ARI KAUPPINEN

Waterborne outbreaks are a constant threat to public health worldwide. The most important waterborne pathogens include enteric viruses, such as noroviruses. This thesis provides new information regarding the occurrence, transport, persistence and control of enteric viruses in water environments. Furthermore, the thesis assesses the suitability of commonly used indicator microbes to describe the water quality as well as the occurrence and fate of enteric viruses in water environments.
PATHOGENIC VIRUSES IN FINNISH WATERS—OCCURRENCE, FATE AND CONTROL
Ari Kauppinen

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ABSTRACT

Waterborne outbreaks occur worldwide, even though knowledge of the health risks posed by microbes in the environment is increasing. In Finland, between 3 and 11 waterborne outbreaks occur every year, and the number of patients may vary from a few to several thousand. Enteric viruses, especially noroviruses, are the main causative agents of waterborne outbreaks, and they end up in the environment mainly through wastewater discharge. The cause of the outbreak is usually connected to deficiencies in the management of water supply and sewerage systems, which results in human wastewater contamination of groundwater or recreational water.

In this thesis, both laboratory and pilot-scale experiments as well as investigations performed during the waterborne outbreaks were utilised in order to study the occurrence, transport, persistence and control of enteric viruses (noroviruses and adenoviruses). In addition, the aim was to study the feasibility of currently used faecal indicator microbes in assessing water quality.

Enteric viruses showed high prevalence in wastewater. Norovirus GI, norovirus GII and adenoviruses were found in 94.7%, 100% and 100%, respectively, of 19 influent samples collected from municipal wastewater treatment plant (WWTP) during a one-year period. The stable occurrence of adenoviruses in wastewater throughout the year supports their use as markers of human wastewater pollution. Although noroviruses were also detected throughout the year in wastewater, their numbers varied, being highest in winter.

High numbers of norovirus GII were detected in groundwater and tap water samples in two drinking water outbreaks (I and II) described in this study. In addition, adenovirus was detected in drinking water outbreak II. On the other hand, only 25% of the water samples collected during seven bathing water outbreaks were positive for noro- and/or adenoviruses. This addresses the temporal and methodological challenges related to detecting contamination.
During a one-year pilot study, sand filters (SFs) used in onsite wastewater treatment systems (OWTSSs) showed high variation in the removal of microbes. The log$_{10}$ removals of enteric viruses ranged from 0.0 to >5.0 depending on the virus, SF and season. In the OWTS that caused drinking water outbreak I, the removal of norovirus GII in SF was 1.1 log$_{10}$. This produced effluent that still contained remarkably high numbers of noroviruses (1 400 GC/mL), which managed to break into groundwater. Thus, current OWTSSs may compromise water safety.

The long persistence of virus genomes in water environments was noted. Noro- and adenovirus genomes were detected in the outbreak water samples stored at 4 °C up to 1 277 and 1 343 days, respectively. In addition, no reduction in norovirus genome numbers was observed in drinking water at 3 °C over the one-year laboratory study. In the laboratory study, significant differences were observed in the decay of the norovirus genome between the temperatures, matrices, and virus strains. The norovirus persisted better in drinking water compared to wastewater, and a cold temperature assisted with its persistence at both matrices. Differences between the persistence of norovirus strains were also evident, and, particularly, indigenous noroviruses persisted better than spiked noroviruses in wastewater. The long persistence of enteric viruses in water underline the importance of active control measures.

The laboratory-scale pipeline system was efficiently decontaminated from adenoviruses with peracetic acid (PAA) and chlorine (within two days). However, in the drinking water outbreak study, noro- and adenoviruses were detected in the distribution network for at least 59 days from the notification of the contamination and 19 days from the start of continuous chlorination. Overall, it took 108 days from the notification of contamination to ascertain the removal of noro- and adenoviruses from the distribution network.

*E. coli* was able to indicate groundwater contamination in two drinking water outbreaks, and intestinal enterococci were detected in groundwater in drinking water outbreak II. In outbreak II, *C. perfringens* was detected for 122 days in the contaminated network, which supports its use for verifying the safety of a drinking water distribution system after decontamination. In bathing water outbreaks, the faecal indicator bacteria (FIB) threshold for management actions was exceeded only in one of eight outbreaks where a clear external contamination source was identified. In other bathing water outbreaks, no statistical difference was noted in the levels of FIB between the outbreak samples and the frequent-monitoring samples. Overall, the value of indicator microbes in the prediction of enteric viruses seems to be case-specific and may require massive contamination. Therefore, direct monitoring of enteric viruses to assess the health risks related to water may be needed.

In summary, this study increases our knowledge of the properties of enteric viruses and why they pose such a great concern for water safety. First, viruses may be present in high numbers in wastewater and contaminated water environments.
Second, they are capable of transporting through soil and may not be effectively removed during wastewater treatment in OWTSs. Third, they can persist for long periods of time in water environments and are resistant to decontamination practices to some extent. This knowledge can be exploited in planning of the prevention and management actions for waterborne outbreaks and contamination cases, e.g. during quantitative microbial risk assessment (QMRA).

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*CAB Thesaurus: Adenoviridae, decontamination, indicators, Norovirus, outbreaks, persistence, risk assessment, wastewater treatment, water, water pollution, water purification, water quality, water supply, waterborne diseases*

*Medical Subject Headings: Adenoviridae; Bathing Beaches; Decontamination; Disease Outbreaks; Drinking Water; Escherichia coli; Genome, Viral; Groundwater; Norovirus; Risk Assessment; Waste Water; Water Pollution; Water Purification; Water Quality; Water Supply; Waterborne Diseases*
TIIVISTELMÄ


Tämän tutkimuksen tavoitteena oli selvittää enteristen virusten (norovirus ja adenovirus) esiintyvyyttä, kohtaloa ja torjuntaa suomalaisissa vesiympäristöissä. Lisäksi testattiin nykyisin käytettävien suolistoperäisten indikaattorimikrobien soveltuvuutta veden laadun mittareina. Tutkimuksessa suoritettiin sekä ennalta suunniteltuja laboratorio- ja pilot-kokeita että käytettiin hyväksytutkimuksen soveltuvia kahta juomavesiepidemiaa ja kahdeksaa uimavesiepidemiaa.

Norov- ja adenoviruksia esiintyi runsaasti yhdyskuntajätevedessä ympäri vuoden. Tutkituista 19:sta jätevedenpuhdistamolle tulevan jäteveden näytteestä adenoviruksia ja noroviruksen genoryhmää II (GII) todettiin 100 %:ssa ja noroviruksen genoryhmää I (GI) 94.7 %:ssa näytteistä. Adenovirusten pitoisuudet olivat tasaiset ympäri vuoden, mikä tukee niiden käyttöä ihmisperäisen jätevesisaastutuksen indikaattorina. Norovirusten esiintyvyydessä puolestaan oli vuodenaikaisvaihtelua ja lukumäärät olivat korkeimmat talvella.

Kahdessa juomavesiepidemiassa (I ja II) tutkituista pohjavesi- ja hanavesinäytteistä todettiin suuri määrä noroviruksia. Lisäksi adenoviruksia todettiin juomavesiepidemiassa II. Toisaalta vain 25 % seitsemän uimavesiepidemian aikana kerätystä näytteistä oli positiivisia noro- ja/tai adenoviruksille. Tämä kuvastaa näytteenottoon liittyviä ajallisia haasteita sekä ympäristönäytteiden analysoinnin menetelmällisiä kehitystarpeita.


Noro- ja adenovirusten genomien todettiin säilyvän pitkään erityisesti viileässä vedessä. Juomavesiepidemianäytteissä noroviruksen genomi säilyi vähintään 1277
päivää ja adenoviruksen genomia 1343 päivää 4 °C:ssa. Lisäksi vuoden kestäneessä
laboratoriotutkimuksessa noroviruksen genomien lukumäärässä ei todettu
vähennemistä juomavedessä 3 °C:ssa. Tulosten perusteella havaittiin tilastollisesti
merkitseviä eroja säilyvyydessä lämpötilan, vesimatriisin ja norovirus-kantojen
välillä. Norovirus säilyi paremmin juomavedessä kuin jätevedessä, ja kylmä
lämpötila edisti säilyvyyttä kummassakin matriisissa. Kantojen välillä erityisesti
jäteveden sisältämät norovirukset säilyivät paremmin kuin laboratoriossa veteen
lisättyt kannat. Tutkimuksessa todettiin enteeristen virusten pitkä säilyvyys osoittaa
asianmukaisten kontrollitoimenpiteiden tärkeyden vesiongelmatilanteiden
hoidossa.

Virusien poistaminen likaantuneesta juomavesiverkostosta voi osoittautua
haasteelliseksi tehtäväksi. Laboratoriokokeessa juomavesiputkisto saatiin
puhdistettua adenoviruksista alle kahdessa päivässä sekä kloorin että
peretikkahapon (PAA) avulla, kun taas juomavesiepidemiassa verkostosta löytyi
noro- ja adenoviruksia vähintään 59 päivää kontaminaation havaitsemisesta ja 19
päivää jatkuvuukokeiston kloraukon aloituksesta. Lopulta juomavesiverkosto
todettiin puhtaaksi viruksista 108 päivää kontaminaation havaitsemisen jälkeen.

Veden hygieenisen laadun tarkkailussa käytetty *E. coli* osiitti veden
likaantumisen juomavesiepidemioissa, joita selvitettiin osana tätä tutkimusta.
Lisäksi juomavesiepidemioissa II todettiin suolistoperäisiä enterokokkeja. *C.
perfringens* -bakteeria todettiin kontaminaation havaitsemisen jälkeen 122 päivää
likaantuneesta verkostosta, mikä tukee tämän bakteerin käyttöä
juomavesiverkoston puhtauden varmistamisessa. Uimavesiepidemioissa
ulosteperäisten indikaattoribakteerien raja-arvot ylittyivät vain yhdessä
epidemiassa kahdeksasta. Kyseinen epidemia oli ainoa, jossa havaittiin selvä
ulkoinen saastelähde. Muissa uimavesiepidemioissa indikaattoribakteerien
lukumäärissä ei havaittu eroa epidemican aikana otettujen ja säännöllisten koko
uimakauden aikana otettujen tarkkailunäytteiden välillä. Kaiken kaikkiaan
indikaattorimikrobiien kyky ennustaa veden likaantumista ja/tai enteeristen
virusten läsnä-oloa näyttää olevan tapaukskohtainen ja vaatia massiivistä
likaantumista. Tämän vuoksi enteeristen virusten suora monitorointi on tarpeellista
arvioitaessa veteen liittyviä terveysriskejä.

Tämä tutkimus lisää tietoa tekijöistä, jotka vaikuttavat enteeristen virusten
kykyyn aiheuttaa vesiepidemioita. Yhteenvetona voidaan todeta, että viruksia voi
esiintyä suuria määrää jätevedessä ja saastuneissa vesiypäristöissä. Virukset
voivat kulkeutua tehokkaasti maaperässä, eikä niiden poistuminen
maasuodattamissa ole välttämättä tehokasta. Lisäksi virukset säilyvät pitkiä aikoja
erilaisissa vesipyäristöissä ja kestävät jossain määrin puhdistustoimenpiteitä.
Saatua tietoa voidaan hyödyntää vesiepidemioiden ja veden saastumistilanteiden
hoitamisessa sekä kyseisten ongelmatilanteiden ennaltaehkäisyssä ja
riskinarvioinnissa.
Yleinen suomalainen asiasanasto: adenovirukset, epidemiat, indikaattorit, juomavesi, jätevesi, mikrobit, norovirus, talousvesi, uimavesi, vedenlaatu, vesihuolto
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Ari Kauppinen
LIST OF ABBREVIATIONS

AGE Acute gastroenteritis
ATCC American type culture collection
PBS Phosphate buffered saline
cDNA Complementary deoxyribonucleic acid
CFU Colony forming unit
Ct Cycle threshold
DEUF Dead-end ultrafiltration
DMEM Dulbecco’s modified eagle medium
dNA Deoxyribonucleic acid
EAC External amplification control
FIB Faecal indicator bacteria
GI, GII Genogroup I, genogroup II
HAV Hepatitis A virus
HEV Hepatitis E virus
ISO International Organization for Standardization
LOD Limit of detection
MPN Most probable number
MWCO Molecular weight cut-off
ORF Open reading frame
OWTS Onsite wastewater treatment system
PAA Peracetic acid
PCR Polymerase chain reaction
PEG Polyethylene glycol
PFU Plaque forming unit
PMA Propidium monoazide
RNA Ribonucleic acid
QMRA Quantitative microbial risk assessment
qPCR Quantitative polymerase chain reaction
RMSE Root mean sum of the squared errors
RT-qPCR Reverse transcription quantitative polymerase chain reaction
SF Sand filter
T90 The time to reduce 90%
TFL Time required to reduce the first log_{10}
USEPA United States Environmental Protection Agency
WHO World Health Organization
WSP Water safety plan
WWTP Wastewater treatment plant
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman Numerals I–V.


The above publications have been included at the end of this thesis with their copyright holders’ permission.
AUTHOR'S CONTRIBUTION

I) The author planned the experiments together with his colleagues. The author was responsible for the virus analyses of the water samples and participated in the laboratory analyses. The author performed the data analyses, interpreted the results and wrote the first draft of the paper. All authors were involved in the preparation and review of the manuscript and approved the final version.

II) The author participated in the national outbreak evaluation panel and the design of the study. The author was responsible for performing the data analyses and participated in the virus analyses of the water samples. He drafted the manuscript together with Ruska Rimhanen-Finne. All authors were involved in the preparation and review of the manuscript and approved the final version.

III) The author participated in the planning of the experiments together with his colleagues. The author was responsible for the enteric virus analyses and participated in laboratory analyses. The author performed the data analyses, interpreted the results and wrote the first draft of the paper. All authors were involved in the preparation and review of the manuscript and approved the final version.

IV) The author planned the experiments and participated in laboratory analyses. The author performed the data analyses, interpreted the results and wrote the paper. The co-author was involved in the preparation and review of the manuscript and approved the final version.

V) The author participated in the planning of the experiments together with his colleagues. The author was responsible for the enteric virus analyses and participated in laboratory analyses. The author performed the data analyses, interpreted the results and wrote the first draft of the paper. All authors were involved in the preparation and review of the manuscript and approved the final version.
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1 INTRODUCTION

Water is an essential component of life and poor water safety is a worldwide problem affecting mainly developing countries but also developed countries. Globally, it has been estimated that in 2010, 1.8 billion people used unsafe water and an additional 1.2 billion used water from sources or systems with significant sanitary risk (Onda et al., 2012). Moreover, in 2015 approximately 2.4 billion people still lacked adequate sanitation and this figure has seen little improvement during the past 13 years (UNICEF & WHO, 2004; UNICEF & WHO, 2015). The combination of unsafe drinking water, inadequate sanitation and poor hygiene is responsible for a vast majority of diarrhoeal disease in the world (WHO, 2002b), with an estimated annual burden of 0.7 million deaths (Walker et al., 2013). This makes diarrhea the fourth leading cause of death among children under five years of age (UN, 2015).

In developed countries, deaths caused by waterborne infections are rare and occur mainly among susceptible populations, such as children, the elderly and immunocompromised patients (Harris et al., 2008; van Asten et al., 2011). However, waterborne illnesses are associated with a substantial socio-economic burden. For example, in the US (with a population of 300 million individuals) it has been estimated that the cost of waterborne illnesses ranges from US$269 to $806 million in medical costs and US$40 to $107 million for absences from work (Grabow, 2007).

A drinking water outbreak may be caused by several factors, including raw water contamination, treatment deficiency and distribution network failure (Moreira & Bondelind, 2017). With recreational waters, the discharge of wastewater, surface run-offs and beach users themselves are among the most important causes of outbreaks (Sanborn & Takaro, 2013).

Finland is famous for its many thousands of lakes, which serve not only as a source of drinking water and/or recreational water, but also as a location for the disposal of wastewater effluents. In developed countries, poor water quality is often linked with contaminated surface water used for irrigation or for recreational purposes (Sinclair et al., 2009; Hlavsa et al., 2014; Kokkinos et al., 2017), since the drinking water produced from surface water is usually efficiently treated (Zacheus & Miettinen, 2011). In addition to surface water discharge, wastewater may end up in the groundwater, e.g. from wastewater pipe breakages and onsite wastewater treatment systems (OWTSS) based on soil infiltration (Scandura & Sobsey, 1997; Moreira & Bondelind, 2017). In Finland, most waterborne outbreaks are associated with small groundwater supplies and private wells located in rural areas (Zacheus & Miettinen, 2011; Klove et al., 2017).

