Premkumar, P., Parashar, U.D., Koopmans, M., Lopman, B.A., 2014. Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. Lancet Infect Dis 14, 725-730.

Ahmed, W., Gyawali, P., Toze, S., 2016. Evaluation of glass wool filters and hollow-fiber ultrafiltration concentration methods for qPCR detection of human adenoviruses and polyomaviruses in river water. Water Air Soil Pollut 227, 327.

Ahmed, W., Harwood, V.J., Gyawali, P., Sidhu, J.P.S., Toze, S., 2015. Comparison of concentration methods for quantitative detection of sewage-associated viral markers in environmental waters. Appl Environ Microbiol 81, 2042-2049.

Albertsson, P. A. 1960. Partitions of cell particles and macro- molecules. John Wiley & Sons, Inc., New York.

Albinana-Gimenez, N., Clemente-Casares, P., Bofill-Mas, S., Hundesa, A., Ribas, F., Girones, R., 2006. Distribution of human polyomaviruses, adenoviruses, and hepatitis E virus in the environment and in a drinking-water treatment plant. Environ Sci Technol 40, 7416-7422.

Albinana-Gimenez, N., Miagostovich, M.P., Calgua, B., Huguet, J.M., Matia, L., Girones, R., 2009. Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants. Water Res 43, 2011-2019.

Anon, 2011. Investigation into the levels of norovirus in influent and treated wastewater samples from a sewage treatment works, in: (FSA), F.S.A.p. (Ed.), Cefas ref: C3027 ed.

Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29 Suppl 1, S49-52.

Artieda, J., Pineiro, L., Gonzalez, M., Munoz, M., Basterrechea, M., Iturzaeta, A., Cilla, G., 2009. A swimming pool-related outbreak of pharyngoconjunctival fever in children due to adenovirus type 4, Gipuzkoa, Spain, 2008. Euro Surveill 14.

Asami, T., Katayama, H., Torrey, J.R., Visvanathan, C., Furumai, H., 2016. 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.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Graham, D.Y., 2008. Norwalk virus shedding after experimental human infection. Emerg Infect Dis 14, 1553-1557.

AWWA, 2006. Waterborne Pathogens. American Water Works Association, Denver, CO.

Baggi, F., Demarta, A., Peduzzi, R., 2001. Persistence of viral pathogens and bacteriophages during sewage treatment: lack of correlation with indicator bacteria. Res Microbiol 152, 743-751.

Bandari, R., Knolle, W., Buchmeiser, M.R., 2008. Comparative study on the separation behavior of monolithic columns prepared via ring-opening metathesis polymerization and via electron beam irradiation triggered free radical polymerization for proteins. J Chromatogr A 1191, 268-273.

Bartram, J., 2009. Water safety plan manual: step-by-step risk management for drinking-water suppliers. World Health Organization.

Beck, S.E., Rodriguez, R.A., Linden, K.G., Hargy, T.M., Larason, T.C., Wright, H.B., 2014. Wavelength dependent UV inactivation and DNA damage of adenovirus as measured by cell culture infectivity and long range quantitative PCR. Environ Sci Technol 48, 591-598.

Berg, G., 1967. Transmission of Viruses by the Water Route, in: Berg, G. (Ed.). John Wiley & Sons Ltd., p. 484.

Berg, G., Safferman, R.s., Dahling, D.R., Berman, D., Hurst, C.J., 1984. USEPA manual of methods for virology (EPA-600/4-84-013). Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency Cincinnati, OH.

Bertrand, I., Schijven, J.F., Sánchez, G., Wyn-Jones, P., Ottoson, J., Morin, T., Muscillo, M., Verani, M., Nasser, A., de Roda Husman, A.M., Myrmel, M., Sellwood, J., Cook, N., Gantzer, C., 2012. The impact of temperature on the inactivation of enteric viruses in food and water: a review. J Appl Microbiol 112, 1059-1074.

Beuret, C., 2003. A simple method for isolation of enteric viruses (noroviruses and enteroviruses) in water. J Virol Methods 107, 1-8.

Bibby, K. Crank, K., Greaves, J., Li, X., Wu, Z., Hamza, I.A., Stachler, E., 2019. Metagenomics and the development of viral water quality tools. Npj Clean Water 2, 9.

Bibby, K., Peccia, J., 2013. Prevalence of respiratory adenovirus species B and C in sewage sludge. Environ Sci Process Impacts 15, 336-338.

Blaise-Boisseau, S., Hennechart-Collette, C., Guillier, L., Perelle, S., 2010. Duplex real-time qRT-PCR for the detection of hepatitis A virus in water and raspberries using the MS2 bacteriophage as a process control. J Virol Methods 166, 48-53.

Blanco Fernández, M.D., Barrios, M.E., Cammarata, R.V., Torres, C., Taboga, O.A., Mbayed, V.A., 2017. Comparison of internal process control viruses for detection of food and waterborne viruses. Appl Microbiol Biotechnol 101, 4289-4298.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M., Girones, R., 2006. Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. Appl Environ Microbiol 72, 7894-7896.

Bofill-Mas, S., Calgua, B., Clemente-Casares, P., La Rosa, G., Iaconelli, M., Muscillo, M., Rutjes, S., de Roda Husman, A.M., Grunert, A., Gräber, I., Verani, M., Carducci, A., Calvo, M., Wyn-Jones, P., Girones, R., 2010. Quantification of Human Adenoviruses in European Recreational Water. Food Environ Virol 2, 101-109.

Bofill-Mas, S., Rusiñol, M., 2020. Recent trends on methods for the concentration of viruses from water samples. Curr. Opin. Environ. Sci. Health 16, 7-13.

Borchardt, M.A., Bertz, P.D., Spencer, S.K., Battigelli, D.A., 2003. Incidence of Enteric Viruses in Groundwater from Household Wells in Wisconsin. Appl Environ Microbiol 69, 1172-1180.

Borrego, J.J., Cornax, R., Preston, D.R., Farrah, S.R., McElhaney, B., Bitton, G., 1991. Development and application of new positively charged filters for recovery of bacteriophages from water. Appl Environ Microbiol 57, 1218-1222.

Borrego, J., Figueras, M., 1997. Microbiological quality of natural waters. Microbiologia (Madrid, Spain) 13, 413-426.

Bosch, A., 1998. Human enteric viruses in the water environment: a minireview. Int Microbiol 1, 191-196.

Bosch, A., Guix, S., Sano, D., Pinto, R.M., 2008. New tools for the study and direct surveillance of viral pathogens in water. Curr Opin Biotechnol 19, 295-301.

Bosch, A., Pinto, R.M., Abad, F.X., 2006. Survival and transport of enteric viruses in the environment, in: Goyal, S.M. (Ed.), Viruses in Food. Springer, New York, pp. 151-187.

Brauch, H.-J., 2010. Sicherheit und Schutz vor Krankheitserregern durch ein multiples Barrierensysteme, In: R. Niessner, Höll - Wasser 2010, 412-426, de Gruyter, Berlin.

Burd, E.M., 2010. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev 23, 550-576.

Burns, M., Valdivia, H., 2007. Modelling the limit of detection in real-time quantitative PCR. Eur Food Res Technol 226, 1513-1524.

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25, 169-193.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55, 611-622.

Calgua, B., Fumian, T., Rusinol, M., Rodriguez-Manzano, J., Mbayed, V.A., Bofill-Mas, S., Miagostovich, M., Girones, R., 2013. Detection and quantification of classic and emerging viruses by skimmed milk flocculation and PCR in river water from two geographical areas. Water Res 47, 2797-2810.

Calgua, B., Mengewein, A., Grunert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A., Wyn-Jones, A.P., Lopez-Pila, J.M., Girones, R., 2008. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. J Virol Methods 153, 79-83.

Carducci, A., Verani, M., 2013. Effects of bacterial, chemical, physical and meteorological variables on virus removal by a wastewater treatment plant. Food Environ Virol 5, 69-76.

Carter, M.J., 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. J Appl Microbiol 98, 1354-1380.

Cashdollar, J.L., Brinkman, N.E., Griffin, S.M., McMinn, B.R., Rhodes, E.R., Varughese, E.A., Grimm, A.C., Parshionikar, S.U., Wymer, L., Fout, G.S., 2013. Development and Evaluation of EPA Method 1615 for Detection of Enterovirus and Norovirus in Water. Appl Environ Microbiol 79, 215-223.