Many pathogens can be transmitted through water. The most important waterborne pathogens include enteric viruses, such as noroviruses, rotaviruses, sapoviruses, hepatitis A-viruses and adenoviruses (Schwab, 2007). These viruses
are shed via the stools of infected person and transmitted through a faecal-oral route. In addition to enteric viruses, pathogenic bacteria and protozoan, such as campylobacteria and giardia, may be transmitted through water and have caused waterborne outbreaks also in Finland (Rimhanen-Finne et al., 2010; Zacheus & Miettinen, 2011). The microbiological quality of water and the possible presence of pathogens has been traditionally estimated by using faecal indicator bacteria (FIB), such as Escherichia coli, intestinal enterococci and Clostridium perfringens. However, the capability of these indicators to measure water quality and predict waterborne viral outbreaks has been widely questioned (Gerba et al., 1979; Payment et al., 1985; Borchardt et al., 2004; Harwood et al., 2005).

The success of enteric viruses in causing a waterborne outbreak is based on several natural properties of these viruses, which may have developed at least partly due to their environmental route of transmission. First, enteric viruses are secreted in faeces in very high numbers (Atmar et al., 2014). Second, the virus genome is protected against environmental stress and, e.g. disinfection, by a persistent protein coat (Mayer et al., 2015). Third, the small size of these viruses enables their transport through soil layers into the groundwater (Pedley et al., 2006). Finally, their low infectious dose makes them highly contagious and enables them to efficiently spread further through person-to-person transmission (Teunis et al., 2008).

To date, the importance of enteric viruses in environmental microbiology and water safety has been well addressed (Grabow, 2007). However, relatively little is known about the site-specific environmental factors that play an important role in, e.g. the development and management of a waterborne outbreak. Also, more knowledge is needed on the inputs of quantitative microbial risk assessments (QMRA) (Haas et al., 1999). Therefore, this thesis studies the occurrence, fate and control of enteric viruses in Finnish water environments. Pre-designed laboratory and pilot-scale experiments as well as investigations carried out during waterborne outbreaks were included in the study. The thesis provides new information regarding the occurrence, transport, persistence and control of enteric viruses in water environments. In addition, the suitability of commonly used indicator microbes to describe the water quality as well as the occurrence and fate of enteric viruses in water environments was tested.
2 REVIEW OF THE LITERATURE

2.1 WATERBORNE VIRUSES

Viruses are the most abundant microorganisms on Earth (Madigan et al., 2006), and they play an important role in biological processes by controlling the natural balance in an ecosystem. They are the smallest microorganisms and can multiply only within the living cell of a host organism. Human viruses capable of being transmitted through water are predominantly members of the group of enteric viruses, which include a large number of pathogenic viruses, such as noroviruses, adenoviruses, rotaviruses, enteroviruses and astroviruses (Schwab, 2007). These viruses are non-enveloped viruses consisting of a nucleic acid (either deoxyribonucleic acid, DNA, or ribonucleic acid, RNA) surrounded by a protective protein coat called a capsid.

Enteric viruses primarily infect cells of the gastrointestinal tract, and more than 150 enteric viruses can be found in human faeces (Gerba, 2008; Wong et al., 2012). In addition to gastroenteritis, illnesses caused by enteric viruses can include hepatitis, conjunctivitis, respiratory infections, encephalitis, paralysis and myocarditis (Fong & Lipp, 2005; Sinclair et al., 2009). Human enteric viruses are highly host specific, and thus far evidence suggests that only the hepatitis E virus infects both humans and certain animals (Khuroo et al., 2016). The host specificity of a virus is due to specific attachment sites, receptors, on the surface of the host cells recognised by the virus.

In addition to enteric viruses, many respiratory viruses are excreted in the faeces and/or in the urine. In particular, respiratory adenoviruses have been shown to be transmitted via recreational waters, suggesting that other than enteric viruses might also be transmitted through water (Mena & Gerba, 2009; Sinclair et al., 2009). More recently, severe disease outbreaks caused by enveloped viruses, such as severe acute respiratory syndrome (SARS) and avian influenza H5N1, have raised concerns about their potential spread and transmission through water environments (Wigginton et al., 2015; Ye et al., 2016).

2.1.1 Adenovirus

Adenoviruses were first described by Rowe et al. (1953) and were named according to their disease presentation (adenoid degeneration, adenoid-pharyngeal conjunctival and acute respiratory disease). However, the first illnesses associated with adenoviruses may have been documented as early as 1926 (Enriquez, 2002). Human adenoviruses are associated with several distinct clinical illnesses involving almost every organ system in the human body. Typical illnesses caused by
adenoviruses include respiratory illnesses, conjunctivitis, cystitis and gastroenteritis (Enriquez, 2002; Mena & Gerba, 2009).

Enteric adenoviruses (40–41) are second only to rotavirus as a leading causative agent of gastroenteritis in infants and young children worldwide (Mena & Gerba, 2009). Asymptomatic infections are also common and healthy people can shed viruses (Wadell, 1984). By the age of two, 50% of children have acquired neutralising antibodies to enteric adenovirus 40 and 41 (Shinozaki et al., 1987). The role of adenoviruses in childhood infections is underestimated because of the high number of asymptomatic infections (Butler et al., 1992).

Human adenoviruses belong to the *Adenoviridae* family. Currently, there are over 60 human adenovirus types divided into seven species (A-G) (Lion, 2014). Human adenoviruses are non-enveloped and approximately 70–100 nm in diameter, consisting of icosahedral nucleocapsid, which contains a linear double-stranded DNA (dsDNA) 26–45 kb in size (Enriquez, 2002).

Because all adenoviruses are excreted in faeces, in theory, contaminated water can spread all types of adenoviruses through ingestion, inhalation or by direct contact with the eyes (Mena & Gerba, 2009). The most common water related illnesses caused by adenoviruses are gastroenteritis, eye infections and pharyngoconjunctival fever (Mena & Gerba, 2009). The US Environmental Protection Agency (USEPA) has included adenoviruses on its list of Drinking Water Candidate Contaminants, which is a list of contaminants that are known or anticipated to occur in public water systems (USEPA, 2016).

Adenoviruses are common causative agents of recreational water outbreaks, including swimming pools (Sinclair et al., 2009; Mena & Gerba, 2009), but they have been associated with only a few drinking water outbreaks (Kukkula et al., 1997; Divizia et al., 2004; Maunula et al., 2009a). Unlike, e.g. noroviruses and rotaviruses, the numbers of adenoviruses show no seasonal variation and they are among the most abundant pathogenic viruses in wastewater (Pina et al., 1998; Bofill-Mas et al., 2006; Katayama et al., 2008; Schlindwein et al., 2010). Adenoviruses also show good thermal stability and they can survive for long periods of time in water environments (Enriquez et al., 1995). In addition, adenovirus 40 is the most UV-resistant waterborne pathogen known (Hijnen et al., 2006). The common occurrence of adenoviruses in wastewater combined with their inherent persistence make adenoviruses very suitable indicators of human sewage pollution.

2.1.2 Norovirus

Human norovirus, previously known as Norwalk virus, is an enteric RNA virus of the family *Caliciviridae* and causes acute gastroenteritis (AGE). Illness associated with the norovirus was described as early as 1929, termed as ‘winter vomiting disease’ due to its seasonal variation (Zahorsky, 1929). However, it was only in the late 1960s that the norovirus was detected as the first viral agent shown to cause...
gastroenteritis during a waterborne outbreak in Norwalk, USA (Kapikian et al., 1972; Kapikian, 2000). Since then, noroviruses have been associated with water and foodborne outbreaks as well as person-to-person outbreaks. Currently, noroviruses are recognised as the most common causative agent of gastroenteritis throughout the world (Koo et al., 2010; CDC, 2011; Ahmed et al., 2014; Belliot et al., 2014). It is estimated that each year, noroviruses are responsible for 64 000 diarrheal episodes requiring hospitalisation, 900 000 clinic visits among children in industrialised countries and up to 200 000 deaths of children over five years of age in developing countries (Patel et al., 2008).

Human noroviruses consist of a non-enveloped icosahedral nucleocapsid approximately 27 to 30 nm in diameter and the viral RNA genome (Green, 2007). The genome of the human norovirus is a linear, positive-sense, single-stranded RNA (ssRNA) approximately 7.5 kb in length (Xi et al., 1990). The genome is organised into three open reading frames (ORFs) that encode several structural and nonstructural proteins (Thorne & Goodfellow, 2014). The classification is most commonly based on the sequence of ORF2, which encodes the major structural capsid protein (VP1) and/or ORF1, which encodes the RNA-dependent RNA polymerase (Vinjé et al., 2004). Currently, noroviruses are classified into six genogroups (GI-GVI); noroviruses in three of the genogroups, GI, GII and GIV, can infect humans (Robilotti et al., 2015). The genogroups are further subdivided into genetic clusters called genotypes, including numerous subgroups. The nomenclature contains information about the genogroup, genotype and subgroup or variant, e.g. the human norovirus GII.4 New Orleans_2009 (GII = genogroup, 4 = genotype and New Orleans_2009 = subgroup or variant) (Kroneman et al., 2013).

The GII.4 genotype is the most prevalent genotype and new variants have emerged every two to three years in recent decades, apparently driven by the selective pressure exerted by the human immune system (Lindesmith et al., 2012; Eden et al., 2013). Although GII.4 variants predominate overall, GI and other GII genotypes than GII.4 may play a more important role in outbreaks that involve food- or waterborne transmission (Lysen et al., 2009; CDC, 2011; Perez-Sautu et al., 2012; Vega et al., 2014). In addition to human noroviruses, noroviruses have also been isolated from other species, such as pigs (GII), cattle and sheep (GIII), mice (GV) and dogs and cats (GVI) (Green, 2007; Martella et al., 2008; Pinto et al., 2012).

Human noroviruses cause AGE in persons of all age groups (Rocks et al., 2002). The incubation period typically varies between 0.5 and 2 days, with a median of 1.2 days (CDC, 2011; Lee et al., 2013). The symptoms include watery diarrhea, vomiting, nausea and abdominal pain. Other symptoms, such as headache, anorexia, malaise and fever, have also been reported with norovirus infection. Asymptomatic infections are also common, especially in children. They have been shown to occur in approximately one third of infected persons in a previous human volunteer study (Graham et al., 1994), and to range between 1% and nearly 50% in excretion studies of asymptomatic individuals (Robilotti et al., 2015). Noroviruses
can be detected in stool for an average of four weeks following infection; however, it is unclear how long the detection of a virus after illness indicates a risk of transmission, given the lack of an infectivity test for noroviruses (Atmar et al., 2008; CDC, 2011).

Although clinical symptoms may be severe, they generally resolve without treatment within 1–3 days. However, more prolonged courses of illness lasting 4–6 days may occur, particularly among young children, elderly persons and immunocompromised persons (Rockx et al., 2002; Lopman et al., 2004). Norovirus-associated deaths have been reported among elderly persons and in long-term care facilities (Harris et al., 2008; van Asten et al., 2011). The clinical picture may also be genotype dependent, e.g. the most prevalent norovirus genotype, GI.4, has been observed to cause more severe gastroenteritis (Huhti et al., 2011). There is no cure for the norovirus infection, but fluid therapy can be used for the treatment of dehydration and an imbalance in bodily salts. The potential benefits of the development of an effective norovirus vaccine are supported by both public health and economic arguments (Robilotti et al., 2015; Cortes-Penfield et al., 2017).

2.1.3 Sapovirus

Sapoviruses were first discovered in diarrheal stool samples via electron microscopy in 1976 in UK (Madeley & Cosgrove, 1976), and the prototype strain was then identified in an outbreak of diarrhea in Sapporo, Japan, in 1977 (Chiba et al., 1979; Chiba et al., 2000). Sapoviruses belong to the same family, *Caliciviridae*, as noroviruses and are an etiologic agent for AGE in humans and animals. Even though the number of sapovirus infections are less than that of norovirus infections (Blanton et al., 2006; Bucardo et al., 2014; Chhabra et al., 2014; Iritani et al., 2014; Wu et al., 2014), an increasing prevalence of sapovirus infections related to both outbreaks and sporadic cases has been described, highlighting the emerging role of sapoviruses as a public health concern (Pang et al., 2009; Svraka et al., 2010; Räsänen et al., 2010; Dey et al., 2012; Harada et al., 2012; Lee et al., 2012b; Bucardo et al., 2014; Nidaira et al., 2014; Wang et al., 2014; Jalava et al., 2014; Franck et al., 2015).

Sapoviruses were initially distinguished from noroviruses by their ‘Star of David’ morphological appearance when viewed with an electron microscope (Caul & Appleton, 1982). Sapoviruses are small (about 30–38 nm in diameter), non-enveloped icosahedral particles (Oka et al., 2015). They have a positive-sense, ssRNA genome, which is approximately 7.1 to 7.7 kb in size and is organised into two ORFs and a predicted third ORF (Oka et al., 2015). Sapoviruses are divided into at least five genogroups (GI-GV) on the basis of their capsid gene sequences (Farkas et al., 2004). Three genogroups, GI, GII, and GIV, have been detected in humans, while GV strains have been detected in humans and animals, and GIII strains have been detected in swine (Oka et al., 2015). Each genogroup is subdivided into
genotypes. Currently, human sapoviruses are subdivided into seven genotypes in
genogroups GI and GII (GI.1 to GI.7 and GII.1 to GII.7), one in genogroup GIV
(GIV.1) and two in genogroup GV (GV.1 and GV.2). Genogroup GV also includes
sapoviruses detected in pigs (GV.3) and sea lions (GV.4) (Oka et al., 2015). Recently,
nine additional sapovirus genogroups (GVI–GXIV) were proposed to exist (Scheuer
et al., 2013).

Human sapoviruses cause AGE in all age groups in both sporadic cases and
outbreaks worldwide (Oka et al., 2015). The clinical symptoms of sapovirus
gastroenteritis are indistinguishable from those caused by noroviruses (Oka et al.,
2015). However, a recent study suggested that diarrhea is the most frequent
symptom in sapovirus outbreaks, whereas in norovirus outbreaks the most
prevalent symptoms include vomiting and fever (Sala et al., 2014). In general, the
clinical severity of sapovirus-associated AGE is milder than that for norovirus and
rotavirus (Oka et al., 2015). Even though the symptoms are self-limiting, sapovirus
AGE may lead to hospitalisation, especially among immunocompromised persons,
small children or elderly people (Medici et al., 2012; Lee et al., 2012b; Sala et al.,
2014). Sapoviruses are common in wastewater (Hata et al., 2013; Fioretti et al.,
2016), and due to the availability of improved methodologies, these viruses are also
now being analysed and detected more often. In the future, the significance of this
emerging virus may increase in waterborne outbreaks.

2.1.4 Rotavirus

Rotaviruses were first identified in the 1970s in children suffering from severe
diarrhea (Bishop et al., 1973). Rotaviruses belong to the Reoviridae family and
contain a uniquely segmented, double-stranded RNA (dsRNA) genome (Estes &
Kapikian, 2007). Since their discovery, rotaviruses have been recognised as one of
the most important causative agents of AGE in children. Serological studies have
shown that at least 95% of children became seropositive for the rotavirus by the age
of five (Velazquez et al., 1996; Glass et al., 1996). Although reinfection may occur
again later in life, most clinically relevant cases involve children under five years of
age (Estes & Kapikian, 2007). Rotaviruses cause AGE all over the world; however,
the consequences are more severe in developing countries.

In 2008, before rotavirus vaccine was available, it was estimated that rotaviruses
caus ed approximately 450 000 deaths annually in children under five years of age,
mostly in developing countries (Tate et al., 2012; WHO, 2013). Currently, two live
attenuated vaccines, administered orally, are available for the rotavirus. In Finland,
the vaccine was included in the national vaccination programme in 2009, and this
has decreased the prevalence of rotavirus infections dramatically in the Finnish
population (Hemming-Harlo et al., 2016; Leino et al., 2017). The efficacy of both
vaccines has also been demonstrated in other countries (Ruiz-Palacios et al., 2006;
Vesikari et al., 2006). Despite the significant reduction in infections due to
vaccinations, a recent study estimates that rotaviruses are still associated with approximately 215,000 deaths of children up to 5 years old annually (Tate et al., 2016). In countries where the vaccination has not been implemented, rotaviruses continue to be a leading cause of severe AGE and childhood hospitalisation (Podkolzin et al., 2009; Liu et al., 2012; Walker et al., 2013; Kotloff et al., 2013).

Rotaviruses have a non-enveloped, triple-layered, icosahedral virus capsid that is approximately 75 nm in diameter (Estes & Kapikian, 2007). The rotavirus dsRNA genome is approximately 18.5 kb in size and consists of eleven segments, which encode six structural and six non-structural proteins (Estes & Kapikian, 2007). Rotaviruses are currently classified into nine different groups, A-I: groups A, B and C are known to cause disease in humans (Estes & Kapikian, 2007; ICTV, 2017). Group A rotaviruses are the most important cause of AGE and are responsible for more than 90% of all rotavirus AGE in humans (Estes & Kapikian, 2007; Tate et al., 2012). Group A rotaviruses are divided into genotypes based on genes encoding the outer capsid proteins, indicated as G- and P-types. Currently, 27 G-types and 37 P-types have been described (Matthijnssens et al., 2011; Trojnar et al., 2013). However, only a small number of genotype combinations, such as G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8], are responsible for the majority of infections in humans (Santos & Hoshino, 2005; Matthijnssens et al., 2011; Iturriza-Gomara et al., 2011).