Cashdollar, J.L., Dahling, D.R., 2006. Evaluation of a method to re-use electropositive cartridge filters for concentrating viruses from tap and river water. J Virol Methods 132, 13-17.

Cashdollar, J.L., Wymer, L., 2013. Methods for primary concentration of viruses from water samples: a review and meta-analysis of recent studies. J Appl Microbiol 115, 1-11.

Chang, L.T., Farrah, S.R., Bitton, G., 1981. Positively charged filters for virus recovery from wastewater treatment plant effluents. Appl Environ Microbiol 42, 921-924.

Chapron, C.D., Ballester, N.A., Fontaine, J.H., Frades, C.N., Margolin, A.B., 2000. Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface water collected and evaluated by the information collection rule and an integrated cell culture-nested PCR procedure. Appl Environ Microbiol 66, 2520-2525.

Chaudhry, R.M., Nelson, K.L., Drewes, J.E., 2015. Mechanisms of pathogenic virus removal in a full-scale membrane bioreactor. Environ Sci Technol 49, 2815-2822.

Chen, Y., Chen, L., Deng, Q., Zhang, G., Wu, K., Ni, L., Yang, Y., Liu, B., Wang, W., Wei, C., Yang, J., Ye, G., Cheng, Z., 2020. The presence of SARS-CoV-2 RNA in the feces of COVID-19 patients. J Med Virol 92, 833-840.

Chigor, V.N., Okoh, A.I., 2012. Quantitative detection and characterization of human adenoviruses in the Buffalo River in the Eastern Cape Province of South Africa. Food Environ Virol 4, 198-208.

Cimino, G.D., Metchette, K.C., Tessman, J.W., Hearst, J.E. Isaacs, S.T., 1990. Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Acids Res 19, 99-107.

Conte, J., Potoczniak, M.J., Tobe, S.S., 2018. Using synthetic oligonucleotides as standards in probe-based qPCR. Biotechniques 64, 177-179.

Coudray-Meunier, C., Fraisse, A. Martin-Latil, S., Guillier, L., Delannoy, S., Fach, P., Perelle, S., 2015. A comparative study of digital RT-PCR and RT-qPCR for quantification of Hepatitis A virus and Norovirus in lettuce and water samples. Int J Food Microbiol 201, 17-26.

Craun, G.F., Brunkard, J.M., Yoder, J.S., Roberts, V.A., Carpenter, J., Wade, T., Calderon, R.L., Roberts, J.M., Beach, M.J., Roy, S.L., 2010. Causes of outbreaks associated with drinking water in the United States from 1971 to 2006. Clin Microbiol Rev 23, 507-528.

da Silva, A.K., Le Saux, J.C., Parnaudeau, S., Pommepuy, M., Elimelech, M., Le Guyader, F.S., 2007. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol 73, 7891-7897.

Dahling, D.R., 2002. An improved filter elution and cell culture assay procedure for evaluating public groundwater systems for culturable enteroviruses. Water Environ Res 74, 564-568.

De Keuckelaere, A., Baert, L., Duarte, A., Stals, A., Uyttendaele, M., 2013. Evaluation of viral concentration methods from irrigation and processing water. J Virol Methods 187, 294-303.

Dhopeshwarkar, G.A., Rao, K.R., Viswanathan, R., 1957. Infectious hepatitis: biochemical studies. Indian J Med Res 45, 125-133.

DIN, 1984. German standard methods for the examination of water, waste water and sludge, general informations (group A), interlaboratory trials in water analysis planning and organization (A41) and evaluation (A42), DIN 38 402, Berlin.

Divizia, M., Santi, A.L., Pana, A., 1989. Ultrafiltration: an efficient second step for hepatitis A virus and poliovirus concentration. J Virol Methods 23, 55-62.

Dongdem, J.T., Soyiri, I., Ocloo, A., 2009. Public health significance of viral contamination of drinking water. Afr J Microbiol Res 3, 856-861.

Dube, S., Qin, J., Ramakrishnan, R., 2008. Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. PLoS One 3, e2876.

El-Senousy, W.M., Costafreda, M.I., Pinto, R.M., Bosch, A., 2013. Method validation for norovirus detection in naturally contaminated irrigation water and fresh produce. Int J Food Microbiol 167, 74-79.

Elsaesser, D., 2017. Combined process for rapid concentration of bacteria and viruses for the inline-monitoring of drinking and raw water, Faculty of Chemistry. Technical University of Munich, Munich.

Elsaesser, D., Ho, J., Niessner, R., Tiehm, A., Seidel, M., 2018. Heterogeneous asymmetric recombinase polymerase amplification (haRPA) for rapid hygiene control of large-volume water samples. Anal Biochem 546, 58-64.

Enriquez, C.E., Gerba, C.P., 1995. Concentration of enteric adenovirus 40 from tap, sea and waste water. Water Res 29, 2554-2560.

Espinosa, A.C., Arias, C.F., Sanchez-Colon, S., Mazari-Hiriart, M., 2009. Comparative study of enteric viruses, coliphages and indicator bacteria for evaluating water quality in a tropical high-altitude system. Environ Health 8, 49.

Farkas, K., Mannion, F., Hillary, L.S., Shelagh, K.M., Walker, D.I., 2020. Emerging technologies for the rapid detection of enteric viruses in the aquatic environment. Curr Opin Environ Sci Health 16, 1-6.

Favier, A.L., Burmeister, W.P., Chroboczek, J., 2004. Unique physicochemical properties of human enteric Ad41 responsible for its survival and replication in the gastrointestinal tract. Virol 322, 93-104.

Feachem, R.G., Garelick, H., Slade, J., 1981. Enteroviruses in the environment. Trop Dis Bull 78, 185-230.

Feachem, R.G., Bank, W., 1983. Sanitation and Disease: Health Aspects of Excreta and Wastewater Management. World Bank.

Figueras, M., Borrego, J.J., 2010. New perspectives in monitoring drinking water microbial quality. Int J of Environ Res and Public Health 7, 4179-4202.

Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K., Morato, J., 2011. Viable real-time PCR in environmental samples: can all data be integrated directly? Microb Ecol 61, 7-12.

Flannery, J., Rajko-Nenow, P., Keaveney, S., O'Flaherty, V., Dore, W., 2013. Simulated sunlight inactivation of norovirus and FRNA bacteriophage in seawater. J Appl Microbiol 115, 915-922.

Foladori, P., Cutrupi, F., Segata, N., Manara, S., Pinto, F., Malpei, F., Bruni, L., La Rosa, G., 2020. SARS-CoV-2 from faeces to wastewater treatment: What do we know? A review. Sci Total Environ, 140444.

Fong, T.T., Phanikumar, M.S., Xagoraraki, I., Rose, J.B., 2010. Quantitative Detection of Human Adenoviruses in Wastewater and Combined Sewer Overflows Influencing a Michigan River. Appl Environ Microbiol 76, 715-723.

Fong, T.T., Lipp, E.K., 2005. Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. Microbiol Mol Biol Rev 69, 357-371.

Fongaro, G., Nascimento, M.A., Viancelli, A., Tonetta, D., Petrucio, M.M., Barardi, C.R., 2012. Surveillance of human viral contamination and physicochemical profiles in a surface water lagoon. Water Sci Technol 66, 2682-2687.

Formiga-Cruz, M., Hundesa, A., Clemente-Casares, P., Albinana-Gimenez, N., Allard, A., Girones, R., 2005. Nested multiplex PCR assay for detection of human enteric viruses in shellfish and sewage. J Virol Methods 125, 111-118.

Forootan, A., Sjoback, R., Bjorkman, J., Sjogreen, B., Linz, L., Kubista, M., 2017. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). Biomol Detect Quantif 12, 1–6.

Fout, G.S., Brinkman, N.E., Cashdollar, J.L., Griffin, S.M., McMinn, B.R., Rhodes, E.R., Varughese, E.A., Karim, M.R., Grimm, A.C., Spencer, S.K., Borchardt, M. A. 2010. Method 1615. Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR. U. S. Environmental Protection Agency, Report EPA/600/R-10/18.

Fout, G.S., III, F.S., Messer, J.W., Dahling, D.R., 1996. ICR Microbial Laboratory Manual, in: EPA, U. (Ed.). US EPA, Washington, D.C.