Rotavirus is one of the most infectious pathogens, with an infectious dose being as low as about 10 virions (Ward et al., 1986). The incubation period for rotavirus is typically from one to four days, with a median of two days (Lee et al., 2013). The symptoms of rotavirus AGE consist of the acute onset of watery diarrhea, vomiting, fever, abdominal discomfort and dehydration (WHO, 2002a). Symptoms show high variation in duration, but usually last for 3 to 5 days (Estes & Kapikian, 2007). Rotaviruses can cause a wide spectrum of symptom severity, albeit infections are usually more severe compared to other common causes of AGE and can lead more often to dehydration and hospitalisation (WHO, 2002a). Asymptomatic infections are also common, especially among infants less than six months of age, who seem to be protected by maternal antibodies (Velazquez et al., 1996; Estes & Kapikian, 2007).

The association of rotaviruses with waterborne outbreaks has been well documented (Villena et al., 2003; Gallay et al., 2006; Martinelli et al., 2007; Maunula et al., 2009a; Koroglu et al., 2011; Mellou et al., 2014).

2.1.5 Enterovirus

Enteroviruses were first discovered by Landsteiner and Popper in 1909 when the poliovirus was identified after inoculating monkeys with specimens from cases of paralytic poliomyelitis (Landsteiner & Popper, 1909). Enteroviruses are members of the Picornaviridae family and cause various diseases, such as conjunctivitis, respiratory infections, hand-foot-and-mouth disease, myocarditis, diabetes, aseptic
meningitis, encephalitis, paralysis and gastroenteritis (Betancourt & Shulman, 2016). Currently, over 100 types of enteroviruses have been isolated from humans and they are divided into four species (EV-A to EV-D), including polioviruses and non-polio enteroviruses, i.e. coxsackieviruses, echoviruses and enteroviruses (Pallansch et al., 2013). Most enterovirus infections are asymptomatic or result in only mild illness, mainly in infants, but adults can also be affected (Kogon et al., 1969; Racaniello, 2013). It is estimated that enteroviruses are responsible for 30 million to 50 million infections per year in the United States, with 30 000 to 50 000 of these resulting in meningitis hospitalisations (Oberste et al., 1999).

Enteroviruses are icosahedral, non-enveloped and 27 nm in diameter. They have a positive-sense ssRNA genome that is 7.5 kb in size and contains single ORF encoding four structural proteins (VP1-VP4) and seven nonstructural proteins implicated in viral replication and maturation (Nasri et al., 2007).

Human enteroviruses are among the most commonly detected viruses in polluted waters (Grabow, 2007). They were also among the first enteric viruses that could be analysed from water samples. Partly as a consequence of historical concern for poliovirus and the availability of cell culture methods, enteroviruses have been proposed and used as a water quality indicator of human faecal pollution in environmental waters (Boehm et al., 2003; Fong et al., 2005; Wong et al., 2012). However, reports describing the link between water and enterovirus infections are limited, with infections mainly having occurred as a result of recreational waterborne outbreaks (Begier et al., 2008; Sinclair et al., 2009; Maunula et al., 2009a).

### 2.1.6 Hepatitis A virus

Hepatitis A virus (HAV) infection is an ancient disease, but was first identified in the faeces of an individual with acute hepatitis as late as 1973 (Feinstone et al., 1973; Cuthbert, 2001). Currently, HAV is the most common agent causing acute liver disease worldwide, and it has been estimated that it is responsible for approximately 1.5 million clinical cases every year (WHO, 2000; Vaughan et al., 2014). The incidence of HAV varies globally and is highly dependent on the quality of sanitation and drinking water (WHO, 2010a). The severity of the disease is strongly associated with age. Adults and older children often exhibit symptoms, whereas infections among young children are usually asymptomatic or mildly symptomatic (Willner et al., 1998; O'Grady, 2000).

HAV is a member of the *Picornaviridae* family. It is a small (27 nm), spherical, non-enveloped, positive-sense ssRNA virus consisting of a 7.5 kb genome coding for a single ORF (Feinstone et al., 1973; Najarian et al., 1985). HAV has been classified into four human (I, II, III and VII) and three simian (IV, V and VI) genotypes (Robertson et al., 1992; Costa-Mattioli et al., 2003). The genotypes are
further divided into several subtypes (Vaughan et al., 2014). Genotype I, with subtype IA is the most prevalent worldwide (Vaughan et al., 2014).

The incubation period of HAV has been seen to vary from 15 to 50 days, with an average of 28 days (Craig & Schaffner, 2004; CDC, 2005). HAV replicates within the liver and is excreted in the bile and shed in stool. Symptoms may include fever, headache, malaise and non-specific gastrointestinal symptoms, followed by jaundice (Lemon, 1985). Symptoms usually last less than two months, although some people may be ill for up to six months (Glikson et al., 1992).

Even though poor water quality is linked to increased HAV prevalence (WHO, 2010a), only limited number of HAV-related waterborne outbreaks have been described worldwide (Bloch et al., 1990; Mahoney et al., 1992; De Serres et al., 1999; Kumar et al., 2016; Shin et al., 2017).

2.1.7 Hepatitis E virus

Hepatitis E virus (HEV) was discovered as the causative agent for a massive waterborne outbreak of jaundice that occurred in Kashmir, India, in November 1978, and was classified as an ‘epidemic non-A, non-B hepatitis’ (Khuroo, 1980). HEV transmits enterically and causes acute liver inflammation in humans, predominantly in developing countries, where the outbreaks are usually associated with the faecal contamination of drinking water (Corwin et al., 1996; Emerson & Purcell, 2003; Guerrero-Latorre et al., 2011; Khuroo & Khuroo, 2016; Khuroo et al., 2016; Kaur et al., 2017).

The global burden of HEV in developing countries was estimated in 2005 to account for approximately 20 million cases of incident HEV infections, resulting in an estimated 3.4 million cases of symptomatic illness, 70,000 deaths and 3,000 stillbirths (Rein et al., 2012). In developed countries, serological studies have shown seropositivity among a small percentage (1.1%–1.4%) of persons, indicating that only sporadic hepatitis E cases occur (Zaaijer et al., 1993; Mast et al., 1997). However, HEV is assumed to be frequently under-reported as a cause of infection, and increased numbers of HEV infections have been reported recently also in developed countries (Pischke et al., 2014; Khuroo & Khuroo, 2016).

HEV is a spherical, non-enveloped virus of the Hepeviridae family, approximately 27–34 nm in diameter (Smith et al., 2014; Khuroo et al., 2016). HEV has a positive-sense ssRNA genome approximately 7.2 kb in length and it contains three discontinuous, partially overlapped ORFs (Tam et al., 1991). HEV has five genotypes (1–4 and 7), all of which can infect humans, but genotypes 3, 4 and 7 can also infect several animals (Khuroo et al., 2016). HEV is a zoonotic disease, and it has been isolated in a number of animals, including domestic pigs, wild boars, Sicca deer, moose, rabbit, dromedaries, chickens, bats, ferrets, mink, rats, mongooses, and cutthroat trout (Pavio et al., 2010; Thiry et al., 2017). HEV causes a disease that
is indistinguishable from the symptoms associated with HAV infections (Hollinger & Emerson, 2007; Emerson & Purcel, 2007).

2.1.8 Astrovirus

Astroviruses were discovered in 1975 in the stools of children with diarrhea (Appleton & Higgins, 1975; Madeley & Cosgrove, 1975) and named based on their characteristic star-like shape when viewed under an electron microscope. Astroviruses belong to the Astroviridae family (Monroe et al., 1993), and together with the Picornaviridae and the Caliciviridae families, comprise a third family of non-enveloped viruses whose genome is composed of linear, positive-sense ssRNA. The Astroviridae family shows a high diversity and zoonotic potential, and astroviruses have been found in the faeces of numerous mammalian and avian species (Bosch et al., 2014). Currently, astroviruses are one of the most important causes of pediatric AGE, after rotaviruses and caliciviruses (Bosch et al., 2014).

Astroviruses are non-enveloped, icosahedral virions that are 28 to 41 nm in diameter (Appleton & Higgins, 1975; Risco et al., 1995). The ssRNA genome of astroviruses is approximately 6.8 kb in length and organised into three ORFs that encode several structural and nonstructural proteins (Bosch et al., 2014). Astroviruses were initially classified into two genera based on their hosts of origin, Mamastrovirus and Avastrovirus, infecting mammalian and avian species, respectively (Bosch et al., 2014). Recent studies based on viral metagenomic analysis have described many new astroviruses infecting different species, including humans (Finkbeiner et al., 2008a; Finkbeiner et al., 2008b; Kapoor et al., 2009; Finkbeiner et al., 2009a; Finkbeiner et al., 2009b). Currently, three divergent groups of human astroviruses (HAstV) are recognised: the classic group, the HAstV-MLB group, and the HAstV-VA/HMO group. Classic HAstVs contain eight serotypes and account for 2 to 9% of all acute nonbacterial gastroenteritis in children worldwide (Bosch et al., 2014).

Human astroviruses primarily infect children worldwide, with very few reported disease cases in normal healthy adults (Belliot et al., 1997; Pager & Steele, 2002; Hwang et al., 2015). Serological studies indicate that most children are infected with astroviruses and develop antibodies to the virus early in life, which are thought to provide protective immunity against future infections (Kriston et al., 1996; Koopmans et al., 1998). In addition to children, immunocompromised persons and the elderly represent high-risk groups.

Human astroviruses cause typical gastrointestinal symptoms: mild, watery diarrhea that lasts for 2 to 3 days, associated with vomiting, fever, anorexia and abdominal pain (Bosch et al., 2014). However, vomiting is less prevalent and the diarrhea is milder in astrovirus infections than in rotavirus or calicivirus(es) infections. Moreover, astroviruses have a longer incubation period (median 4.5 days) compared to rotavirus and calicivirus(es) (Lee et al., 2013). Astrovirus
infections can also be asymptomatic (Kurtz et al., 1979; Maldonado et al., 1998; Mendez-Toss et al., 2004).

Human astroviruses have been associated with several foodborne as well as institutional outbreaks (Oishi et al., 1994; Mitchell et al., 1995; Abad et al., 2001; Gallimore et al., 2005), but the number of reports associating astroviruses with waterborne outbreaks is limited (Maunula et al., 2004; Maunula et al., 2009a; Sezen et al., 2015).

2.1.9 Other potential waterborne viruses

Severe disease outbreaks caused by enveloped viruses, such as Ebola, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS) and avian influenza H5N1, have raised concerns about their potential spread and transmission through water environments (Ye et al., 2016). In a large virus pandemic scenario, wastewater and drinking water treatment utilities would possibly be challenged, with these viruses posing potential occupational and public health risks. The main transmission routes of these viruses are direct person-to-person contact or indirect contact with contaminated objects (Couch et al., 1966; Bausch et al., 2007). Often, these enveloped viruses are presumed to exist in low numbers in human faeces and excreted in nonviable form or else they undergo rapid inactivation in water environments. However, these assumptions are not always evidence based (Ye et al., 2016).

Previous studies have demonstrated the occurrence of coronaviruses and avian influenza viruses in the faeces of infected individuals (Metcalf et al., 1995; Leung et al., 2003; Poon et al., 2004; Chan et al., 2004; de Jong et al., 2005; To et al., 2010; Esper et al., 2010; Arena et al., 2012; Jevsnik et al., 2013). In addition, it has been suggested that these viruses survive long enough (for several days to several weeks) to be of concern for wastewater treatment facilities, during stormwater overflow events and in cases of wastewater intrusion in drinking water (Casanova et al., 2009; Gundy et al., 2009; Wigginton et al., 2015; Ye et al., 2016). As evidence of this finding, a SARS outbreak in a housing complex in Hong Kong in 2003 was attributed to the transport of viruses in wastewater to the air shaft (Yu et al., 2004). However, more knowledge is needed about the potential role of a water environment in the spread of enveloped viruses to recognise and prepare for potential future risks of a deadly viral pandemic (Wigginton et al., 2015).

2.2 INDICATORS OF WATERBORNE ENTERIC VIRUSES

Indicator microbes have been applied for tracking the presence and sources of faecal pollution and to assess efficacy of microbial removal and disinfection treatments. Ideally, a good indicator microbe of waterborne enteric viruses should fulfil the following criteria, as stated by Bosch (1998): (I) it should be associated
with the source of the pathogen and should be absent in unpolluted areas, (II) it should occur in greater numbers than the pathogen, (III) it should not multiply out of the host, (IV) it should be at least equally resistant to natural and artificial inactivation as the viral pathogen, (V) it should be detectable by means of easy, rapid and inexpensive procedures, and (VI) it should not be pathogenic.

Traditionally, the microbiological quality of water has been estimated by using faecal indicator bacteria (FIB), such as *Escherichia coli*, intestinal enterococci and *Clostridium perfringens* (Table 1). These bacteria are part of the normal flora in the intestinal tract of humans and other vertebrates, and thus they are consistently present in wastewater. Their occurrence in the environment indicates that faecal pathogens may also be present. However, the capability of these conventional FIB to measure water quality and predict waterborne viral outbreaks has been widely questioned for at least two reasons. First, there is often a lack of correlation between the occurrence of FIB and viruses in water samples (Gerba et al., 1979; Borchardt et al., 2004; Harwood et al., 2005), and second, viruses are more resistant to environmental stress and disinfection processes than FIB (Payment et al., 1985). To overcome these shortcomings in the use of traditional FIB, alternative indicators of waterborne viruses have been explored.

Bacterial viruses, especially somatic and F-specific coliphages, which infect *E. coli*, have been suggested as more suitable indicators of contamination. Phages share many features with waterborne enteric viruses, such as size, composition, structure, morphology and resistance to environmental conditions (Leclerc et al., 2000; Jofre, 2007; USEPA, 2015). Among F-specific coliphages, MS2 has been widely used as a surrogate virus to model the environmental persistence and fate of enteric viruses, particularly noroviruses. Compared to enteric virus analysis, standardised, simple and cheap methods are available for coliphages (ISO, 1995; ISO, 1998; USEPA, 2001a; USEPA, 2001b). However, there are also shortcomings in using coliphages, and especially specificity is an issue since coliphages can originate from both humans and other animals as well as outside the gut (Jofre, 2007).

Even though there has been a huge effort to find a universal indicator for pathogenic enteric viruses during the last few decades, no single good candidate exists. Thus, for human-specific faecal source tracking, abundant human enteric viruses, such as adenoviruses, polyomaviruses and enteroviruses, have been used and also suggested as potential indicators for enhanced monitoring (Boehm et al., 2009; Fujioka et al., 2015; Updyke et al., 2015). In addition, host-specific *Bacteroidales*, such as HF183, have been used for the detection of human wastewater pollution (Ahmed et al., 2016). More recently, new candidates have been proposed as indicators of enteric viruses, such as the pepper mild mottle virus, whose abundance and persistence in water environments supports its use as an indicator of faecal pollution (Rosario et al., 2009b; Hamza et al., 2011; Kuroda et al., 2015; Symonds et al., 2016). In the future, a metagenomic approach has been proposed to
serve as a superior method for comprehensively evaluating the microbiological quality of water (Nieuwenhuijse & Koopmans, 2017).

Table 1. Microbiological parametric values for management actions according to Finnish legislation (CFU = colony forming unit, MPN = most probable number)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>E. coli (CFU/MPN/100 ml)</th>
<th>Intestinal enterococci (CFU/MPN/100 ml)</th>
<th>Coliform bacteria (CFU/100 ml)</th>
<th>C. perfringens* (CFU/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drinking water</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inland bathing water</td>
<td>1000</td>
<td>400</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coastal bathing water</td>
<td>500</td>
<td>200</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*If the water originates from or is influenced by surface water.

2.3 ROUTES OF ENTERIC VIRUS TRANSMISSION

The faecal-oral route is the most important route of transmission among enteric viruses (Fong & Lipp, 2005). Enteric viruses are excreted in high numbers in faeces (Table 2), and they must reach the gastrointestinal tract to reproduce, or uptake in the case of hepatitis A and E viruses. The faecal-oral route may occur directly from person to person or via contaminated vehicles, such as food, water or surfaces. The number of viral particles may also be high in fomites, up to 12 million genome copies (GC)/mL, (Atmar et al., 2014), and, e.g. noroviruses and rotavirus have been shown to spread by droplets (Estes & Kapikian, 2007; Bonifait et al., 2015). Airborne transmission is possible by ingesting aerosols generated by vomiting or, e.g. flushing the toilet (Lopman, 2011; CDC, 2011; Bonifait et al., 2015). The potential risk of occupational airborne transmission of enteric viruses in wastewater treatment plants (WWTPs) has also been addressed (De Serres & Laliberté, 1997; Masclaux et al., 2014; Carducci et al., 2016).

A low infectious dose is typical of enteric viruses, e.g. for rotaviruses and noroviruses it has been estimated that as few as 10 and 18 viral particles, respectively, may result in infection (Ward et al., 1986; Teunis et al., 2008). Considering the high numbers of viruses excreted into the faeces and the low infectious dose, the spread of these viruses is efficient and difficult to control. Water should be considered as an important vehicle for the spread of enteric viruses, and outbreaks related to water, including drinking water and recreational water, are being detected worldwide.
Table 2. The numbers of enteric viruses in the stools of an infected person and in wastewater influent (GC = genome copies, PFU = plaque forming units)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Stool</th>
<th>Reference</th>
<th>Wastewater influent</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus</td>
<td>≤10^{12} GC/g</td>
<td>Atmar et al., 2014</td>
<td>≤10^{8} GC/L</td>
<td>da Silva et al., 2007; Nordgren et al., 2009; Hewitt et al., 2011</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>≤10^{11} GC/g</td>
<td>Oka et al., 2015</td>
<td>≤10^{7} GC/L</td>
<td>Hata et al., 2013; Fioretti et al., 2016</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>≤10^{10} GC/g</td>
<td>Zhang et al., 2006</td>
<td>≤10^{7} GC/L</td>
<td>Aw &amp; Gin, 2010; Hata et al., 2013; Victoria et al., 2014</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>≤10^{12} PFU/g</td>
<td>WHO, 2002a</td>
<td>≤10^{7} GC/L ≤10^{7} PFU/L</td>
<td>Kitajima et al., 2014; Victoria et al., 2014; Lodder &amp; de Roda Husman, 2005</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>≤10^{11} PFU/g</td>
<td>Allard &amp; Vantarakis, 2017</td>
<td>≤10^{9} GC/L</td>
<td>La Rosa et al., 2010; Kitajima et al., 2014</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>≤10^{9} PFU/g</td>
<td>Hollinger &amp; Emerson, 2007</td>
<td>≤10^{7} GC/L</td>
<td>Villar et al., 2007; Ouardani et al., 2016</td>
</tr>
<tr>
<td>Hepatitis E</td>
<td>≤10^{8} GC/mL</td>
<td>Takahashi et al., 2007</td>
<td>≤10^{8} GC/L</td>
<td>Masciaux et al., 2013; Miura et al., 2016</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>≤10^{7} PFU/g</td>
<td>Betancourt &amp; Shulman, 2016</td>
<td>≤10^{6} GC/L ≤10^{3} PFU/L</td>
<td>Hewitt et al., 2011; Kitajima et al., 2014; Lodder &amp; de Roda Husman, 2005; Hewitt et al., 2011</td>
</tr>
</tbody>
</table>

### 2.4 ENTERIC VIRUSES IN WATER ENVIRONMENTS

#### 2.4.1 Sources of enteric viruses

Enteric viruses can end up in the environment from different sources. Due to their host specificity, the common feature of these sources is that they all contain human wastewater or stool. HEV constitutes an exception, showing also zoonotic transmission (Khuroo et al., 2016). Enteric viruses are commonly excreted in high numbers from both symptomatic and asymptomatic individuals during the infection and for a variable time after recovery from the illness (Table 2). For example, norovirus numbers in faeces peak within a few days following inoculation, decline slowly for several weeks and might be detectable for more than two months (Atmar et al., 2008; Atmar et al., 2014; Teunis et al., 2015). Fomites may also spread viruses in the environment, though the numbers are lower (a median of 41 000 GC/mL for norovirus) compared to stool (Atmar et al., 2014). The foremost source of enteric viruses is municipal WWTPs and their effluents, which are constantly discharged into the environment, usually into surface waters. The high numbers of enteric viruses in effluents have been widely reported in the literature.
In addition to communal WWTPs, decentralised OWTSs also spread viruses into the environment. Other sources of enteric viruses include leakages in sewerage systems, surface run-offs and individual persons in, e.g. recreational settings (Gerba, 2000; Sanborn & Takaro, 2013; Moreira & Bondelind, 2017). It is worth mentioning that, in theory, 1 g of stool from a norovirus-infected person can contain 5 billion infective doses (CDC, 2011), which accounts for the potential infection of the whole population of the Earth. Therefore, human faeces originating from decentralised systems or individual persons may also be considered as significant sources.

2.4.2 Occurrence of enteric viruses

The occurrence and numbers of enteric viruses in water environments is highly dependent on the epidemic situation of the population in the specific geographical area. In municipal settings, enteric viruses usually end up in WWTPs, and raw wastewater generally contains high numbers of these viruses (Table 2). Despite wastewater treatment, the effluent may still contain high numbers of enteric viruses (Nordgren et al., 2009; Hewitt et al., 2011; Hata et al., 2013; Kitajima et al., 2014; Victoria et al., 2014). Typically, the effluent is discharged into a lake, river or sea near the WWTP, and the presence of enteric viruses has been proved in these water environments (Hörman et al., 2004; van Heerden et al., 2005; Sinclair et al., 2009; Hamza et al., 2009; Fongaro et al., 2012; Maunula et al., 2012; Hokajärvi et al., 2013).

In Finland, Hokajärvi et al. (2013) found that adenoviruses were present in 59% (20/34) of the wastewater effluents taken from 17 locations. In the same study, 12% (6/50) of the samples collected from the bathing waters near the wastewater discharge location were positive for adenoviruses. Similarly, Maunula et al. (2012) detected noroviruses in 30.8% of river water samples (20/65) and 40.5% of the wastewater effluents (17/45) discharged into the same river. In addition, Hörman et al. (2004) detected noroviruses (GI and GII) in 9.4% (13 of 139) of the surface water samples from seven lakes and 15 rivers in 2000 and 2001. Concerns have arisen about the infectivity risks to humans predisposed to use waters under the influence of wastewater.

Enteric viruses have also been detected in groundwater and drinking water, usually in connection with a waterborne outbreak (Anderson et al., 2003; Parshionikar et al., 2003; Maunula et al., 2005; Riera-Montes et al., 2011; Blanco et al., 2017). However, a study done in the United States found that 16% of groundwater samples and 72% of 29 groundwater sites tested positive for human enteric viruses (Fout et al., 2003). Similarly in Korea, 8.7% of 160 groundwater samples and 15% of 12 groundwater sites tested positive for human noroviruses (Lee et al., 2012a). Groundwater may receive viral contamination directly or by the
infiltration of wastewater through the protective soil layers. Contaminations may occur especially in areas where the groundwater protecting soil layers are shallow or fragile (USEPA, 2006a). Artificial groundwater recharge with reclaimed wastewater may also cause viral contamination (Asano & Cotruvo, 2004). On the other hand, drinking water originating from surface water may be contaminated due to, e.g. inefficient treatment processes or a malfunction in the waterworks (Moreira & Bondelind, 2017).

Seasonality of enteric viruses
Some of the enteric viruses show seasonality in their occurrence, meaning that they are more abundant during certain seasons. In particular, noroviruses and rotaviruses have been shown to be more abundant during the winter months in both the northern and southern hemispheres, especially in Europe, North America and Oceania (D’Souza et al., 2008; Nordgren et al., 2009; Ahmed et al., 2013; Patel et al., 2013; Eftim et al., 2017). In developing countries, such seasonal patterns are usually not evident or are undefinable. On the other hand, the occurrence of, e.g. adenoviruses, is more consistent throughout the year (Pina et al., 1998; Bofill-Mas et al., 2006; Katayama et al., 2008; Schlindwein et al., 2010). The reason for the seasonality of some viruses is not yet completely understood. Factors such as temperature, humidity, rainfall, altitude, low population immunity and the emergence of new variants have been used to explain the seasonality of enteric viruses (Lopman et al., 2009; Marshall & Bruggink, 2011; Ahmed et al., 2013; Patel et al., 2013). Moreover, factors related to human behavior, such as increased crowding indoors during winter, are also believed to explain the seasonality. The significance of crowding is supported by the high prevalence of gastroenteritis outbreaks in nursing homes, hospitals, day care centers, schools and cruise ships (Friesema et al., 2009; ECDC, 2013; Kambhampati et al., 2015; Freeland et al., 2016).

2.4.3 Waterborne outbreaks caused by enteric viruses
Water may cause outbreaks directly via contaminated drinking water (Carrique-Mas et al., 2003; Maunula et al., 2009a; Riera-Montes et al., 2011; Braeye et al., 2015) and recreational water (Sartorius et al., 2007; Yoder et al., 2008; Sinclair et al., 2009; Hlavsa et al., 2014), or indirectly through foodborne outbreaks, e.g. seafood originating from contaminated water (Le Guyader et al., 2000; Gentry et al., 2009; EFSA, 2012) or ready-to-eat vegetables or berries irrigated with contaminated water (Hjertqvist et al., 2006; Maunula et al., 2009b; Mathijis et al., 2012; Kokkinos et al., 2017). These food products usually are consumed with little or without any processing, and hence, they may act as vehicles for enteric virus transmission. The role of water in the spread of viruses is summarised in Figure 1. In this section, the focus is on waterborne outbreaks caused directly by contaminated drinking water and recreational water.
Enteric viruses are common causative agents of waterborne outbreaks. Globally, the most severe problems related to the waterborne spread of pathogenic enteric viruses are being faced by developing countries, which are experiencing high mortality and morbidity rates (WHO, 2002b; Onda et al., 2012; Walker et al., 2013). However, water contaminated with viruses may cause large community outbreaks with up to thousands of illness cases also in developed countries (Table 3). The numbers of reported illness cases are usually underestimated due to the fact that they represent the number of patients needing medical consultation and/or are based on a retrospective study. In addition to mortality and morbidity, waterborne outbreaks cause socio-economic burden. For example, in a large waterborne outbreak causing illness in more than 8 000 people in Finland in 2007 (Laine et al., 2011), the costs for the municipality and the insurance company were more than 4 million euros. In addition, the estimated costs of lost workdays due to the outbreak were 1.8–2.1 million euros (Halonen et al., 2012). Similarly, in Sweden the estimated total cost of a large waterborne norovirus outbreak (2 400 patients) was 0.87 million euros (Larsson et al., 2014).

A drinking water outbreak may be caused by several factors, including raw water contamination, treatment deficiencies and distribution network failure (Moreira & Bondelind, 2017). With recreational water, the discharge of wastewater, surface run-offs and beach users themselves are among the most important sources of contamination (Sanborn & Takaro, 2013). In addition, both drinking water and recreational water outbreaks have been associated with climatic conditions, especially with increased precipitation and heavy rainfall events (Curriero et al., 2001; Fong et al., 2007; Vantarakis et al., 2011; Mellou et al., 2014; Wallender et al., 2014; Moreira & Bondelind, 2017).

In Finland, drinking water outbreaks have been systemically documented since 1997, after the launching of the national outbreak surveillance system (Zacheus & Miettinen, 2011). Since 2012, the reporting of all suspected bathing water outbreaks has also been mandatory (Finnish Decree, 2011). Based on this data, on average from 3 to 11 waterborne outbreaks occur every year (Zacheus & Miettinen, 2011).
Usually, drinking water outbreaks are related to small communities serving less than 500 consumers or private households (Zacheus & Miettinen, 2011; Guzman-Herrador et al., 2015). However, the largest waterborne outbreaks have resulted in the sickness of several thousand people (Maunula et al., 2005; Maunula et al., 2009a; Laine et al., 2011; Zacheus & Miettinen, 2011). Currently, the norovirus is recognised as the most common viral agent in waterborne outbreaks (Zacheus & Miettinen, 2011; Guzman-Herrador et al., 2015). In addition to noroviruses, the potential spread of other enteric viruses, such as adenoviruses (Kukkula et al., 1997; Maunula et al., 2009a), sapoviruses (Räsänen et al., 2010; Jalava et al., 2014), enteroviruses (Maunula et al., 2009a), astroviruses (Maunula et al., 2004) and rotaviruses (Maunula et al., 2009a) through water have been documented in Finland. The seroprevalence of HAV and HEV is low in the Finnish population (WHO, 2010a; WHO, 2010b), and no waterborne outbreaks related to these viruses have been reported.

Most of the waterworks (about 60%) in Finland use groundwater or artificial groundwater as raw water (Guzman-Herrador et al., 2015; Klove et al., 2017). The aquifers are typically shallow consisting of unconfined sand and gravel formations and the groundwater table is commonly only 3–5 m below the soil surface (Katko et al., 2006; SYKE, 2016). The groundwater is usually of good quality and mostly meets the requirements set for household water quality (Katko et al., 2006). However, especially the small groundwater waterworks, which do not treat the water, are vulnerable to contamination. Since 1998, most of the waterborne outbreaks (over 90%) have occurred in these small plants (Zacheus & Miettinen, 2011). The vulnerability of groundwater is mainly due to the thin layer of soil protecting the groundwater and/or inadequate well construction and maintenance (Miettinen et al., 2001; Pitkänen et al., 2011). On the other hand, raw drinking water from surface water is generally well treated before distribution and outbreaks seldom occur and may be due to, e.g. treatment deficiencies or distribution network failures (Lahti & Hisivirta, 1995; Miettinen et al., 2001).

It has been estimated that swimming in wastewater-polluted bathing waters globally accounts for approximately 120 million cases of gastrointestinal disease and approximately 50 million cases of respiratory disease each year (Shuval, 2003). Viruses have caused an increasing number of waterborne outbreaks associated with recreational activities (Maunula et al., 2004; Hoebe et al., 2004; Gibson, 2014; Zlot et al., 2015), and according to a survey of 55 viral outbreaks, noroviruses were the most prevalent causative agent in 45% of all cases (Sinclair et al., 2009). Interestingly, a literature survey revealed only one reported bathing water outbreak caused by enteric viruses in Finland before 2014 (Maunula et al., 2004). However, Finland is rich in surface waters, with approximately 56 000 lakes larger than one hectare and over 21 000 km of rivers in length (SYKE, 2005). The shallowness of lakes combined with relatively low discharges of rivers makes them vulnerable to pollution (SYKE, 2005).
Table 3. Examples of waterborne outbreaks caused by enteric viruses in Finland and elsewhere

<table>
<thead>
<tr>
<th>Country (Year)</th>
<th>Estimated number of patients</th>
<th>Water supply or source</th>
<th>Enteric virus findings in water</th>
<th>Reported/suggested cause of the outbreak</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland (2007)</td>
<td>8 000</td>
<td>Groundwater</td>
<td>Norovirus, astrovirus, rotavirus, adenovirus and enterovirus</td>
<td>Wastewater contamination into distribution network</td>
<td>Maunula et al., 2009a; Laine et al., 2010</td>
</tr>
<tr>
<td>Finland (1998)</td>
<td>1 700–3 000</td>
<td>Surface water</td>
<td>Norovirus GII</td>
<td>Inefficient treatment</td>
<td>Kukkula et al., 1999</td>
</tr>
<tr>
<td>Finland (2000)</td>
<td>300</td>
<td>Groundwater</td>
<td>Norovirus GII</td>
<td>Sewage pipe breakage</td>
<td>Maunula et al., 2005</td>
</tr>
<tr>
<td>Korea (2015)</td>
<td>12</td>
<td>Groundwater</td>
<td>Hepatitis A virus</td>
<td>Sewage-contaminated groundwater</td>
<td>Shin et al., 2017</td>
</tr>
<tr>
<td>China (2014)</td>
<td>1 614</td>
<td>Direct drinking water</td>
<td>Norovirus GI and GII</td>
<td>Contaminated drinking water</td>
<td>Zhou et al., 2016</td>
</tr>
<tr>
<td>Spain (2016)</td>
<td>4 136</td>
<td>Bottled spring water</td>
<td>Norovirus GI and GII</td>
<td>Sewage contamination of the spring aquifer</td>
<td>Blanco et al., 2017</td>
</tr>
<tr>
<td>Sweden (2009)</td>
<td>200</td>
<td>Groundwater</td>
<td>Norovirus GI</td>
<td>Contamination of the well by snowmelt or network due to leaks</td>
<td>Riera-Montes et al., 2011</td>
</tr>
<tr>
<td>Italy (2006)</td>
<td>2 860</td>
<td>Groundwater</td>
<td>Norovirus and rotavirus</td>
<td>Possible problems with chlorination</td>
<td>Martinelli et al., 2007</td>
</tr>
<tr>
<td>France (2000)</td>
<td>264</td>
<td>Groundwater</td>
<td>Rotavirus</td>
<td>Contamination of the source, combined with a failure of the water chlorination system</td>
<td>Gallay et al., 2006</td>
</tr>
<tr>
<td>Finland (2001)</td>
<td>242</td>
<td>Wading pool</td>
<td>Norovirus GII and astrovirus</td>
<td>Pool contaminated with human faecal material</td>
<td>Maunula et al., 2004</td>
</tr>
<tr>
<td>The Netherlands (2002)</td>
<td>90</td>
<td>Recreational fountain</td>
<td>Norovirus GI</td>
<td>Faecal contamination</td>
<td>Hoebe et al., 2004</td>
</tr>
<tr>
<td>The Netherlands (2015)</td>
<td>73</td>
<td>Canal water</td>
<td>Norovirus GI and rotavirus</td>
<td>Ingestion of contaminated water</td>
<td>Parkkali et al., 2017</td>
</tr>
</tbody>
</table>
2.4.4 Transport and fate of viruses in soil

Viruses are capable of transporting through soil, and their removal during transport occurs either by inactivation or attachment. The attachment rate is viewed as the main removal mechanism due to the low inactivation rate of viruses (Schijven & Hassanizadeh, 2000; Pedley et al., 2006; Lusk et al., 2017). There is great spatial and temporal heterogeneity in the physicochemical and biological factors that define the transport properties and fate of a virus in soil (Lusk et al., 2017). The most important factors controlling virus transport through soil are soil type, pH, the presence of organic matter and metal oxides, the water saturation state, ionic strength and the characteristics of the microorganism (Schijven & Hassanizadeh, 2000; Pedley et al., 2006; Lusk et al., 2017).