Fout, G.S., Martinson, B.C., Moyer, M.W., Dahling, D.R., 2003. A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater. Appl Environ Microbiol 69, 3158-3164.

Fox, J.P., Hall, C.E., Cooney, M.K., 1977. The Seattle Virus Watch. VII. Observations of adenovirus infections. Am J Epidemiol 105, 362-386.

Francy, D.S., Stelzer, E.A., Brady, A.M., Huitger, C., Bushon, R.N., Ip, H.S., Ware, M.W., Villegas, E.N., Gallardo, V., Lindquist, H.D., 2013. Comparison of filters for concentrating

microbial indicators and pathogens in lake water samples. Appl Environ Microbiol 79, 1342-1352.

Fumian, T.M., Leite, J.P.G., Castello, A.A., Gaggero, A., Caillou, M.S.L. de, Miagostovich, M.P., 2010. Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration. J Virol Methods 170, 42-46.

Futch, J.C., Griffin, D.W., Lipp, E.K., 2010. Human enteric viruses in groundwater indicate offshore transport of human sewage to coral reefs of the Upper Florida Keys. Environ Microbiol 12, 964-974.

Gajra, B., Patel, M., Patel, D., 2011. Validation of Analytical Procedures: Methodology ICH-Q2B. International J Pharma Innov 2249-1031 1, 45-50.

Gantzer, C., Maul, A., Audic, J.M., Schwartzbrod, L., 1998. Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages, and Bacteroides fragilis phages in treated wastewater. Appl Environ Microbiol 64, 4307-4312.

Gantzer, C., Senouci, S., Maul, A., Levi, Y., Schwartzbrod, L., 1997. Enterovirus genomes in wastewater: concentration on glass wool and glass powder and detection by RT-PCR. J Virol Methods 65, 265-271.

Garcia, L.A., Viancelli, A., Rigotto, C., Pilotto, M.R., Esteves, P.A., Kunz, A., Barardi, C.R., 2012. Surveillance of human and swine adenovirus, human norovirus and swine circovirus in water samples in Santa Catarina, Brazil. J Water Health 10, 445-452.

Gentry, J., Vinje, J., Guadagnoli, D., Lipp, E.K., 2009. Norovirus distribution within an estuarine environment. Appl Environ Microbiol 75, 5474-5480.

Gerba, C.P., 1984. Applied and theoretical aspects of virus adsorption to surfaces. Adv Appl Microbiol 30, 133-168.

Gerba, C.P., Farrah, S.R., Goyal, S.M., Wallis, C., Melnick, J.L., 1978. Concentration of enteroviruses from large volumes of tap water, treated sewage, and seawater. Appl Environ Microbiol 35, 540-548.

Gerba, C.P., Goyal, S.M., LaBelle, R.L., Cech, I., Bodgan, G.F., 1979. Failure of indicator bacteria to reflect the occurrence of enteroviruses in marine water. Am J Public Health 69, 1116-1119.

Gerba, C.P., Gramos, D.M., Nwachuku, N., 2002. Comparative inactivation of enteroviruses and adenovirus 2 by UV light. Appl Environ Microbiol 68, 5167-5169.

Giacobbo, A., Rodrigues, M., Zoppas Ferreira, J., Bernardes, A.M., de Pinho, M.N., 2021. A critical review on SARS-CoV-2 infectivity in water and wastewater. What do we know? Sci Total Environ 774, 145721.

Gibbons, C.D., Rodriguez, R.A., Tallon, L., Sobsey, M.D., 2010. Evaluation of positively charged alumina nanofibre cartridge filters for the primary concentration of noroviruses, adenoviruses and male-specific coliphages from seawater. J Appl Microbiol 109, 635-641.

Gibson, K.E., Opryszko, M.C., Schissler, J.T., Guo, Y., Schwab, K.J., 2011. Evaluation of human enteric viruses in surface water and drinking water resources in southern Ghana. Am J Trop Med Hyg 84, 20-29.

Gibson, K.E., Schwab, K.J., 2011a. Tangential-flow ultrafiltration with integrated inhibition detection for recovery of surrogates and human pathogens from large-volume source water and finished drinking water. Appl Environ Microbiol 77, 385-391.

Gibson, K.E., Schwab, K.J., 2011b. Detection of bacterial indicators and human and bovine enteric viruses in surface water and groundwater sources potentially impacted by animal and human wastes in Lower Yakima Valley, Washington. Appl Environ Microbiol 77, 355-362.

Girones, R., Ferrus, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., Correa Ade, A., Hundesa, A., Carratala, A., Bofill-Mas, S., 2010. Molecular detection of pathogens in water--the pros and cons of molecular techniques. Water Res 44, 4325-4339.

Gonzales-Gustavson, E., Cardenas-Youngs, Y., Calvo, M., Figueira Marques da Silva, M., Hundesa, A., Amoros, I., Moreno, Y., Moreno-Medonero, L., Rosell, R., Ganges, L., Araujo, R., Girones, R., 2017. Characterization of the efficiency and uncertainty of skimmed milk flocculation for the simultaneous concentration and quantification of water-borne viruses, bacteria and protozoa. J Microbiol Methods 134, 46-53.

Grabow, W.O., Taylor, M.B., de Villiers, J.C., 2001. New methods for the detection of viruses: call for review of drinking water quality guidelines. Water Sci Technol 43, 1-8.

Graham, D.Y., Jiang, X., Tanaka, T., Opekun, A.R., Madore, H.P., Estes, M.K., 1994. Norwalk virus infection of volunteers: new insights based on improved assays. J Infect Dis 170, 34-43.

Grilo, A.L., Aires-Barros, M.R., Azevedo, A.M., 2016. Partitioning in Aqueous Two-Phase Systems: Fundamentals, Applications and Trends, Sep Purif Reviews 45, 68-80

Grondahl-Rosado, R.C., Yarovitsyna, E., Trettenes, E., Myrmel, M., Robertson, L.J., 2014. A One Year Study on the Concentrations of Norovirus and Enteric Adenoviruses in Wastewater and A Surface Drinking Water Source in Norway. Food Environ Virol 6, 232-245.

Guerrero-Latorre, L., Ballesteros, I., Villacrés-Granda, I., Granda, M. G., Freire-Paspuel, B., Ríos-Touma, B., 2020. SARS-CoV-2 in river water: Implications in low sanitation countries. Sci Total Environ 743, 140832.

Hamza, I.A., Jurzik, L., Stang, A., Sure, K., Uberla, K., Wilhelm, M., 2009. Detection of human viruses in rivers of a densly-populated area in Germany using a virus adsorption elution method optimized for PCR analyses. Water Res 43, 2657-2668.

Hamza, I.A., Jurzik, L., Überla, K., Wilhelm, M., 2011. Evaluation of pepper mild mottle virus, human picobirnavirus and Torque teno virus as indicators of fecal contamination in river water. Water Res 45, 1358-1368.

Haramoto, E., Katayama, H., Oguma, K., Ohgaki, S., 2005. Application of Cation-Coated Filter Method to Detection of Noroviruses, Enteroviruses, Adenoviruses, and Torque Teno Viruses in the Tamagawa River in Japan. Appl Environ Microbiol 71, 2403-2411.

Haramoto, E., Katayama, H., Oguma, K., Ohgaki, S., 2007. Quantitative analysis of human enteric adenoviruses in aquatic environments. J Appl Microbiol 103, 2153-2159.

Haramoto, E., Katayama, H., Oguma, K., Yamashita, H., Tajima, A., Nakajima, H., Ohgaki, S., 2006. Seasonal profiles of human noroviruses and indicator bacteria in a wastewater treatment plant in Tokyo, Japan. Water Sci Technol 54, 301-308.

Haramoto, E., Katayama, H., Ohgaki, S., 2004. Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater. Appl Environ Microbiol 70, 2154-2160.

Haramoto, E., Katayama, H., Utagawa, E., Ohgaki, S., 2009. Recovery of human norovirus from water by virus concentration methods. J Virol Methods 160, 206-209.

Haramoto, E., Kitajima, M., Hata, A., Torrey, J.R., Masago, Y., Sano, D., Katayama, H., 2018. A review on recent progress in the detection methods and prevalence of human enteric viruses in water. Water Res. 135, 168–186.