Soils with a small grain size tend to adsorb viruses more readily than coarsely textured soils, which have large pores that promote rapid water flow and few binding sites for virus removal. In addition, soils with high clay content are believed to adsorb viruses effectively and better compared to sandy soils (Sobsey et al., 1980; Gerba et al., 1981). However, clay soils can shrink and crack during dry periods, which leads to the development of macropores and preferential flow paths.

Soil pH affects the surface charge of the virus. In slightly acidic ambient conditions, viruses are usually negatively charged, thus bound by positively charged material in the soil (Sobsey et al., 1980). In neutral and alkaline conditions, the surface charge of viruses changes from negative to positive, and thus, they are not bound anymore. Organic matter may increase the surface area and provide binding sites for virus adsorption during transport. On the other hand, organic matter competes with the virus for the same soil adsorption sites and reduces virus binding (Sobsey & Hickey, 1985; Powelson et al., 1991). The presence of cationic metal oxides negatively affects transport by providing binding sites for virus attachment (Moore et al., 1981; Zhao et al., 2008).

In saturated soil, the pores are filled with water, which diminishes the virus’s contact with the soil and allows for faster transport. In unsaturated conditions, the viruses are in closer contact with the soils, thus allowing for adsorption (Powelson et al., 1990; Powelson & Gerba, 1994). Climatic conditions, such as heavy rainfall, may affect water flow and the saturation state, thus enhancing the transport of viruses (Bradbury et al., 2013; Gotkowitz et al., 2016). In a diluted environment, increased detachment rates may also be attributed to the lower ionic strength (Schijven & Hassanizadeh, 2000).

With respect to different virus types, the differences in transport are mainly determined by such adsorptive characteristics as the surface charge and hydrophobicity of the outer capsid layer (Goyal & Gerba, 1979; Schijven & Hassanizadeh, 2000; Michen & Graule, 2010; Dika et al., 2013).

The transport of pathogens in soil and the vulnerability of groundwater have increased the need to establish safe setback distances between the contamination...
source and drinking water supply (Blaschke et al., 2016). Blaschke et al. (2016) have postulated that to ensure safe drinking water (less than $10^{-4}$ enteric virus infections/year/person), a target removal efficiency of 12-log$\log_{10}$ is needed from raw wastewater to tap water. Their simulation yielded setback distances ranging from 39 to 144 m in sand aquifers, 66–289 m in gravel aquifers and 1–2.5 km in coarse gravel aquifers. Likewise, larger setback distances, of up to 8 km, have been suggested for fast-flow aquifers like coarse gravels, fractured rocks and karst limestones (Pang et al., 2005; Masciopinto et al., 2007; Masciopinto et al., 2008). USEPA has addressed the following aquifer types vulnerable to pathogen contamination: gravel aquifers, limestone aquifers and aquifers with fractured rocks (USEPA, 2006a). In these vulnerable aquifers, a high level of treatment at the contamination source, e.g. the OWTS, is needed to secure the safety of groundwater.

2.4.5 Transport and fate of viruses in surface water

The transport and fate of viruses in water, especially in surface water, are greatly affected by the process of settling with particles. The general hypothesis regarding virus behaviour in surface water includes the fluffy sediments that contain suspended solids-associated viruses that have settled out of the water phase, and resuspension of this portion may occur via mild turbulence or water movement (Rao et al., 1986). Previous studies have estimated the proportion of sedimentation in different waters, and according to these studies, the majority of viruses tend to remain suspended in the water column either as free or associated with particles smaller than 0.3 μm (Gerba et al., 1978; Hejkal et al., 1981; Payment et al., 1988; Characklis et al., 2005).

A recent study of F-specific RNA coliphages suggests that the status of viruses in surface water would depend on the hydro-climatological conditions, such as rainfall and flow rate (Fauvel et al., 2017). In periods of low flow and at the rising phase of a rainfall-runoff event, viruses will more probably be free or associated with small solid particles remaining in the water column. During the action of rainfall, viruses can be released from the sediment into the water column and also increasingly be associated with the solids, which favours later sedimentation following the rainfall event (Fauvel et al., 2017).

2.4.6 Persistence of enteric viruses

Enteric viruses are considered to show high persistence in water (Murphy, 2017). Their prolonged survival in aquatic environment is probably a result of their adaptation to the waterborne route of transmission (Gerba, 2007). Virus decay in water is a complex process and both the intrinsic properties of viruses and site-specific environmental conditions are expected to affect the persistence and
survival of a virus (Table 4) (John & Rose, 2005; Pedley et al., 2006; Gerba, 2007; USEPA, 2015; Yates, 2017).

Table 4. Factors affecting the persistence of viruses in water: adapted from Pedley et al. (2006) and Gerba (2007)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus outer shell</td>
<td>Viruses containing lipid envelope are suggested to be more fragile than non-enveloped viruses</td>
</tr>
<tr>
<td>Virus genome</td>
<td>Genome type may affect virus persistence. In general, dsDNA viruses are believed to be more resistant than ssRNA viruses to environmental stress</td>
</tr>
<tr>
<td>Temperature</td>
<td>Low temperature favours virus persistence</td>
</tr>
<tr>
<td>Light</td>
<td>Exposure to sunlight negatively affects virus persistence</td>
</tr>
<tr>
<td>Organic matter</td>
<td>The presence of organic matter may enhance persistence</td>
</tr>
<tr>
<td>Microflora</td>
<td>The presence of indigenous microorganisms may have a negative effect on persistence (antiviral compounds, nucleases, proteases, and predation)</td>
</tr>
<tr>
<td>pH</td>
<td>Most enteric viruses are stable at the pH-values (3–9) of most environmental waters</td>
</tr>
<tr>
<td>Salts</td>
<td>Certain cations may prolong survival</td>
</tr>
<tr>
<td>Metals</td>
<td>Metals such as copper and silver have antiviral properties, but their concentrations in water are usually too low</td>
</tr>
<tr>
<td>Moisture content</td>
<td>Longer persistence in moist soils due to inactivation at the air-water interface</td>
</tr>
<tr>
<td>Suspended solids or sediments</td>
<td>Association with solids prolong survival</td>
</tr>
</tbody>
</table>

Previous studies have shown the high persistence of the norovirus genome in a water environment (Bae & Schwab, 2008; Charles et al., 2009; Skraber et al., 2009; Seitz et al., 2011). For example, Seitz et al. (2011) demonstrated that norovirus genomes can be detected in groundwater for 1,266 days at 25 °C. In addition, a challenge study with human volunteers showed that noroviruses can remain infective for at least 61 days (last observation point) in water (Seitz et al., 2011). Similarly, human adenovirus 41 has remained infectious in both drinking water and surface water after 70 days of incubation at 4 °C and 20 °C (Prevost et al., 2016), and human adenovirus 2 has remained infectious in groundwater for at least 364 days at 12 °C (Charles et al., 2009). In addition, human astroviruses and rhesus rotaviruses have survived in groundwater for up to four and seven months, respectively (Espinosa et al., 2008).
There is still a limited amount of information regarding the survival of enteric viruses in different water environments. This may partly be due to methodological restrictions, such as the difficulty and laboriousness of the methods being used as well as the lack of an infectivity test for certain viruses like noroviruses. One must bear in mind that the results obtained with molecular methods, such as PCR, may not reliably assess the infectious state of a virus (Rodriguez et al., 2009).

2.5 WATER TREATMENT TECHNOLOGIES FOR VIRUS REMOVAL

2.5.1 Wastewater treatment

The objective of wastewater treatment is to reduce the amount of harmful substances before discharge into the environment. The main focus has been on the removal of nutrients, aimed at preventing eutrophication in water, whereas less attention has been paid to the removal of microbes. The different treatment methods have shown high variation in their efficiencies at removing microbes. Importantly, none of the treatment methods are 100% efficient in removing enteric viruses, allowing for a significant load of them to be released into the environment. In addition to the pollution released into surface water, groundwater safety may be compromised due to the OWTS or, e.g. sewer pipe breakage or blockage and overflow in groundwater areas.

Centralised systems

Traditionally, the wastewater treatment processes in municipal settings have been divided into primary treatment and secondary treatment. More recently, a tertiary treatment has been established in order to enhance the purification results. Primary treatment removes mainly suspended solids and floating organic material, and it has only a minor effect on the removal of microbes in the liquid phase. It has been showed that virus removal during primary treatment may vary from 0 to 2.5 log10 (Nordgren et al., 2009; Flannery et al., 2012; Campos et al., 2016).

Secondary treatment may be carried out with different processes, such as activated sludge systems, media filters, anaerobic sludge blanket reactors, membrane bioreactors, waste stabilisation ponds and constructed wetlands. In Finland, activated sludge systems are the most commonly used process and their virus removal typically ranges from 0.3 to 3.5 log10 (Koivunen et al., 2003; Naughton & Rousselot, 2017). The high variation in the removal of viruses is dependent on the influent characteristics as well as the site-specific environmental and operational parameters of the WWTP (Naughton & Rousselot, 2017).

Tertiary treatment follows secondary treatment and usually targets the additional removal of suspended solids, nutrients and pathogenic microbes
(Naidoo & Olaniran, 2014). It may include processes such as filtration, coagulation and flocculation. Tertiary treatment before wastewater disinfection is generally recommended to improve the efficacy of UV or to decrease the required dose of chlorine.

**Decentralised systems**
In rural areas, which are often outside the sewer networks, wastewater treatment is generally managed with small-scale OWTSs. In Finland, about one million people (19% of the population) rely on OWTSs (Vilpas & Santala, 2007). For comparison, approximately 25 million US homes (25% of the population) depend on decentralised wastewater treatment systems (USEPA, 2003; USEPA, 2005). In these systems, the treatment can also be divided into primary and secondary treatment.

Primary treatment usually takes place in septic tanks, which allows for the settlementation of the suspended solids present in raw wastewater. Usually two to three septic tanks are utilised before the secondary treatment unit. The removal of viruses in septic tanks is negligible and unlikely to exceed 50% (Gerba, 2008). Secondary treatment is usually carried out either in a soil absorption field based on natural soil or by more advanced systems, including sand filters (SFs), peat filters or constructed wetlands. In addition, alternative systems, such as sequencing batch reactors, may be applied (Vilpas & Santala, 2007). The removal of viruses in different OWTSs is still poorly understood and varies greatly depending on the system (Olson et al., 2005).

### 2.5.2 Drinking water treatment

The purpose of drinking water treatment is to produce safe water for consumers, and, as such, drinking water should not contain any substances in numbers or concentrations that could potentially be harmful to human health (EU, 1998). Waterworks produce drinking water from surface or groundwater sources or by using artificial groundwater recharge technique. In Finland, the proportions are as follows: surface water 44%, groundwater 41% and artificial recharged groundwater 15% (Guzman-Herrador et al., 2015). The quality of the source water used for drinking water production usually determines the required treatment processes (Crittenden et al., 2005). Generally, the drinking water produced from surface water demands more processing than the water produced from groundwater source.

Conventional treatments used worldwide for the treatment of surface waters include chemical and physical processes such as coagulation, flocculation and sand filtration (Hijnen & Medema, 2010). In addition, chemical adjustment and disinfection are often performed in order to ensure the safety and good aesthetic quality of drinking water (Bonton et al., 2012). The drinking water produced from groundwater or artificial groundwater sources is generally processed less and in many cases used as unprocessed. In particular, in small waterworks drinking water
originating from groundwater is usually assumed to meet the quality requirements without treatment (Katko et al., 2006).

The treatment processes and their capability to remove enteric viruses show high variation, and they are dependent on many factors, including site-specific conditions and the type of virus (Hijnen & Medema, 2010; WHO, 2011). For example, coagulation and flocculation have been shown to remove viruses from 0.1 to 4.3 log10, rapid sand filtration from 0 to 3.7 log10, slow sand filtration from 0.25 to 4.0 log10 and membrane filtration from <1 to <6.5 log10 (Hijnen & Medema, 2010; WHO, 2011).

2.5.3 Disinfection

Disinfection of water is a common practice in the drinking water industry. Chlorine in its different forms (Cl₂, HOCl, ClO₂, NH₂Cl, NHCl₂, NCl₃), ozone and UV are among the most commonly used disinfection methods. Also, alternative oxidants, such as peracetic acid (PAA), have been suggested as potential disinfectants (Kitis, 2004). The objective of disinfection is to secure safe drinking water even in exceptional situations resulting from, e.g. raw water contamination or pipe breakage. More recently, there has been an increasing interest in wastewater disinfection because of the well-addressed risk of effluents in water environments (Naidoo & Olaniran, 2014). Wastewater disinfection is often carried out as a tertiary treatment process following such processes as filtration, coagulation and flocculation.

The different disinfection methods have shown varying efficiencies against a broad range of microbes, and the suitability of these methods to control enteric viruses have also been addressed (Stanfield et al., 2003; Hijnen & Medema, 2010; WHO, 2011; Malayeri et al., 2016). With chemical disinfection, such as chlorination and ozonation, CT values are used to describe the inactivation rate of the microbe. The CT value is defined as the concentration of disinfectant (C) and contact time (T), and it is expressed in mg*min/L. With chlorination, the CT values (mg*min/L) required for 2 log10 reduction vary from 2 to 30 for viruses, 0.04 to 0.08 for bacteria and 25 to 245 for protozoa (LeChevallier & Au, 2004; WHO, 2011). For example, norovirus genomes have been reduced 2 log10 after 3 min with 1 mg/L of chlorine (Shin & Sobsey, 2008). Ozone is also an efficient disinfectant, and a CT value as low as 0.5 mg*min/L has been presented for 2 log10 reduction of viruses (Stanfield et al., 2003).

PAA has been reported to be effective against a large variety of micro-organisms (Koivunen & Heinonen-Tanski, 2005; Zanetti et al., 2007). PAA is completely biodegradable and does not produce significant amounts of harmful DBPs (Monarca et al., 2002). Therefore, PAA is an attractive choice for the disinfection of matrices with a high organic load, e.g. wastewater, and it is included in the USEPA’s list of candidate disinfectants for use in sewer overflows (USEPA, 1999).
However, the higher cost of PAA disinfection compared to chlorine hinders its broader use, and more studies are needed to better assess the efficiency of PAA disinfection.

UV has been shown to be effective against enteric viruses (Hijnen et al., 2006; USEPA, 2006b). However, adenoviruses are resistant to UV and the doses required for reductions of 1 log_{10} and 4 log_{10} are 42 mJ/cm^2 and 167 mJ/cm^2, respectively. In addition, in tertiary wastewater treatment UV has been observed to achieve variable (0.05–2.00 log_{10}) removals for noroviruses (Campos et al., 2016).

2.6 DETECTION METHODS FOR ENTERIC VIRUSES IN WATER

Many enteric viruses cause indistinguishable clinical symptoms; therefore, laboratory diagnosis is essential to identify the pathogen. Methods for virus detection in water are needed for identifying the contamination and enabling comparisons between clinical and environmental samples to ensure the cause of the outbreak. In addition to outbreak investigations, such methods are being applied in different studies on, e.g. water safety planning for drinking water works or optimising different treatment processes, such as wastewater purification.

Traditionally, enteric viruses have been detected in clinical samples via electron microscopy, cell culture or antibody-based methods (Atmar & Estes, 2001). Even though the sensitivity of the methods has been good enough for clinical samples, which usually may contain high numbers of viruses, these methods are often unsuitable for environmental samples with low numbers of viruses. Cell culture-based quantitative methods for the detection of certain viruses in water became available in the 1970s. However, many enteric viruses are still difficult or impossible to propagate in a cell culture. In addition, working with cell cultures can be laborious and time-consuming and the recognition of cytopathic effects is subjective (Teunis et al., 2005).