Haramoto, E., Kitajima, M., Katayama, H., Ohgaki, S., 2010. Real-time PCR detection of adenoviruses, polyomaviruses, and torque teno viruses in river water in Japan. Water Res 44, 1747-1752.

Haramoto, E., Kitajima, M., Kishida, N., Katayama, H., Asami, M., Akiba, M., 2012. Occurrence of viruses and protozoa in drinking water sources of Japan and their relationship to indicator microorganisms. Food Environ Virol 4, 93-101.

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci Total Environ 737, 140405.

Haas, C.N., Rose, J.B., Gerba, C., Regli, S., 1993. Risk assessment of virus in drinking water. Risk Anal 13, 545-552.

Hauri, A.M., Schimmelpfennig, M., Walter-Domes, M., Letz, A., Diedrich, S., Lopez-Pila, J., Schreier, E., 2005. An outbreak of viral meningitis associated with a public swimming pond. Epidemiol Infect 133, 291-298.

He, X., Wei, Y., Cheng, L., Zhang, D., Wang, Z., 2012. Molecular detection of three gastroenteritis viruses in urban surface water in Beijing and correlation with levels of fecal indicator bacteria. Environ Monit Assess 184, 5563-5570.

Heerden, J., Ehlers, M.M., Vivier, J.C., Grabow, W.O., 2005. Risk assessment of adenoviruses detected in treated drinking water and recreational water. J Appl Microbiol 99, 926-933.

Hellmer, M., Paxeus, N., Magnius, L., Enache, L., Arnholm, B., Johansson, A., Bergstrom, T., Norder, H., 2014. Detection of pathogenic viruses in sewage provided early warnings of hepatitis A virus and norovirus outbreaks. Appl Environ Microbiol 80, 6771-6781.

Hernroth, B.E., Conden-Hansson, A.C., Rehnstam-Holm, A.S., Girones, R., Allard, A.K., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, Mytilus edulis: the first Scandinavian report. Appl Environ Microbiol 68, 4523-4533.

Hess, S., Niessner, R., Seidel, M., 2021. Quantitative detection of human adenovirus from river water by monolithic adsorption filtration and quantitative PCR. J Virol Methods, 114128.

Hewitt, J., Greening, G.E., Leonard, M., Lewis, G.D., 2013. Evaluation of human adenovirus and human polyomavirus as indicators of human sewage contamination in the aquatic environment. Water Res 47, 6750-6761.

Higuchi, R., Dollinger, G., Walsh, P.S., Griffith, R., 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y) 10, 413-417.

Hill, V.R., Polaczyk, A.L., Kahler, A.M., Cromeans, T.L., Hahn, D., Amburgey, J.E., 2009. Comparison of hollow-fiber ultrafiltration to the USEPA VIRADEL technique and USEPA method 1623. J Environ Qual 38, 822-825.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A 88, 7276-7280.

Hot, D., Legeay, O., Jacques, J., Gantzer, C., Caudrelier, Y., Guyard, K., Lange, M., Andreoletti, L., 2003. Detection of somatic phages, infectious enteroviruses and enterovirus genomes as indicators of human enteric viral pollution in surface water. Water Res 37, 4703-4710.

Hund, E., Massart, D.L., Smeyers-Verbeke, J., 2000. Inter-laboratory studies in analytical chemistry. Anal Chim Acta 423, 145-165.

Hunter, M.E., Dorazio, R.M., Butterfield, J.S., Meigs-Friend, G., Nico, L.G., Ferrante, J.A., 2017. Detection limits of quantitative and digital PCR assays and their influence in presence-absence surveys of environmental DNA. Mol Ecol Resour 17, 221-229.

Hryniszyn, A., Skonieczna, M., Wiszniowski, J., 2013. Methods for detection of viruses in water and wastewater. AiM 03, 442-449.

Iker, B.C., Bright, K.R., Pepper, I.L., Gerba, C.P., Kitajima, M., 2013. Evaluation of commercial kits for the extraction and purification of viral nucleic acids from environmental and fecal samples. J Virol Methods 191, 24–30.

Ikner, L.A., Gerba, C.P., Bright, K.R., 2012. Concentration and recovery of viruses from water: a comprehensive review. Food Environ Virol 4, 41-67.

Ikner, L.A., Soto-Beltran, M., Bright, K.R., 2011. New method using a positively charged microporous filter and ultrafiltration for concentration of viruses from tap water. Appl Environ Microbiol 77, 3500-3506.

Jiang, S.C., 2006. Human adenoviruses in water: occurrence and health implications: a critical review. Environ Sci Technol 40, 7132-7140.

Jiang, S.C., Chu, W., 2004. PCR detection of pathogenic viruses in southern California urban rivers. J Appl Microbiol 97, 17-28.

Jiang, S.C., Chu, W., He, J.W., 2007. Seasonal detection of human viruses and coliphage in Newport Bay, California. Appl Environ Microbiol 73, 6468-6474.

Jiang, X., Estes, M.K., Metcalf, T.G., 1987. Detection of hepatitis A virus by hybridization with single-stranded RNA probes. Appl Environ Microbiol 53, 2487-2495.

Johnson, G., Nolan, T., Bustin, S.A., 2013. Real-time quantitative PCR, pathogen detection and MIQE. Methods Mol Biol 943, 1-16.

Jurzik, L., Hamza, I.A., Puchert, W., Uberla, K., Wilhelm, M., 2010. Chemical and microbiological parameters as possible indicators for human enteric viruses in surface water. Int J Hyg Environ Health 213, 210-216.

Karim, M.R., Rhodes, E.R., Brinkman, N., Wymer, L., Fout, G.S., 2009. New electropositive filter for concentrating enteroviruses and noroviruses from large volumes of water. Appl Environ Microbiol 75, 2393-2399.

Karmakar, S., Rathore, A.S., Kadri, S.M., Dutt, S., Khare, S., Lal, S., 2008. Post-earthquake outbreak of rotavirus gastroenteritis in Kashmir (India): an epidemiological analysis. Public Health 122, 981-989.

Kataki, S., Chatterjee, S., Vairale, M. G., Sharma, S. & Dwivedi, S. K. 2021. Concerns and strategies for wastewater treatment during COVID-19 pandemic to stop plausible transmission. Resour Conserv Recycl 164, 105156.

Katayama, H., Haramoto, E., Oguma, K., Yamashita, H., Tajima, A., Nakajima, H., Ohgaki, S., 2008. One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan. Water Res 42, 1441-1448.

Katayama, H., Shimasaki, A., Ohgaki, S., 2002. Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. Appl Environ Microbiol 68, 1033-1039.

Katzenelson, E., Fattal, B., Hostovesky, T., 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl Environ Microbiol 32, 638-639.

Kern, A., Kadar, M., Szomor, K., Berencsi, G., Kapusinszky, B., Vargha, M., 2013. Detection of enteric viruses in Hungarian surface water: first steps towards environmental surveillance. J Water Health 11, 772-782.

Kidd, A.H., Chroboczek, J., Cusack, S., Ruigrok, R.W., 1993. Adenovirus type 40 virions contain two distinct fibers. Virol 192, 73-84.

Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 in wastewater: State of the knowledge and research needs. Sci Total Environ 739, 139076.

Klymus, K.E., Merkes, C.M., Allison, M.J., Goldberg, C.S., Helbing, C.C., Hunter, M.E., Jackson, C.A., Lance, R.F., Mangan, A.M., Monroe, E.M., Piaggio, A.J., Stokdyk, J.P., Wilson, C.C., Richter, C.A., 2020. Reporting the limits of detection and quantification for environmental DNA assays. Environ DNA 2, 271-282.

Kocwa-Haluch, R., 2001. Waterborne enteroviruses as a hazard for human health. Polish J Environ Studies 77, 485-487.

Kozyra, I., Kaupke, A., Rzezutka, A., 2004. Seasonal occurence of human enteric viruses in river water samples collected from rural areas of South-East Poland. Food Environ Virol 3, 115-120.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K., Sindelka, R., Sjoback, R., Sjogreen, B., Strombom, L., Stahlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. Mol Aspects Med 27, 95-125.

Kunze, A., Pei, L., Elsaesser, D., Niessner, R., Seidel, M., 2015. High performance concentration method for viruses in drinking water. J Virol Methods 222, 132-137.

La Rosa, G., Pourshaban, M., Iaconelli, M., Muscillo, M., 2009. Quantification of Norovirus Genogroups I and II in Environmental and Clinical Samples Using TaqMan Real-Time RT-PCR. Food Environ Virol 1, 15-22.

La Rosa, G., Pourshaban, M., Iaconelli, M., Muscillo, M., 2010. Quantitative real-time PCR of enteric viruses in influent and effluent samples from wastewater treatment plants in Italy. Ann I Super Sanita 46, 266-273.

Lambertini, E., Spencer, S.K., Bertz, P.D., Loge, F.J., Kieke, B.A., Borchardt, M.A., 2008. Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. Appl Environ Microbiol 74, 2990-2996.

Leclerc, H., Schwartzbrod, L., Dei-Cas, E., 2002. Microbial agents associated with waterborne diseases. Crit Rev Microbiol 28, 371-409.

Leifels, M, Shoults, D., Wiedemeyer, A., Ashbolt, N.J, Sozzi, E., Hagemeier, A., Jurzik, L., 2019. Capsid integrity qPCR – an azo-dye based and culture-independent approach to estimate adenovirus infectivity after disinfection and in the aquatic environment. Water 11, 1196.

Lemiale, F., Haddada, H., Nabel, G.J., Brough, D.E., King, C.R., Gall, J.G., 2007. Novel adenovirus vaccine vectors based on the enteric-tropic serotype 41. Vaccine 25, 2074-2084.

Levidiotou, S., Gartzonika, C., Papaventsis, D., Christaki, C., Priavali, E., Zotos, N., Kapsali, E., Vrioni, G., 2009. Viral agents of acute gastroenteritis in hospitalized children in Greece. Clin Microbiol Infect 15, 596-598.

Levin, R.E., 2012. PCR detection of aflatoxin producing fungi and its limitations. Int J Food Microbiol 156, 1-6.

Lewis, G. D., Loutit, M. W., Austin, F. J., 1985. A method for detecting human enteroviruses in aquatic sediments. J Virol Methods 10, 153-162.

Liang, L., Goh, S.G., Vergara, G.G., Fang, H.M., Rezaeinejad, S., Chang, S.Y., Bayen, S., Lee, W.A., Sobsey, M.D., Rose, J.B., Gin, K.Y., 2015. Alternative fecal indicators and their empirical relationships with enteric viruses, Salmonella enterica, and Pseudomonas aeruginosa in surface water of a tropical urban catchment. Appl Environ Microbiol 81, 850-860.

Lipfert, J., Doniach, S., Das, R., Herschlag, D., 2014. Understanding nucleic acid-ion interactions. Annuc Rev Biochem 83, 813-814.

Lipp, E.K., Farrah, S.A., Rose, J.B., 2001. Assessment and impact of microbial fecal pollution and human enteric pathogens in a coastal community. Mar Pollut Bull 42, 286-293.

Lipp, E.K., Rose, J.B., 1997. The role of seafood in foodborne diseases in the United States of America. Rev Sci Tech 16, 620-640.

Lodder, W.J., de Roda Husman, A.M., 2005. Presence of noroviruses and other enteric viruses in sewage and surface water in The Netherlands. Appl Environ Microbiol 71, 1453-1461.

Love, D.C., Rodriguez, R.A., Gibbons, C.D., Griffith, J.F., Yu, Q., Stewart, J.R., Sobsey, M.D., 2014. Human viruses and viral indicators in marine water at two recreational beaches in Southern California, USA. J Water Health 12, 136-150.

Lukasik, J., Scott, T.M., Andryshak, D., Farrah, S.R., 2000. Influence of salts on virus adsorption to microporous filters. Appl Environ Microbiol 66, 2914-2920.

Lund, E., Hedstrom, C.E., 1966. The use of an aqueous polymer phase system for enterovirus isolations from sewage. Am J Epidemiol 84, 287-291.

Ma, J.F., Naranjo, J., Gerba, C.P., 1994. Evaluation of MK filters for recovery of enteroviruses from tap water. Appl Environ Microbiol 60, 1974-1977.

Maalouf, H., Pommepuy, M., Le Guyader, F.S., 2010. Environmental Conditions Leading to Shellfish Contamination and Related Outbreaks. Food Environ Virol 2, 136-145.

Maier, R.M., Pepper, I.L., Gerba, C.P., 2008. Environmental microbiology. San Diego: Academic Press.

Malik, A., Yasar, A., Tabinda, A.B., Abubakar, M., 2012. Water-borne diseases, costs of illness and willingness to pay for diseases interventions in rural communities of developing countries. Iran J Public Health 41, 39-49.

Masclaux, F.G., Hotz, P., Friedli, D., Savova-Bianchi, D., Oppliger, A., 2013. High occurrence of hepatitis E virus in samples from wastewater treatment plants in Switzerland and comparison with other enteric viruses. Water Res 47, 5101-5109.

Matthes-Martin, S., Boztug, H., Lion, T., 2013. Diagnosis and treatment of adenovirus infection in immunocompromised patients. Expert Rev Anti Infect Ther 11, 1017-1028.

Maunula, L., Klemola, P., Kauppinen, A., Söderberg, K., Nguyen, T., Pitkänen, T., Kaijalainen, S., Simonen, M.L., Miettinen, I.T., Lappalainen, M., Laine, J., Vuento, R., Kuusi, M., Roivainen, M., 2009. Enteric Viruses in a Large Waterborne Outbreak of Acute Gastroenteritis in Finland. Food Environ Virol 1, 31-36.

McMinn, B.R., 2013. Optimization of adenovirus 40 and 41 recovery from tap water using small disk filters. J Virol Methods 193, 284-290.

Melnick, J.L., 1957. A water-borne urban epidemic of hepatitis, Hepatitis Frontiers. Little, Brown & Co., Boston, MA.

Melnick, J.L., 1984. Etiologic agents and their potential for causing waterborne virus diseases, in: Melnick, J.L. (Ed.), Enteric Viruses in Water. Karger, Basel, Switzerland, pp. 1-16.

Melnick, J.L.; Gerba, C.P., Berg, G., 1980. The ecology of enteroviruses in natural waters. Crit Rev Environ Sci Technol 10, 65-93.

Melnick, J.L., Safferman, R., Rao, V.C., Goyal, S., Berg, G., Dahling, D.R., Wright, B.A., Akin, E., Stetler, R., Sorber, C., et al., 1984. Round robin investigation of methods for the recovery of poliovirus from drinking water. Appl Environ Microbiol 47, 144-150.

Miagostovich, M.P., Ferreira, F.F., Guimaraes, F.R., Fumian, T.M., Diniz-Mendes, L., Luz, S.L., Silva, L.A., Leite, J.P., 2008. Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream water of Manaus, central Amazonia, Brazil. Appl Environ Microbiol 74, 375-382.

Michen, B., Graule, T., 2010. Isoelectric points of viruses. J Appl Microbiol 109, 388-397.

Montazeri, N., Goettert, D., Achberger, E.C., Johnson, C.N., Prinyawiwatkul, W., Janes, M.E., 2015. Pathogenic enteric viruses and microbial indicators during secondary treatment of municipal wastewater. Appl Environ Microbiol 81, 6436-6445.

Monteiro, S., Santos, R., 2017. Nanofluidic digital PCR for the quantification of Norovirus for water quality assessment. PLoS ONE 12, e0179985.

Morales-Morales, H.A., Vidal, G., Olszewski, J., Rock, C.M., Dasgupta, D., Oshima, K.H., Smith, G.B., 2003. Optimization of a Reusable Hollow-Fiber Ultrafilter for Simultaneous Concentration of Enteric Bacteria, Protozoa, and Viruses from Water. Appl Environ Microbiol 69, 4098-4102.

Morens, D.M., Folkers, G.K., Fauci, A.S., 2004. The challenge of emerging and re-emerging infectious diseases. Nature 430, 242-249.

Morris, J., 1996. Filtered or waivered, you need source protection. J N Engl Water Work Assoc 110, 182-191.

Myrmel, M., Berg, E.M.M., Grinde, B., Rimstad, E., 2006. Enteric viruses in inlet and outlet samples from sewage treatment plants. J Water Health 4, 197-209.