The invention of the polymerase chain reaction (PCR) enabled the specific detection of non-cultivable viruses (Xi et al., 1990), and quantitative PCR (qPCR) revolutionised the detection of enteric viruses in 2000. The detection of enteric viruses from water samples is currently based on molecular methods. The most common detection method is real-time qPCR. Other methods include (reverse transcription) loop-mediated isothermal amplification ((RT)-LAMP) (Mori & Notomi, 2009), nucleic acid sequence-based amplification (NASBA) (Compton, 1991), and digital PCR (Morley, 2014). The benefits of molecular methods are their sensitivity, specificity and fast turnaround time. However, the main drawback is that they do not discriminate between infectious and inactive viruses (Rodriguez et al., 2009). Moreover, they require specialised laboratory equipment and personnel.

Even though enteric viruses end up in high numbers in the environment through wastewater discharge, due to dilution the numbers per volume in environmental waters are usually low. Considering the low infectious dose of
enteric viruses, a high degree of sensitivity is required for the methods. Therefore, the detection of viruses in water requires efficient concentration and consists of several steps. The process usually includes primary concentration, secondary concentration, extraction of viral nucleic acids and, finally, detection of the virus genome via, e.g. PCR. High degrees of variation in the extraction efficiencies have been presented in the existing literature, depending on the methods, virus and matrix used. According to an international standard, the minimum acceptable extraction efficiency for a process control virus in water is 1% (ISO/TS, 2013).

2.6.1 Concentration

Primary concentration

Typically, the concentration volume required for enteric virus analysis varies from tens or hundreds of millilitres to hundreds or thousands of litres, depending largely on the properties of the water. For example, for unclean waters like wastewater and surface water, usually up to a ten-litre sample volume is sufficient. In contrast, clean drinking water may require samples up to 1 000 litres of volume. There are several methods for concentrating the numbers of enteric viruses and many of them are based on membrane filtration. The most commonly used approaches include the adsorption-elution of viruses using positively or negatively charged filters or virus retention by pore size exclusion using ultrafilters (Shi et al., 2017).

The use of charged membranes is based on electrostatic differences between the membrane and virus capsid. The positively charged membranes can adsorb negatively charged viruses from waters with near neutral pH levels (Sobsey & Jones, 1979). The use of negatively charged membranes requires the addition of salts (MgCl$_2$ or AlCl$_3$) and adjusting water’s pH to 3.5 prior to filtration (Wallis et al., 1972). When the pH of the sample is lower than the isoelectric point of a virus, the electrostatic potential of the virus capsid is positive. However, electrostatic filters usually have a clogging problem when large volumes of water need to be studied or the water contains high amounts of organic matter. To relieve the clogging problem, prefilters (e.g. fibre glass) or additional larger diameter membrane filters have been used to increase the flow rate (Wu et al., 2011).

To overcome the problems related to clogging and enable examination of larger volumes of water samples (up to 1 000 L), hollow-fiber ultrafiltration has been applied (Winona et al., 2001; Hill et al., 2005; Polaczyk et al., 2008; Smith & Hill, 2009; Rhodes et al., 2011). With this method, simultaneous recovery of diverse microbes can be carried out with large volumes of different types of water (Olszewski et al., 2005; Leskinen et al., 2010; Gibson & Schwab, 2011). This method captures and concentrates the viruses as well as other microbes by size exclusion (molecular weight cut-off, MWCO, e.g. 30 kDa). Currently, an alternative approach to the traditional tangential flow ultrafiltration setup called dead-end ultrafiltration (DEUF) has been developed and employed in several field studies (Kearns et al., 2011).
2008; Leskinen et al., 2009; Smith & Hill, 2009; Francy et al., 2013; Rhodes et al., 2016). Some of the advantages of concentrating large volumes of water may be compromised by the co-concentration of inhibitory substances (Hata et al., 2011).

Other filters have also been applied for virus concentration from water, including different materials like glass wool (Lambertini et al., 2008) and nanoalumina fibre (Li et al., 2010).

Elution
Viruses concentrated either with charged filters or ultrafilter cartridges must be eluted from the membranes. For this purpose, different elution buffers have been developed depending on the filter properties (Shi et al., 2017). For charged filters, an alkaline elution solution with a pH of up to 9.5 and containing beef extract and sometimes buffered with glycine is commonly used to elute the viruses from the filter (Shi et al., 2017). An alkaline pH increases the electrostatic repulsion between the virus and the membrane and facilitates desorption. Beef extract improves virus release from the membrane by disrupting hydrophobic interactions and promotes bioflocculation during the following secondary concentration (Shi et al., 2017). For ultrafilters, an elution solution containing 0.01% of NaPP together with 0.01% of Tween 80 has been commonly used and proved effective (Shi et al., 2017).

Secondary concentration
Despite the method used for primary concentration, secondary concentration is usually required to further reduce the sample volume. The eluents or concentrates from the primary concentration usually range from a few millilitres to a few hundred millilitres. For molecular detection, this volume must be decreased to obtain a sufficient amount of sample for the nucleic acid extraction and PCR reaction. With certain samples, e.g. wastewater, secondary concentration methods may also be used as the primary concentration method when smaller volumes are analysed.

Secondary concentration may be carried out by several methods. Two commonly used secondary concentration methods are organic flocculation (Katzenelson et al., 1976) and polyethylene glycol (PEG) precipitation (Lewis & Metcalf, 1988). Celite concentration has also been applied as a secondary concentration method (Dahling & Wright, 1986; McMinn et al., 2012). One option for secondary concentration is to use centrifugal devices, which are based on the MWCO of the ultrafilter (Haramoto et al., 2004; Hill et al., 2007; Li et al., 2010). Viruses that are larger than the MWCO of the filter (typically 50 to 100 kDa) will be retained, while liquids and low molecular mass particles are passaged. Ultracentrifugation has also been used for virus extractions (Stals et al., 2012; Martín-Díaz & Lucena, 2018).
2.6.2 Nucleic acid extraction

The purity of nucleic acids is important in environmental samples, which may contain inhibitory substances affecting the molecular detection of viruses (Hata et al., 2011). There are several methods for the extraction of nucleic acids (Ali et al., 2017). One of the most commonly used methods exploits the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate together with the nucleic acid-binding properties of silica particles (Vogelstein & Gillespie, 1979; Boom et al., 1990). Nowadays, commercial kits based on this principle utilise the silica particles built on a membrane in a column or exploit the nucleic acid-binding magnetic beads and they are most commonly used in viral nucleic acid extraction. In addition, extraction robots exist to provide a convenient way to process a large batch of samples.

2.6.3 PCR detection

PCR is a powerful technology that utilises specific sequences within DNA or complementary DNA (cDNA) to be amplified using sequence-specific oligonucleotides, heat-stable DNA polymerase and thermal cycling. Theoretically, PCR amplifies DNA exponentially by doubling the number of target sequences with each amplification cycle. In reverse transcription PCR (RT-PCR), RNA is first converted into cDNA by using a reverse transcriptase enzyme prior to the actual PCR amplification. The RT-PCR can be performed separately in two steps or in the same test tube, referred to as one-step RT-PCR. In traditional PCR, detection and quantification of the amplified sequence are performed at the end of the reaction, and post-PCR analysis such as gel electrophoresis and image analysis are needed.

Nowadays, real-time (RT-)PCR is a golden standard for the detection and quantification of enteric viruses from environmental samples. In real-time (RT-)PCR, the amount of target DNA (or RNA) is measured after each amplification cycle via fluorescent probes or dyes that produce an increasing fluorescent signal in direct proportion to the number of target amplicons generated (Arya et al., 2005). The fluorescent signal is measured during the PCR by a real-time PCR instrument. The most widely used chemistries are TaqMan assays and SYBR Green Dye-based assays. In real-time (RT-)PCR, if a particular sequence is abundant in the sample, then amplification is observed in earlier cycles, whereas amplification of the scarce sequence is observed in later cycles. Quantitation is most commonly conducted by using standards (e.g. plasmids, RNA transcripts, synthesised linearised dsDNA) and comparing the Ct values of the sample to the serially-diluted standard curves.

2.6.4 Methods for estimating enteric virus infectivity

Traditionally, a cell culture has been used to assess the infectivity of enteric viruses. The growing need to improve the detection methods and to analyse different
Enteric viruses from water has led to the widespread use of a molecular approach, despite its inherent inability to discriminate between infectious and non-infectious viral particles. Since PCR often targets a rather short region of the genome, a positive signal may be produced even though the genome or viral capsid is damaged. For some enteric viruses, an integrated cell-culture PCR (ICC-PCR) has been applied to obtain information about the infectious state of the virus (Reynolds, 2004; Ryu et al., 2015). However, new molecular approaches for evaluating the integrity of both viral genome and capsid are needed to rapidly reveal the infectivity of the virus.

One attempt has been to analyse a longer target region of the genome to increase the probability of copying intact genomes (Rodriguez et al., 2009). Also, immunocapture (IC) qPCR, which is based on the binding of a structurally intact virus to a specific antibody, porcine gastric mucins or histo-blood group antigens (HBGAs) recognised as receptors for human norovirus have been used to detect only infectious viruses (Rodriguez et al., 2009; Dancho et al., 2012; Ogorzaly et al., 2013; Wang & Tian, 2014).

More recently, the enzymatic pre-treatment of samples prior to nucleic acid extraction with proteinase and/or RNAse has been tested in order to detect only viruses with intact capsids (Nuanualsuwan & Cliver, 2002; Topping et al., 2009; Seitz et al., 2011). The integrity of a viral capsid has also been studied using intercalating dyes (propidium monoazide, PMA, or ethidium monoazide, EMA) (Fittipaldi et al., 2010; Parshionikar et al., 2010; Leifels et al., 2015; Lee et al., 2018). With the PMA approach, a virus with a damaged capsid intakes the dyes, which bind to the virus genome upon exposure to light so that the enzymes in PCR cannot work.

Another promising method is the use of nucleic acid aptamers to selectively detect infectious virus particles (Escudero-Abarca et al., 2014; Moore et al., 2016). However, none of the mentioned new approaches have been universally accepted as effective (Knight et al., 2013; Knight et al., 2016), and more testing and validation is needed for evaluating the applicability of these methods for enteric virus infectivity assessment. The possible negative effects of virus concentration procedures on infectivity must also be carefully studied (Blanco Fernandez et al., 2017).

Since a norovirus is not easily propagated in a cell culture, despite the recent progress (Jones et al., 2015; Ettayebi et al., 2016), infectivity can only be reliably examined via human volunteer studies (Richards, 2012). To overcome the obstacles related to volunteer studies, many surrogate viruses have been used to model the persistence and inactivation of noroviruses. Bacteriophage MS2, Feline calicivirus (FCV), Murine norovirus (MuNoV) and Tulane virus have all been used as model viruses, especially for norovirus (Richards, 2012). These viruses can easily be propagated in a culture, and they are genetically, physically and chemically related to human noroviruses. However, in most cases the survival or persistence of these
surrogates does not mimic human noroviruses under various environmental conditions or processing procedures (Richards, 2012; Cook et al., 2016).

2.6.5 Quality control

The detection of enteric viruses in water samples is a multistep process and the yield is dependent on the efficacy of the selected methods and the sample itself. The multiple steps (primary concentration, elution, secondary concentration, nucleic acid extraction and final detection with PCR) require analysing viruses in large volumes, which increases the chance for viral loss. Therefore, to ensure the reliability of the results, the quality control should meet certain requirements and cover all the steps in the analysis from concentration to PCR. The negative and positive process and/or extraction controls are necessary to minimise the risk of false negative and positive results (ISO/TS, 2013).

PCR is a very sensitive method and prone to contamination. To minimise the risk of contamination, several issues need to be taken into consideration. Ideally, separate rooms for nucleic acid extraction, PCR set-up (pre-PCR) and PCR-amplification (post-PCR) should be arranged. The usage of filtered pipet tips, cleaning of the tables and instruments with proper substances (alcohol, DNA and RNA-removal, RNase removal, UV light), and working in safety/or pipetting cabinets are all part of good molecular biology laboratory practice. False positive results must be ruled out with negative PCR controls. In addition, scheduled contamination tests are recommended.

One limitation of molecular methods is the sensitivity of PCR to the inhibitory compounds naturally occurring in environmental samples (Hata et al., 2011). Inhibitors are often concentrated and extracted together with targeted viruses, and this may cause problems especially when large amounts of water or complex matrices are studied. Despite the development of more robust PCR kits for environmental samples containing inhibitors, sample-specific inhibition should be determined to avoid false negatives and increase the reliability of the results. The amount of inhibition and the efficiency of the target amplification can be estimated by, e.g. diluting the sample and using internal or external amplification controls (Hoorfar et al., 2004; ISO/TS, 2013).

2.6.6 New applications in enteric virus analysis

The recent development of digital PCR (dPCR) to detect and quantify nucleic acids offers an alternate methodology to real-time qPCR (Morley, 2014; Rački et al., 2014; Zhang & Jiang, 2016; Monteiro & Santos, 2017). In dPCR, a sample is partitioned into thousands of individual reactions running in parallel, and the total number of target molecules is calculated via Poisson statistics. The advantage of this
technology is that it does not need external reference standards and it may decrease the levels of PCR inhibitors (Rački et al., 2014).

The most novel and promising method in environmental virology is metagenomic analysis by next-generation sequencing (Nieuwenhuijse & Koopmans, 2017). The advantage of this method is that it detects all viruses in a sample, including previously unknown viruses. Several metagenomics studies have been carried out in water environments and the amount of knowledge is increasing tremendously (Djikeng et al., 2009; Rosario et al., 2009a; Cantalupo et al., 2011; Bibby & Peccia, 2013; Aw et al., 2014; Kim et al., 2016; Fernandez-Cassi et al., 2017). Nevertheless, several issues must be resolved before this methodology can replace the current enteric virus detection methods (Nieuwenhuijse & Koopmans, 2017).

First, sample preparation needs to be improved to get rid off background originating from host and bacterial genomes. Second, efficient and automated processing of metagenomic data is required. Third, interpretation of metagenomic data requires more knowledge about the virome in the environmental samples.

2.7 QUANTITATIVE MICROBIAL RISK ASSESSMENT

Quantitative microbial risk assessment (QMRA) is a method that can be used to estimate the health risks of microbes in water (Haas et al., 1999). With the QMRA approach, the magnitude of risk, e.g. infections/person/year, is calculated based on information about the raw water quality, treatment efficiency, dose-response relationships of pathogens and exposure data.

The QMRA framework consists of four steps: problem formulation, exposure assessment, health effects assessment and risk characterisation (WHO, 2016). The problem formulation includes careful definition of the scope and purpose of the risk assessment. In the exposure assessment, the dose of pathogens for the defined exposure pathway is estimated. The health effects assessments involves evaluation and quantification of the health effects associated with exposure to pathogens. Finally, in the risk characterisation the exposure and health information is combined to produce quantitative measures of risk for the expected health effects of the estimated dose.

QMRA can provide valuable input for the development of the water safety plans (WSPs) in order to systemically assess and manage risks related to a specific water supply (Petterson & Ashbolt, 2016). Currently, computational QMRA tools have been developed to help conduct risk assessments for, e.g. the entire drinking water production chain from raw water to drinking water (Schijven et al., 2011).
3 AIMS OF THE STUDY

The overall objective of this study was to describe and reveal the factors affecting the success of enteric viruses at causing waterborne outbreaks. In addition, the aim was to provide valuable information on how such outbreaks can be prevented and managed efficiently. The specific aims were to:

1. Study the occurrence of pathogenic enteric viruses (noro- and adenoviruses) in Finnish waters and waterborne outbreaks (I, II, III).
2. Investigate the removal of enteric viruses during soil filtration-based wastewater treatment and transport of viruses through soil into the groundwater (I, III).
3. Examine the persistence of enteric viruses in water (I, IV, V).
4. Assess how enteric viruses are removed from contaminated drinking water distribution network (I, V).
5. Evaluate the value of indicator microbes in assessing risks related to waterborne enteric viruses (I, II, III, V).
4 MATERIALS AND METHODS

4.1 ENTERIC VIRUSES (IV, V)

The persistence of the norovirus genome in drinking water and wastewater at different temperatures (IV) was studied by employing the human norovirus GII. Two norovirus GII.Pg/GII.1 inoculums (GII_A and GII_B) were extracted from human stools stored at -20 °C by making a 10–20% (w/v) suspension in nuclease-free water. The suspension was centrifuged at 10 000× g for 2 min and the supernatant was either used immediately or stored at ≤−75 °C.