Naidu, S.S., Viswanathan, R., 1957. Infectious hepatitis in pregnancy during Delhi epidemic. Indian J Med Res 45, 71-76.

Nairn, C., Clements, G.B., 1999. A study of enterovirus isolations in Glasgow from 1977 to 1997. J Med Virol 58, 304-312.

Nakanishi, K., Tsugawa, T., Honma, S., Nakata, S., Tatsumi, M., Yoto, Y., Tsutsumi, H., 2009. Detection of enteric viruses in rectal swabs from children with acute gastroenteritis attending the pediatric outpatient clinics in Sapporo, Japan. J Clin Virol 46, 94-97.

Nestola, P., Peixoto, C., Silva, R.R., Alves, P.M., Mota, J.P., Carrondo, M.J., 2015. Improved virus purification processes for vaccines and gene therapy. Biotechnol Bioeng 112, 843–57.

Newby, D.T., Marlowe, E.M., Maier, R.M., 2009. Nucleic acid-based methods of analysis. In: Maier, R.M., Pepper, I.L., Gerba, C.P. (Eds), Environmental Microbiology. Elsevier Inc. p. 42.

Nordgren, J., Matussek, A., Mattsson, A., Svensson, L., Lindgren, P.E., 2009. Prevalence of norovirus and factors influencing virus concentrations during one year in a full-scale wastewater treatment plant. Water Res 43, 1117-1125.

Nupen, E. M., 1970. Virus studies on the Windhoek wastewater reclamation plant (southwest Africa). Water Res 4, 661-672.

Ogorzaly, L., Bertrand, I., Paris, M., Maul, A., Gantzer, C., 2010. Occurrence, survival, and persistence of human adenoviruses and F-specific RNA phages in raw groundwater. Appl Environ Microbiol 76, 8019-8025.

Okoh, A.I., Sibanda, T., Gusha, S.S., 2010. Inadequately treated wastewater as a source of human enteric viruses in the environment. Int J Environ Res Public Health 7, 2620-2637.

Olszewski, J., Winona, L., Oshima, K.H., 2005. Comparison of 2 ultrafiltration systems for the concentration of seeded viruses from environmental water. Can J Microbiol 51, 295-303.

Osei Sekyere, J., Govinden, U., Essack, S.Y., 2015. Review of established and innovative detection methods for carbapenemase-producing Gram-negative bacteria. J Appl Microbiol 119, 1219-1233.

Pang, X.L. Lee, B.E., Pabbaraju, K., Gabos, S., Craik, S., Payment, P., Neumann, N., 2012. Pre-analytical and analytical procedures for the detection of enteric viruses and enterovirus in water samples. J Virol Methods 184, 77-83.

Park, G.W., Barclay, L., Macinga, D., Charbonneau, D., Pettigrew, C.A., Vinje, J., 2010. Comparative efficacy of seven hand sanitizers against murine norovirus, feline calicivirus, and GII.4 norovirus. J Food Prot 73, 2232-2238.

Parks, G.A., 1965. Isoelectric points of solid oxides solid hydroxides and aqueous hydroxo complex systems. Chem Rev 65, 177-198.

Patti, A.M., Aulicino, F.A., Santi, A.L., Muscillo, M., Orsinv, P., Bellucci, C., La Rosa, G., Mastroeni, I., Volterra, L., 1996. Enteric virus pollution of tyrrhenian areas. Water Air Soil Poll 88, 261-267.

Payne, D.C., Vinje, J., Szilagyi, P.G., Edwards, K.M., Staat, M.A., Weinberg, G.A., Hall, C.B., Chappell, J., Bernstein, D.I., Curns, A.T., Wikswo, M., Shirley, S.H., Hall, A.J., Lopman, B., Parashar, U.D., 2013. Norovirus and medically attended gastroenteritis in U.S. children. N Engl J Med 368, 1121-1130.

Pei, L., Rieger, M., Lengger, S., Ott, S., Zawadsky, C., Hartmann, N.M., Selinka, H.C., Tiehm, A., Niessner, R., Seidel, M., 2012. Combination of crossflow ultrafiltration, monolithic affinity filtration, and quantitative reverse transcriptase PCR for rapid concentration and quantification of model viruses in water. Environ Sci Technol 46, 10073-10080.

Pepper, I.L., Gerba, C.P., 2015. Environmental sample collection and processing. In: Pepper, I.L., Gerba, C.P., Gentry, T.J. (Eds.), Environmental Microbiology. Elsevier, pp. 157–175.

Perez-Sautu, U., Sano, D., Guix, S., Kasimir, G., Pinto, R.M., Bosch, A., 2012. Human norovirus occurrence and diversity in the Llobregat river catchment, Spain. Environ Microbiol 14, 494-502.

Peskoller, C., Niessner, R., Seidel, M., 2009. Development of an epoxy-based monolith used for the affinity capturing of Escherichia coli bacteria. J Chromatogr A 1216, 3794-3801.

Philipson, L., Albertsson, P. A., Frick, G., 1960. The purification and concentration of viruses by aqueous polymer phase systems. Virology 11, 553-571.

Pina, S., Buti, M., Cotrina, M., Piella, J., Girones, R., 2000. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. J Hepatol 33, 826-833.

Pina, S., Puig, M., Lucena, F., Jofre, J., Girones, R., 1998. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. Appl Environ Microbiol 64, 3376-3382.

Pinheiro, L.B., Coleman, V.A., Hindson, C.M., Herrmann, J., Hindson, B.J., Bhat, S., Emslie, K.R., 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem 84, 1003–1011.

Pinto, R.M., Gajardo, R., Abad, F.X., Bosch, A., 1995. Detection of fastidious infectious enteric viruses in water. Environ Sci Technol 29, 2636-2638.

Polaczyk, A.L., Narayanan, J., Cromeans, T.L., Hahn, D., Roberts, J.M., Amburgey, J.E., Hill, V.R., 2008. Ultrafiltration-based techniques for rapid and simultaneous concentration of multiple microbe classes from 100-L tap water samples. J Microbiol Methods 73, 92-99.

Prado, T., Silva, D.M., Guilayn, W.C., Rose, T.L., Gaspar, A.M., Miagostovich, M.P., 2011. Quantification and molecular characterization of enteric viruses detected in effluents from two hospital wastewater treatment plants. Water Res 45, 1287-1297.

Preston, D.R., Vasudevan, T.V., Bitton, G., Farrah, S.R., Morel, J.L., 1988. Novel approach for modifying microporous filters for virus concentration from water. Appl Environ Microbiol 54, 1325-1329.

Prevost, B., Lucas, F.S., Goncalves, A., Richard, F., Moulin, L., Wurtzer, S., 2015. Large scale survey of enteric viruses in river and waste water underlines the health status of the local population. Environ Int 79, 42-50.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G., Girones, R., 1994. Detection of adenoviruses and enteroviruses in polluted water by nested PCR amplification. Appl Environ Microbiol 60, 2963-2970.

Pusch, D., Oh, D.Y., Wolf, S., Dumke, R., Schroter-Bobsin, U., Hohne, M., Roske, I., Schreier, E., 2005. Detection of enteric viruses and bacterial indicators in German environmental water. Arch Virol 150, 929-947.

Qiu, Y., Lee, B.E., Neumann, N., Ashbolt, N., Craik, S., Maal-Bared, R., Pang, X.L., 2015. Assessment of human virus removal during municipal wastewater treatment in Edmonton, Canada. J Appl Microbiol 119, 1729-1739.

Qiu, Y., Lee, B.E., Ruecker, N.J., Neumann, N., Ashbolt, N., Pang, X., 2016. A one-step centrifugal ultrafiltration method to concentrate enteric viruses from wastewater. J Virol Methods 237, 150-153.

Racki, N., Morisset, D., Gutierrez-Aguirre, I., Ravnikar, M., 2014a. One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. Anal Bioanal Chem 406, 661-667.

Racki, N., Dreo, T., Gutierrez-Aguirre, I., Blejec, A., Ravnikar, M., 2014b. Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. Plant Methods 10, 42.

Rao, V.C., 2013. Environmental Virology. Springer US.

Rao, V.C., Melnick, J.L., 1986. Environmental virology, in: Cole, J.A., Knowles, C.J., Chlessinger, D. (Eds.), Aspects of Microbiology 13. American Society for Microbiology, Washington, D.C.

Rawsthorne, H., Phister, T.G., Jaykus, L.A., 2009. Development of a fluorescent in situ method for visualization of enteric viruses. Appl Environ Microbiol 75, 7822-7827.

Ren, J.-G., Li, D.-Y., Wang, C.-F., Wu, J.-H., Wang, Y, Sun, Y.-J., Zhang, Q., Wang, Y.-Y., Chang, X.-J., 2020. Positive RT-PCR in urine from asymptomatic patient with novel coronavirus 2019 infection: a case report. Infec Dis 52, 571-574.

Reynolds, K.A., 2004. Adenoviruses: balancing water treatment challenges. Water Cond Purif 46, 42-43.

Rhodes, E.R., Huff, E.M., Hamilton, D.W., Jones, J.L., 2016. The evaluation of hollow-fiber ultrafiltration and celite concentration of enteroviruses, adenoviruses and bacteriophage from different water matrices. J Virol Methods 228, 31-38.

Richards, G.P., Cliver, D.O., Greening, G.E., 2015. 44. Foodborne Viruses, Compendium of Methods for the Microbiological Examination of Foods.

Rigotto, C., Kolesnikovas, C., Moresco, V., Simoes, C. and Barardi, C., 2009. Evaluation of HA negatively charged membranes in the recovery of human adenoviruses and hepatitis A virus in different water matrices. Memorias do Instituto Oswaldo Cruz 104, 970-974.

Rimoldi, S.G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Cappelli, F., Roscioli, C., Moja, L., Gismondo, M. R., Salerno, F., 2020. Presence and infectivity of SARS-CoV-2 virus in wastewaters and rivers. Sci Total Environ 744, 140911.

Rock, C., Alum, A., Abbaszadegan, M., 2010. PCR inhibitor levels in concentrates of biosolid samples predicted by a new method based on excitation-emission matrix spectroscopy. Appl Environ Microbiol 76, 8102-8109.

Rodriguez, R.A., Gundy, P.M., Rijal, G.K., Gerba, C.P., 2012. The impact of combined sewage overflows on the viral contamination of receiving water. Food Environ Virol 4, 34-40.

Rodriguez-Lazaro, D., Cook, N., Ruggeri, F.M., Sellwood, J., Nasser, A., Nascimento, M.S., D'Agostino, M., Santos, R., Saiz, J.C., Rzezutka, A., Bosch, A., Girones, R., Carducci, A., Muscillo, M., Kovac, K., Diez-Valcarce, M., Vantarakis, A., von Bonsdorff, C.H., de Roda Husman, A.M., Hernandez, M., van der Poel, W.H., 2012. Virus hazards from food, water and other contaminated environments. FEMS Microbiol Rev 36, 786-814.

Ruiz-Villalba, A., van Pelt-Verkuil, E., Gunst, Q.D., Ruijter, J.M., van den Hoff, M.J., 2017. Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). Biomol Detect Quantif 14, 7-18.

Russell, W.C., 2009. Adenoviruses: update on structure and function. J Gen Virol 90, 1-20.

Rutjes, S.A., Italiaander, R., van den Berg, H.H., Lodder, W.J., de Roda Husman, A.M., 2005. Isolation and detection of enterovirus RNA from large-volume water samples by using the

NucliSens miniMAG system and real-time nucleic acid sequence-based amplification. Appl Environ Microbiol 71, 3734-3740.

Samandoulgou, I., Fliss, I., Jean, J., 2015. Zeta Potential and Aggregation of Virus-Like Particle of Human Norovirus and Feline Calicivirus Under Different Physicochemical Conditions. Food Environ Virol 7, 249-260.

Sato, K., Inaba, Y., Shinozaki, T., Fujii, R., Matumoto, M., 1981. Isolation of human rotavirus in cell cultures. Arch Virol 69, 155-160.

Schaudies, P., Robinson, D.A., 2007. Literature review of molecular methods for simultaneous detection of pathogens in water. U.S. Environmental Protection Agency., Report 600/R-07/128 Washington D.C.

Schiff, G., Stefanovic, G., Young, B., and Pennekamp, J., 1984. Minimum human infectious dose of enteric virus (Echovirus-12) in drinking water, in Enteric viruses in water. Karger Publishers, 222-228.

Schlindwein, A.D., Rigotto, C., Simoes, C.M., Barardi, C.R., 2010. Detection of enteric viruses in sewage sludge and treated wastewater effluent. Water Sci Technol 61, 537-544.

Sedmak, G., Bina, D., MacDonald, J., 2003. 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.

Seidel, M., Jurzik, L., Brettar, I., Höfle, M., Griebler, C., 2016. Microbial and viral pathogens in freshwater: current research aspects studied in Germany. Environ Earth Sci 75, 1384-1403.

Seitz, S.R., Leon, J.S., Schab, K.J., Lyon, G.M., Dowd, M., McDaniels, M., Abdulhafid, G., Fernandez, M.L., Lindesmith, L.C., Baric, R.S., Moe, C.L., 2011. Norovirus infectivity in humans and persistence in water. Appl Environ Microbiol 77, 6884-6888.

Shortridge, K. F., Alexander, D. J., Collins, M. S., 1980. Isolation and properties of viruses from poultry in Hong Kong, which represent a new (sixth) distinct group of avian paromyxoviruses. J Gen Virol 49, 255-262.

Shrivastava, A., Gupta, V.B., 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chron Young Sci 2, 21-25.

Shuval, H. I., Cymbalista, S., Fattal, B., Goldblum, N., 1967. Concentration of enteric viruses in water by hydroextraction and two phase separation, p. 45-55. In G. Berg (ed.), Transmission of viruses by the water route. JohnWiley& Sons,Inc., New York.

Sidhu, J.P.S., Ahmed, W., Toze, S., 2013. Sensitive detection of human adenovirus from small volume of primary wastewater samples by quantitative PCR. J. Virol. Methods 187, 395–400.

Siebenga, J.J., Vennema, H., Zheng, D.P., Vinje, J., Lee, B.E., Pang, X.L., Ho, E.C., Lim, W., Choudekar, A., Broor, S., Halperin, T., Rasool, N.B., Hewitt, J., Greening, G.E., Jin, M., Duan, Z.J., Lucero, Y., O'Ryan, M., Hoehne, M., Schreier, E., Ratcliff, R.M., White, P.A., Iritani, N., Reuter, G., Koopmans, M., 2009. Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007. J Infect Dis 200, 802-812.

Silverman, A.I., Akrong, M.O., Amoah, P., Drechsel, P., Nelson, K.L., 2013. Quantification of human norovirus GII, human adenovirus, and fecal indicator organisms in wastewater used for irrigation in Accra, Ghana. J Water Health 11, 473-488.

Simmons, F.J., Kuo, D.H., Xagoraraki, I., 2011. Removal of human enteric viruses by a full-scale membrane bioreactor during municipal wastewater processing. Water Res 45, 2739-2750.

Sinclair, R.G., Jones, E.L., Gerba, C.P., 2009. Viruses in recreational water-borne disease outbreaks: a review. J Appl Microbiol 107, 1769-1780.

Skraber, S., Gassilloud, B., Gantzer, C., 2004a. Comparison of coliforms and coliphages as tools for assessment of viral contamination in river water. Appl Environ Microbiol 70, 3644-3649.

Skraber, S., Gassilloud, B., Schwartzbrod, L., Gantzer, C., 2004b. Survival of infectious Poliovirus-1 in river water compared to the persistence of somatic coliphages, thermotolerant coliforms and Poliovirus-1 genome. Water Res 38, 2927-2933.

Sobsey, M.D., 1982. Quality of currently available methodology for monitoring viruses in the environment. Environ Internat 7, 39-51.

Sobsey, M.D., Glass, J.S., 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl Environ Microbiol 40, 201-210.

Sobsey, M.D., Glass, J.S., 1984. Influence of water quality on enteric virus concentration by microporous filter methods. Appl Environ Microbiol 47, 956-960.

Soto-Beltran, M., Ikner, L.A., Bright, K.R., 2013. Effectiveness of poliovirus concentration and recovery from treated wastewater by two electropositive filter methods. Food Environ Virol 5, 91-96.