The persistence and decontamination of adenovirus in drinking water pipelines (V) was studied by employing enteric human adenovirus 40 (ATCC VR-931). In addition to using stock adenovirus 40, the strain was also propagated in 293 cells, as described by Mautner (1998). Briefly, 293 cells grown in 75-cm² cell culture flasks in monolayers at 37 °C and 5% CO₂ were infected with a ten-fold virus dilution, and the viruses were allowed to adsorb for 1 h at 37 °C before the medium was added. After being incubated for 5–7 days, the cells were pooled and harvested, and the viruses were released by three freeze-thaw cycles, collected by centrifugation (1 500× g for 2 min) and stored at ≤−75 °C.

4.2 INDICATOR MICROBES (V)

The persistence and decontamination of E. coli in drinking water pipelines (V) was studied by employing an environmental E. coli strain isolated during a drinking water outbreak (Laine et al., 2011). The E. coli was cultured aerobically at 36 ± 2 °C on tryptone soya agar (Oxoid, Basingstoke, UK), and the contaminant suspension was prepared in a nutrient broth, washed and centrifuged, as previously described by Lehtola et al. (2007).

4.3 EXPERIMENTAL SET-UP AND SAMPLES

4.3.1 Outbreak descriptions (I, II)

In this study, two drinking water outbreaks (I) and eight bathing water outbreaks (II) were described and investigated. Both drinking water outbreaks were defined with a strong strength of association (class A) between water and illness (Anonymous, 1996; Pihlajasaaari et al., 2016). The strength of association for bathing waterborne outbreaks were classified as strong, probable or possible based on classification criteria modified from those presented by Tillett et al. (1998).
Information regarding the outbreaks was collected from the local investigation reports and personal communications with the people concerned.

**Drinking water outbreak I. Transport of Microbes Through the Sand Filter of an OWTS and Soil (I)**

In drinking water outbreak I, two family members had suffered AGE on 20 June 2011 in southern Finland. In addition, 15 visitors to the private property had caught the disease during a three-week period from the day of primary illness. Symptoms appeared approximately one day after the drinking water ingestion, lasted for about one day and mainly involved vomiting. The use of water originating from a private dug well of about 2.5–3 m in depth was prohibited in the second week of July 2011, and the suspicion was that an OWTS located on the same property had contaminated the dug well.

The OWTS consisted of three separate septic tanks where the settled wastewater was piped through distribution box and infiltration tubing to the SF. Percolated wastewater was collected in a collection tank via tubing located at the bottom of the SF. From the collection tank, the treated wastewater was discharged into a nearby open ditch, which transported the wastewater at closest within a 10 m distance from the groundwater well. The distance between the groundwater well and the OWTS was approximately 45 m.

The cause of the outbreak and the treatment efficiency ($\log_{10}$ removal) of the SF of the OWTS were investigated using five water samples collected from groundwater well on 8 August 2011 and 23 August 2011, and from a septic tank (influent), collection tank (effluent), and tap water on 23 August 2011. All the water samples were analysed for norovirus GI and GII. Human adenoviruses were analysed from the septic tank and collection tank samples taken on 23 August 2011, and faecal indicator microbes (*E. coli*, coliform bacteria, intestinal enterococci, spores of *C. perfringens*, somatic and F-specific coliphages) were analysed from the groundwater well, septic tank, and collection tank samples taken on 23 August 2011.

Drinking water was not treated or disinfected before use. The control measures during the outbreak included the restriction on using the water for drinking purposes and the maintenance and cleaning of the groundwater well by the local company on 16 August 2011. In addition, septic tanks were emptied between the onset of outbreak (20 June 2011) and the first sampling on 8 August 2011.

**Drinking water outbreak II. Cleaning of the Contaminated Distribution Network (I)**

In drinking water outbreak II, ten people fell ill from contaminated water in northern Finland in May 2011. The outbreak was caused by the blockage of the sewer collection drainpipe of four private houses, leading to wastewater overflow directly into the drilled groundwater well serving as a drinking water source for 14
households. The contaminated drinking water distribution network consisted of a 450 m water main pipe and 600 m household distribution pipes.

In total, four water samples were collected from the contaminated groundwater well and 17 tap water samples from several points along the distribution network over a four-month period (3 June–12 October 2011). Sodium thiosulphate was used to inactivate chlorine from the samples prior to microbiological analyses. The samples were analysed for enteric viruses (norovirus GI and GII and adenoviruses) and indicator microbes (E. coli, coliform bacteria, intestinal enterococci and C. perfringens).

**Bathing water outbreaks (II)**

In July 2014, several outbreaks linked to bathing water were reported in different parts of Finland. In total, eight suspected bathing water outbreaks (I–VIII) were included in this study. Water samples were collected for noro- and adenovirus analyses in seven outbreaks. In addition, FIB results (E. coli and intestinal enterococci) from the water were obtained from the municipal health authorities.

**4.3.2 Pilot-scale sand filters (III)**

Three different pilot-scale SFs (SF1-3) and a separate phosphorus removal unit (Nordkalk Filtra P, Nordkalk, Parainen, Finland) connected at the end of SF-3 (SF-3+P) were tested (Figure 2). The SFs were located in an insulated container whose temperature was kept above zero degrees with an automatic heat blower during winter and cooled to approximately 15 °C during summer.

The influent loaded into the SFs was raw municipal post-screen wastewater from Kuopio (Finland), allowed to sediment for two days in a 1 000 L tank. The normal load of each SF was 33.3 L/d and the daily flow pattern was according to the European standard (EN, 2005). Periods of overloading and underloading were 125% and 50% of the normal load and continued for one week, and both were carried out three times during the study.

Every effluent sample was collected over a 24 h period and taken at three-week intervals as well as one week after the underloading and overloading periods. In total, 20 influent and effluent samples from SF-1–3 were collected over a one-year period (12 October 2010–25 October 2011). The effect of the cold period on the purification efficiencies was studied by comparing eight winter samples collected between 23 November 2010 and 29 March 2011 to the rest of the samples (summer). In addition, ten effluent samples from SF-3+P were collected over a six-month period (18 April–25 October 2011). The samples were analysed for noroviruses, adenoviruses and indicator microbes (E. coli, intestinal enterococci, spores of sulphite-reducing clostridia, somatic and F-specific coliphages).
4.3.3 Persistence of enteric viruses and indicator microbes in water (I, IV, V)

Persistence was studied in the laboratory experiments (IV, V) and by utilising samples obtained from the drinking water outbreaks (I). In the laboratory study (IV), the persistence of two norovirus GII strains (GII_A and GII_B) in drinking water and three norovirus GII strains (GII_A, GII_B and indigenous GII (GII_ind)) in wastewater were examined. The drinking water was tap water from the city of Kuopio, Finland, where the chlorine was quenched with sodium thiosulfate prior to the experiments. Wastewater was settled influent taken from the distribution box of a three-tank septic system of a private OWTS serving five people. Tests were carried out in the dark at 3 °C, 21 °C and 36 °C. Temperature was monitored every five min with an automated monitoring system (Labo Line, Helsinki, Finland). With the drinking water, the average temperatures and standard deviations were 3.2 ± 0.8 °C, 21.0 ± 0.8 °C and 35.9 ± 0.1 °C, while for the wastewater they were 3.0 ± 0.8 °C, 20.9 ± 0.3 °C and 35.8 ± 0.1 °C.

The initial numbers in the drinking water experiment for GII_A and GII_B were 7.4 × 10^6 GC/mL and 3.7 × 10^6 GC/mL, respectively. The initial numbers for GII_A, GII_B, and GII_ind in the wastewater experiment were 5.4 × 10^6 GC/mL, 2.6 × 10^6 GC/mL and 5.2 × 10^3 GC/mL, respectively. In the wastewater test calculations, the numbers of GII_ind were subtracted from the numbers of spiked GII_A and GII_B. Duplicate samples were taken after 0, 5, 10, 20, 40, 80, 160, 251, 320 and 365 days in the drinking water experiment and after 0, 5, 10, 14 (15), 20, 40 (50), 63, 80, 101, 120 and 140 days in the wastewater experiment.

In the drinking water outbreak study (I), the persistence of norovirus GII, adenoviruses and C. perfringens was assessed with four samples collected from a contaminated groundwater well over a 108-day period. Moreover, persistence of E. coli, coliform bacteria and intestinal enterococci was studied from the three
groundwater well samples over a 59-days period. The long-term persistence of norovirus GII and adenovirus genomes was studied by storing samples from drinking water outbreaks in dark conditions at 4 °C. The persistence of the norovirus GII genome was studied using three wastewater samples from outbreak I, two drinking water samples from outbreak I and one drinking water sample from outbreak II. The persistence of the adenovirus genome in drinking water was studied using six samples from drinking water outbreak II. The virus analyses were performed for the selected samples after 966, 1 029, 1 262, 1 277, 1 332 and 1 343 days of storage.

The persistence of adenovirus genomes and *E. coli* in water and biofilm samples from the laboratory-scale distribution network were studied in the non-decontaminated control pipelines (V). Water and biofilms samples were collected before contamination and after 1, 24, 25, 48, 72 and 96 h from the start of the contamination.

### 4.3.4 Decontamination of a drinking water distribution network (I, V)

In drinking water outbreak II study (I), decontamination was carried out by first dosing dissolved calcium hypochlorite tablets into the groundwater well during a 71-h period (7 June–10 June 2011). This resulted in an average of 71.5 mg/L chlorine in the network, and subsequent flushing of the network was performed (10 June–13 June 2011). Second chlorination was carried out with sodium hypochlorite dosed into the well on 6 July 2011, but continued only for a few hours due to pump failure. Finally, continuous chlorination with sodium hypochlorite dosed into the network was carried out between 13 July and 12 October 2011, with chlorine levels varying between 0.02 and 4.2 mg/L.

In the laboratory study (V), two parallel pipeline systems consisting of 24 individual polyvinyl chloride (PVC) pieces (diameter 10 mm, length 10 cm) were constructed, one for decontamination and the other for non-decontamination control. The tap water from Kuopio, which contained on average 0.05 mg/L chlorine, was run through the pipeline system (0.2 L/min). Biofilms in the pipelines were allowed to grow for four weeks prior to contamination. The contaminant (5 L) was added at a flow rate of 0.1 L/min into the main stream, and the overall water flow was kept at 0.2 L/min. The contamination was conducted over a time period of 25 min, after which only tap water was run into the pipelines. The concentration of adenovirus 40 in the contaminant was approximately $3 \times 10^6$ GC/L (ATCC stock) and $5 \times 10^6$ GC/L (ATCC stock and propagated adenovirus 40) in the PAA and chlorine experiments, respectively. The *E. coli* concentrations in the contaminant were $7 \times 10^8$ cfu/L in the PAA experiment and $3 \times 10^{10}$ cfu/L in the chlorine experiment. Decontamination with PAA (Solvay Chemicals Finland Oy, Kouvol, Finland) or chlorine (sodium hypochlorite, Merck KgaA, Darmstadt, Germany) was started 24 h after spiking the contaminant in order to achieve final concentrations of
1 mg/L in outlet water. The decontaminant was quenched from the samples using sodium thiosulfate (20 mg/L). At every sampling point, single water samples and two to four replicate 10 cm PVC pieces for biofilm analyses were taken from both pipeline systems. Biofilms were detached using glass beads, as previously described by Zacheus et al. (2000).

4.4 DETECTION OF ENTERIC VIRUSES

4.4.1 Concentration (I–III, V)

Noro- and adenoviruses were concentrated from 0.45 to 2 L groundwater well and tap water samples by the adsorption–elution method (Gilgen et al., 1997). Briefly, the water sample was filtered using positively charged Sartolon polyamide membranes (diameter 47 mm, pore size 45 μm; Sartorius, Göttingen, Germany) or Zetapor membranes (diameter 47 mm, pore size 45 μm; AMF-Cuno, Meriden, CO, USA). Viruses were eluted from the membranes in a 50 mM glycine buffer, pH 9.5, containing 1% beef extract (MP Biomedicals, Solon, OH, USA), and the eluate was immediately neutralised with HCl. A secondary concentration with a microconcentrator (Vivaspin 2, Sartorius, Stonehouse, Gloucestershire, UK) was carried out to reduce the volume to about 200 μL.

In study II, samples from bathing water outbreaks IV, V and VIII were analysed using membrane disk HA (Millipore, Billerica, MA, USA) and Nanoceram (Argonide, Sanford, FL, USA) to filter a total volume of 4.5 L of water. When necessary, a prefilter (Waterra) was used; otherwise, the protocol was as described above.

Wastewater samples were concentrated from a volume of 500 mL by a two-phase separation method (WHO, 2003). Briefly, a neutralised wastewater sample (pH 7–7.5) was mixed with 39.5 mL of 22% dextran (Pharmacosmos, Holbaek, Denmark), 287 mL of 29% PEG6000 (Sigma-Aldrich, Steinheim, Germany) and 35 mL of 5 N NaCl, and it was kept under constant agitation for 1 h at 4 °C. After overnight incubation at 4 °C in a separation funnel, the lower layer and the interphase were collected and extracted with a 20% volume of chloroform (Merck KgaA, Darmstadt, Germany).

4.4.2 Nucleic acid extraction (I–V)

Viral DNA and RNA were extracted from a 200 μL volume using the High Pure Viral Nucleic Acid and High Pure Viral RNA Kit, respectively, according to the manufacturer’s instructions (Roche Molecular Biochemicals Ltd., Mannheim, Germany). The extracted nucleic acids were stored at ≤-75 °C.
4.4.3 Real-time (RT-)qPCR (I–V)

The primers and probes used for the real-time (RT-)qPCR are shown in Table 5. In the design of the norovirus GI genogroup-specific primers and probe, multiple sequences from the GenBank were aligned using MEGA4 software (Tamura et al., 2007). The forward primer (NVGIF) and reverse primer (NVGIR) amplified a 95bp fragment of the ORF1-ORF2 junction region.

For noroviruses, the real-time RT-qPCR assays were carried out in one step, separately for genogroups I and II, using the QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany; studies I–III) or the TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Austin, TX, USA; study IV). In studies I–III, the amplification reaction mixtures contained 12.5 µL 2x QuantiTect Probe RT-PCR Master Mix, 0.25 µL QuantiTect RT Mix, 0.4 µM primers, 0.2 µM probe and 5 µL of RNA sample or control in a final volume of 25 µL. The real-time RT-PCR amplification was carried out in a Rotor-Gene™ 3000 real-time rotary analyser (Qiagen, Hilden, Germany), running at 50 °C for 30 min and 95 °C for 15 min, followed by 45 cycles at 94 °C for 15 s and 60 °C for 1 min. In study IV, the following updated RT-qPCR method was applied for norovirus GII. The amplification reaction mixtures contained 6.25 µL 4x TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Austin, TX, USA), 0.4 µM primers, 0.2 µM probe and 5 µL of RNA sample or control in a final volume of 25 µL. The real-time RT-qPCR assays were carried out using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), running at 50 °C for 5 min and 95 °C for 20 s, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min.

For adenoviruses, real-time qPCR assays were carried out in a Rotor-Gene™ 3000 real-time rotary analyser (Qiagen, Hilden, Germany). Each PCR mix included 12.5 µl of 2x TaqMan Universal Master mix (Applied Biosystems, Branchburg, NJ, USA; studies I, III and V) or Taqman Environmental Master Mix 2.0 (Life Technologies, Warrington, UK; study II), 0.3 µM primers, 0.2 µM TaqMan probe and 5 µl of DNA sample or control in a final volume of 25 µL. The real-time qPCR running programme was 50 °C for 2 min and 95 °C for 15 min, followed by 45 cycles at 95 °C for 10 s, 55 °C for 30 s and 72 °C for 15 s.

In study II, samples from bathing water outbreaks IV, V and VIII were analysed for noro- and adenoviruses according to the real-time (RT-)PCR protocol described by Maunula et al. (2009a). As a modification, Taqman primer–probe sets for norovirus GI and GII were applied, as published in ISO/TS 15216–1 (ISO/TS, 2013).
Table 5. The primers and probes used for real-time (RT)-qPCR in this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Oligonucleotide</th>
<th>Sequence and label (5' - 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus GI</td>
<td>NVGIF</td>
<td>GCYATGGTCCGCTGGATG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>NVGIR</td>
<td>CCTTAGACGCCATCATCATT</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>NVGIP-MGB</td>
<td>VIC-TGGACAGGAGAYCGC-MGB-NFQ</td>
<td>This study</td>
</tr>
<tr>
<td>Norovirus GII</td>
<td>QNIF2d</td>
<td>ATGTTCAGRTGATGAGRTTCTCAG</td>
<td>Loisy et al., 2005</td>
</tr>
<tr>
<td></td>
<td>COG2R</td>
<td>TCGACGCCATCTTCATACCA</td>
<td>Kageyama et al., 2003</td>
</tr>
<tr>
<td></td>
<td>RING2-TP</td>
<td>FAM-TGGGAGGGCGATCGCAATCT-BHQ1</td>
<td>Kageyama et al., 2003</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>JTVXF</td>
<td>GGACGCCTCGGAGTACCTGAG</td>
<td>Jothikumar et al., 2005</td>
</tr>
<tr>
<td></td>
<td>JTVXR</td>
<td>ACIGTGGGTTTCTGAACCTTGTT</td>
<td>Jothikumar et al., 2005</td>
</tr>
<tr>
<td></td>
<td>JTVXP</td>
<td>FAM-CTGGTGCAAGTCCGTGCA-BHQ1</td>
<td>Jothikumar et al., 2005</td>
</tr>
</tbody>
</table>

Quantitation of each virus was determined by comparing the Ct values of the sample to the serially-diluted standard curves previously established or included in the run. For norovirus GI and GII genogroups, the plasmid standards were used in studies I–III (Nordgren et al., 2008). In study IV, standard curves were generated using gBlocks® Gene Fragments (Integrated DNA Technologies, Leuven, Belgium) containing the sequences for the target amplicon. For adenovirus quantitation, a 379-bp fragment of the hexon region of human adenovirus 40 was amplified using the forward primer 5’-TGGCCACCCCCTCGATGA-3’ and reverse primer 5’-TTTGGGGGCCAGGGAGTTGA-3’ (Jothikumar et al., 2005). The insert was cloned into a pGEMT-Easy vector and transformed into JM109 competent cells using the pGEMT-Easy Vector System II kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The concentration of PstI-linearised plasmid was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Madison, WI, USA).