Soule, H., Genoulaz, O., Gratacap-Cavallier, B., Chevallier, P., Liu, J., Seigneurin, J., 2000. Ultrafiltration and reverse transcription-polymerase chain reaction: an efficient process for poliovirus, rotavirus and hepatitis A virus detection in water. Water Res 34, 1063-1067.

Stewart, J.R., Boehm, A.B., Dubinsky, E.A., Fong, T.-T., Goodwin, K.D., Griffith, J.F., Nobel, R.T., Shanks, O.Q., Vijayavel, K., Weisberg, S. B., 2013. Recommendations following a multi-laboratory comparison of microbial source tracking methods. Water Res 47, 6829–6838.

Straub, T.M., Chandler, D.P., 2003. Towards a unified system for detecting waterborne pathogens. J Microbiol Methods 53, 185-197.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B., Koopmans, M., 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J Clin Microbiol 45, 1389-1394.

Tao, C.W., Hsu, B.M., Kao, P.M., Huang, W.C., Hsu, T.K., Ho, Y.N., Lu, Y.J., Fan, C.W., 2016. Seasonal difference of human adenoviruses in a subtropical river basin based on 1-year monthly survey. Environ Sci Pollut Res Int 23, 2928-2936.

Thompson, S.S., Jackson, J.L., Suva-Castillo, M., Yanko, W.A., El Jack, Z., Kuo, J., Chen, C.L., Williams, F.P., Schnurr, D.P., 2003. Detection of infectious human adenoviruses in tertiary-treated and ultraviolet-disinfected wastewater. Water Environ Res 75, 163-170.

Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Riley, K., Gerba, C.P., 2003. Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. Appl Environ Microbiol 69, 577-582.

Tong, H.I., Lu, Y., 2011. Effective detection of human adenovirus in Hawaiian water using enhanced PCR methods. Virol J 8, 57.

Vaidya, S.R., Chitambar, S.D., Arankalle, V.A., 2002. Polymerase chain reaction-based prevalence of hepatitis A, hepatitis E and TT viruses in sewage from an endemic area. J Hepatol 37, 131-136.

van Heerden, J., Ehlers, M.M., Grabow, W.O., 2005. Detection and risk assessment of adenoviruses in swimming pool water. J Appl Microbiol 99, 1256-1264.

van Heerden, J., Ehlers, M.M., van Zyl, W.B., Grabow, W.O., 2003. Incidence of adenoviruses in raw and treated water. Water Res 37, 3704-3708.

van Heerden, J., Ehlers, M.M., van Zyl, W.B., Grabow, W.O., 2004. Prevalence of human adenoviruses in raw and treated water. Water Sci Technol 50, 39-43.

Vantarakis, A., Papapetropoulou, M., 1999. Detection of Enteroviruses, Adenoviruses and Hepatitis A Viruses in Raw Sewage and Treated Effluents by Nested-PCR. Water Air Soil Poll 114, 85-93.

Vergara, G., Rose, J.B., Gin, K.Y.H., 2016. Risk assessment of noroviruses and human adenoviruses in recreational surface water. Water Res 103, 276-282.

Verheyen, J., Timmen-Wego, M., Laudien, R., Boussaad, I., Sen, S., Koc, A., Uesbeck, A., Mazou, F., Pfister, H., 2009. Detection of adenoviruses and rotaviruses in drinking water sources used in rural areas of Benin, West Africa. Appl Environ Microbiol 75, 2798-2801.

Victoria, M., Guimaraes, F., Fumian, T., Ferreira, F., Vieira, C., Leite, J.P., Miagostovich, M., 2009. Evaluation of an adsorption-elution method for detection of astrovirus and norovirus in environmental water. J Virol Methods 156, 73-76.

Victoria, M., Rigotto, C., Moresco, V., de Abreu Correa, A., Kolesnikovas, C., Leite, J.P., Miagostovich, M.P., Barardi, C.R., 2010. Assessment of norovirus contamination in environmental samples from Florianopolis City, Southern Brazil. J Appl Microbiol 109, 231-238.

Vilaginès, P., Sarrette, B., Husson, G., Vilaginès, R., 1993. Glass Wool for Virus Concentration at Ambient Water pH Level. Water Sci Technol 27, 299-306.

Viswanathan, R., Sidhu, A.S., 1957. Infectious hepatitis; clinical findings. Indian J Med Res 45, 49-58.

Vivier, J.C., Ehlers, M.M., Grabow, W.O., 2004. Detection of enteroviruses in treated drinking water. Water Res 38, 2699-2705.

Wallis, C., Melnick, J.L., 1967. Concentration of enteroviruses on membrane filters. J Virol 1, 472-477.

Ward, R.L., Bernstein, D.I., Young, E.C., Sherwood, J.R., Knowlton, D.R., Schiff, G.M., 1986. Human Rotavirus Studies in Volunteers: Determination of Infectious Dose and Serological Response to Infection. J Infect Dis 154, 871-880.

Whelan, J.A., Russell, N.B., Whelan, M.A., 2003. A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods 278, 261-269.

White, K.E., Osterholm, M.T., Mariotti, J.A., Korlath, J.A., Lawrence, D.H., Ristinen, T.L., Greenberg, H.B., 1986. A foodborne outbreak of Norwalk virus gastroenteritis. Evidence for post-recovery transmission. Am J Epidemiol 124, 120-126.

WHO, 2008. Guidelines for drinking-water quality: third edition incorporating the first and second addenda. World Health Organization, Geneva.

WHO, 2011. Guidelines for drinking-water quality: fourth edition. World Health Organization, Geneva.

WHO, 2017. Guidelines for drinking-water quality: fourth edition incorporating the first addendum. World Health Organization, Geneva.

WHO, 2020. Naming the Coronavirus Disease (COVID-19) and the Virus That Causes It. 2020. World Health Organization.

Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N., Topp, E., Lapen, D.R., 2009. Seasonal relationships among indicator bacteria,

pathogenic bacteria, Cryptosporidium oocysts, Giardia cysts, and hydrological indices for surface water within an agricultural landscape. Water Res 43, 2209-2223.

Williams Jr., F.P., Akin, E.W., 1986. Waterborne Viral Gastroenteritis. J Am Water Works Ass 78, 34-39.

Wolf, S., Hewitt, J., Greening, G.E., 2010. Viral Multiplex Quantitative PCR Assays for Tracking Sources of Fecal Contamination. Appl Environ Microbiol 76, 1388-1394.

Wunderlich, A., Torggler, C., Elsaesser, D., Luck, C., Niessner, R., Seidel, M., 2016. Rapid quantification method for Legionella pneumophila in surface water. Anal Bioanal Chem 408, 2203-2213.

Wyn-Jones, A.P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Holler, C., de Roda Husman, A.M., Kay, D., Kozyra, I., Lopez-Pila, J., Muscillo, M., Nascimento, M.S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R., Wyer, M., 2011. Surveillance of adenoviruses and noroviruses in European recreational water. Water Res 45, 1025-1038.

Wyn-Jones, A.P., Sellwood, J., 2001. Enteric viruses in the aquatic environment. J Appl Microbiol 91, 945-62.

Xagoraraki, I., Kuo, D.H.-W., Wong, K., Wong, M., Rose, J.B., 2007. Occurrence of Human Adenoviruses at Two Recreational Beaches of the Great Lakes. Appl Environ Microbiol 73, 7874-7881.

Yang, N., Qi, H., Wong, M.M., Wu, R.S., Kong, R.Y., 2012. Prevalence and diversity of norovirus genogroups I and II in Hong Kong marine water and detection by real-time PCR. Mar Pollut Bull 64, 164-168.

Yang, S., Rothman, R.E., 2004. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis 4, 337-348.

Ye, X.Y., Ming, X., Zhang, Y.L., Xiao, W.Q., Huang, X.N., Cao, Y.G., Gu, K.D., 2012. Real-time PCR detection of enteric viruses in source water and treated drinking water in Wuhan, China. Curr Microbiol 65, 244-253.

Yeh, H.Y., Pieniazek, N., Pieniazek, D., Gelderblom, H., Luftig, R.B., 1994. Human adenovirus type 41 contains two fibers. Virus Res 33, 179-198.

Zamxaka, M., Pironcheva, G., Muyima N.Y.O., 2004. Microbiological and physico-chemical assessment of the quality of domestic water sources in selected rural communities of the Eastern Cape Province, South Africa. Water SA 30, 333-340.