4.4.4 Controls (I–V)

The virus extraction process was controlled by positive and negative controls. Spiked norovirus stool suspension and human adenovirus 40 (ATCC VR-931) were used as positive process controls, and sterile deionised or nuclease-free water as a negative process control. Inhibition in (RT)-qPCR was assessed by running undiluted and ten-fold dilutions of the samples (studies I–V) and by using an external amplification control (EAC) in norovirus GII RT-PCR (ISO/TS, 2013) (study...
EAC was produced from the plasmid standard used for norovirus RT-qPCR quantification. Briefly, a norovirus plasmid was digested with NotI (Promega, Madison, WI, USA), and purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. RNA transcripts were obtained and DNase treated using the Riboprobe in vitro Transcription Systems (Promega, Madison, WI, USA) and purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers’ protocols. A non-template control (nuclease-free water) was included in each PCR run.

4.5 SEQUENCING AND VIRUS TYPING (I, VI, V)

Virus sequencing was performed using BigDye v. 3.1 terminator chemistry and analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA). Noroviruses were sequenced with the primers MJV12 and RegA (Vinjé et al., 2004) targeting the polymerase region (studies I and II). In addition, samples from drinking water outbreak II (study I) and study IV were sequenced with primers targeting the capsid region (Vinjé et al., 2004), and ORF1-ORF2 junction region using norovirus GI primers NVGIF and G1SKR and norovirus GII primers QNIF2d and G2SKR (this study; Kojima et al., 2002; Loisy et al., 2005). The adenovirus hexon gene region was sequenced with the primers hex1deg and hex2deg (Allard et al., 2001). The norovirus sequences were assigned using the Norovirus Genotyping Tool (Kroneman et al., 2011), and the adenovirus sequences were compared with the adenovirus hexon gene sequences in the NCBI database using BLAST (basic local alignment search tool).

4.6 DETECTION OF INDICATOR MICROBES (I–III, V)

E. coli was analysed (III, V) on Chromocult® Coliform Agar (Merck KgaA, Darmstadt, Germany) according to the manufacturer’s instructions and the international standard ISO 8199 (ISO, 2005) using spread plating and membrane filtration techniques. Moreover, E. coli and coliform bacteria were analysed (I) using membrane filtration with LES Endo (Merck KgaA, Darmstadt, Germany) and Chromocult Coliform Agar media (ISO, 2014), or by using the MPN method (I, II) based on defined substrate technology according to standard ISO 9308-2 (ISO, 2012).

Intestinal enterococci were determined (I–III) using membrane filtration on a Slanetz and Bartley medium (Oxoid, Basingstoke, UK) according to the standard method ISO 7899-2 (ISO, 2000).

Spores of sulphite-reducing clostridia were analysed according to the standard SFS-EN 26461-2 (SFS-EN, 1993) after pretreatment at 75 °C for 15 min on sulphite iron agar incubated in anaerobic jars at 37 °C (III). Additionally, vegetative cells and
spores of C. perfringens were enumerated (I) on tryptose sulphite cycloserine agar (Oxoid, Basingstoke, UK) incubated in anaerobic jars at 44 °C following the principles of the standard method ISO 14189 (ISO, 2013).

Somatic coliphages (host E. coli ATCC 13706) and F-specific coliphages (host E. coli ATCC 15597) were determined (III) with the double agar technique following ISO/DIS 10705-2.2 (ISO, 1998) and ISO 10705-1 (ISO, 1995), respectively, or with the single layer agar technique (Grabow & Coubrough, 1986) using the corresponding hosts. In study I, somatic and F-specific coliphages were analysed using the single agar layer method (USEPA, 2001b) and/or two-step enrichment method (USEPA, 2001a).

4.7 MODELLING OF NOROVIRUS DECAY CURVES (IV)

GlnaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool) (Geeraerd et al., 2005), a freeware add-in for Microsoft Excel 2010, was used for testing different microbial survival models. The models were selected based on the root mean sum of the squared errors (RMSE). The RMSE can be considered the simplest and most informative measure of goodness-of-fit, both for linear and non-linear models (Ratkowsky, 2003). The model with the lowest RMSE with a comparable experimental precision was considered the best fit. If the same or similar RMSE values were obtained, the less complex model was considered to fit best (Geeraerd et al., 2005).

In addition to the log-linear model (Equation (1)), three non-linear models; log-linear shoulder tail (Equation (2)), Weibull (Equation (3)) and double Weibull (Equation (4)) models were applied to describe the decay patterns. The log-linear model equation is described as follows:

\[
\log_{10}(N) = \log_{10}(N(0)) - \frac{k_{\text{max}} t}{\ln(10)}
\]

where \( t \) is the time, \( N \) represents the microbial cell density, \( N(0) \) the initial microbial cell density and \( k_{\text{max}} \) the first-order inactivation constant.

The log-linear shoulder tail equation (Geeraerd et al., 2000) is described as follows:

\[
\log_{10}(N) = \log_{10} \left(10^{\log_{10}(N(0))} - 10^{\log_{10}(N_{\text{res}})} \right) \times e^{-k_{\text{max}} t} \times \left(\frac{e^{k_{\text{max}} S_1}}{1 + (e^{k_{\text{max}} S_1})e^{-k_{\text{max}} t}}\right) + 10^{\log_{10}(N_{\text{res}})}
\]

where \( N_{\text{res}} \) is the residual population density and \( S_1 \) represents the shoulder length.

The Weibull model equation (Mafart et al., 2002; van Boekel, 2002) is described as follows:

\[
\log_{10}(N) = \log_{10}(N(0)) - \left(\frac{t}{\delta}\right)^p
\]

where \( \delta \) is a scale parameter representing the time for achieving a 1 log reduction and \( p \) is a shape parameter.
The double Weibull model equation (Coroller et al., 2006) is described as follows:

\[
\log_{10}(N) = \log_{10}\left[\frac{10^{\log_{10}(N(0))}}{1+10^\alpha} \times \left(10^{-\left(\frac{t}{\delta_1}\right)^p} + 10^{-\left(\frac{t}{\delta_2}\right)^p}\right)\right]
\]  

(4)

where the subscripts 1 and 2 indicate the two different subpopulations and \(\alpha\) is a parameter varying from negative infinity to positive infinity (Equation (5)): 

\[
\alpha = \log_{10}\left(\frac{\hat{f}}{1-\hat{f}}\right)
\]

(5)

where \(f\) is the fraction of subpopulation 1 in the population.

4.8 STATISTICAL ANALYSIS (I–V)

The statistical analyses were conducted using SPSS, versions 19, 20, 22 and 24 software for Windows (SPSS Inc., Chicago, IL, USA). Differences were considered significant if the \(p\) value was < 0.05. The theoretical method detection limit values were used for the calculations when a below limit of detection (LOD) result was obtained.

In the study of two drinking water outbreaks (I), prior to the calculations the microbial numbers were converted to their ten-base logarithms. The \(\log_{10}\) removal for the SF of an OWTS was determined by subtracting the effluent numbers from the numbers of microbes in the influent. Similarly, the additional removal was determined by subtracting the microbial numbers in a groundwater well from the numbers in the effluent. The \(\log_{10}\) reduction was calculated by subtracting the number of viruses at a given time point from the number of viruses on day 0. In the long-term persistence study, average reduction rates (\(\log_{10}/d\)) were calculated from the positive samples providing quantitative data. Statistical significances in the reductions of noro- and adenovirus numbers in the network were analysed by the independent samples Kruskal–Wallis test. Differences between the \(\log_{10}\) reductions of noro- and adenoviruses in the network were tested using the related-samples Wilcoxon signed-rank test.

In the bathing water outbreak study (II), the related samples Wilcoxon signed-rank test was used to test the significance of the FIB analyses, while comparing the bathing water outbreak samples with frequent-monitoring samples collected during the summer.

With the data from pilot SFs (III), the Spearman rank correlation coefficients \((r_s)\) were used to test the associations between the microbes in the influent and between the removals of different microbes in each SF. Only positive samples providing quantitative data were used in the correlation analyses. The comparisons between the removals of different microbes within an SF (only quantitative data counted) and a microbe between SFs (detection limits used) were carried out with the related samples Wilcoxon signed rank test. The statistical differences between different groups of samples (summer vs. winter) were calculated using the Independent-Samples Mann–Whitney U Test.
In the statistical analyses for the norovirus persistence study (IV), the related-samples Wilcoxon signed rank test was used to assess the statistical significance of differences in log$_{10}$ reductions between temperatures, matrices and norovirus strains. For the decay rate comparison, T90 and T99.99 (the time to reduce 90% and 99.99% of the initial numbers) were determined using the log-linear decay model. For non-linear decay, the time required to reduce the first log$_{10}$ (TFL) and the time required to reduce the fourth log$_{10}$ (T4L) were determined. Only positive samples providing quantitative data were used to determine the decay rates.

The statistical differences between the samples before and after the decontamination of a pipeline system (V) were calculated using the Independent-Samples Mann–Whitney U Test.
5 RESULTS

5.1 OCCURRENCE OF ENTERIC VIRUSES AND INDICATOR MICROBES IN WATER

5.1.1 Occurrence in wastewater (I, III)

The numbers of noroviruses and adenoviruses in the influent of municipal WWTP and effluents of the pilot SFs was determined during a one-year study (Table 6, III). In addition, the numbers of noro- and adenoviruses were determined from the influent and effluent wastewater of a private OWTS during drinking water outbreak I (Table 6, I).

The presence of enteric viruses in municipal influent, ranked from highest to lowest, was as follows: norovirus GII > adenoviruses > norovirus GI. Seasonality was observed with noroviruses, with the numbers being highest at the end of winter and declining at the end of the summer and autumn, except for a peak in the GI level in autumn. The GII genogroup predominated over GI for almost the entire year. The numbers of adenoviruses were stable and did not show seasonality. The occurrence of viruses in effluents of the pilot SFs loaded with municipal influent varied depending on the system and season (Table 6).

The mean numbers of faecal indicator microbes, except for clostridia, were higher compared to enteric viruses in the influent of municipal WWTP (Table 6). During a one-year study, a statistically significant positive correlation was noted in the influent between the occurrence of norovirus GII and intestinal enterococci ($r_s = 0.66$, $p = 0.002$). A lower correlation was noted between the occurrence of norovirus GII and $E. coli$ ($r_s = 0.50$, $p = 0.03$).

In the drinking water outbreak, high numbers of norovirus GII were found in the wastewater samples of a private OWTS, whereas norovirus GI and adenoviruses were not detected. In the influent of a private OWTS, the numbers of norovirus GII were comparable with the numbers of $E. coli$, whereas in the effluent norovirus GII was clearly the most abundant microbe.
Table 6. Occurrence of enteric viruses (GC/mL), faecal indicator bacteria (CFU/mL) and coliphages (PFU/mL) in wastewater in a pilot study (III) and outbreak study (I)

<table>
<thead>
<tr>
<th>Municipal WWTP influent and effluents of pilot SFs*</th>
<th>SF in outbreak</th>
</tr>
</thead>
<tbody>
<tr>
<td>WW influent</td>
<td>SF-1</td>
</tr>
<tr>
<td>Norovirus GI</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>18/19</td>
</tr>
<tr>
<td>Mean</td>
<td>80</td>
</tr>
<tr>
<td>Range</td>
<td>&lt;0.6–4 900</td>
</tr>
<tr>
<td>Norovirus GII</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>19/19</td>
</tr>
<tr>
<td>Mean</td>
<td>2 300</td>
</tr>
<tr>
<td>Range</td>
<td>43–42 000</td>
</tr>
<tr>
<td>Adenovirus</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>19/19</td>
</tr>
<tr>
<td>Mean</td>
<td>980</td>
</tr>
<tr>
<td>Range</td>
<td>210–2 800</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>20/20</td>
</tr>
<tr>
<td>Mean</td>
<td>33 000</td>
</tr>
<tr>
<td>Range</td>
<td>4 600–310 000</td>
</tr>
<tr>
<td>Intestinal enterococci</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>20/20</td>
</tr>
<tr>
<td>Mean</td>
<td>4 100</td>
</tr>
<tr>
<td>Range</td>
<td>500–85 000</td>
</tr>
<tr>
<td>Clostridia</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>20/20</td>
</tr>
<tr>
<td>Mean</td>
<td>400</td>
</tr>
<tr>
<td>Range</td>
<td>36–7 300</td>
</tr>
<tr>
<td>Somatic coliphages</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>20/20</td>
</tr>
<tr>
<td>Mean</td>
<td>4 900</td>
</tr>
<tr>
<td>Range</td>
<td>450–61 000</td>
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<tr>
<td>F-specific coliphages</td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>20/20</td>
</tr>
<tr>
<td>Mean</td>
<td>3 600</td>
</tr>
<tr>
<td>Range</td>
<td>140–50 000</td>
</tr>
</tbody>
</table>

*The number of positives/no. of experiments, geometric mean and range (min-max) are shown for municipal WWTP influent and effluents of pilot sand filters (SFs).

5.1.2 Occurrence in bathing water outbreak samples (II)

Noro- and/or adenoviruses were detected in water in three of the seven bathing water outbreaks (II). Norovirus GI was present in water collected during the investigation of outbreak II, norovirus GII in outbreak V and adenoviruses in outbreaks II and VIII. In total, 25% of the studied water samples (4/16) were positive for noro- and/or adenoviruses.

Elevated levels of *E. coli* and intestinal enterococci in bathing water were found in two of the outbreaks (VII and VIII), but only in outbreak VII did the number of *E. coli* (1 100 CFU/100 mL) exceed the limit for management actions (500 CFU/100 mL). In addition, elevated levels of enterococci were noted in outbreak I. In the
remaining outbreaks, the levels of FIB were low, and overall, no statistical difference in the levels of *E. coli* (p = 0.8) or enterococci (p = 0.086) were noted between the water samples taken during the outbreaks (n = 14) and frequent monitoring (n = 42), excluding the samples from outbreak VII, where a clear contamination source was noted.

5.1.3 Occurrence in groundwater and tap water during outbreaks (I)

The occurrences of enteric viruses and faecal indicators in groundwater and tap water were determined in two drinking water outbreaks. In outbreak I, where an OWTS caused the contamination of the groundwater well, the numbers of norovirus GII in the groundwater was 3 600 GC/100 mL on 8 August 2011 and 1 500 GC/100 mL on 23 August 2011 (sixteen days later). In tap water, norovirus GII was detected at 4 100 GC/100 mL on 23 August 2011. *E. coli* (1 CFU/100 mL) and coliform bacteria (2 CFU/100 mL) were detected in the groundwater well at low numbers compared to the norovirus GII numbers. Norovirus GI, intestinal enterococci, *C. perfringens*, somatic and F-specific coliphages were not detected in the groundwater samples.

In drinking water outbreak II, wastewater overflowed from a blocked drainpipe directly into the groundwater well. Before decontamination measures were enacted, norovirus GII was found in groundwater well at 390 GC/100 mL and in tap water at 950 ± 300 GC/100 mL, while adenoviruses were found at 190 GC/100 mL and 660 ± 220 GC/100 mL in groundwater well and tap water, respectively. In the same samples, *E. coli* (36 CFU/100 mL and 100 ± 44 CFU/100 mL), coliform bacteria (62 CFU/100 mL and 150 ± 45 CFU/100 mL) and intestinal enterococci (2 CFU/100 mL and 10 ± 5 CFU/100 mL) were detected in the groundwater well and tap water, respectively. *C. perfringens* was not detected in these samples when using a 100 mL volume.

5.2 TRANSPORT AND REMOVAL OF ENTERIC VIRUSES AND INDICATOR MICROBES IN SAND FILTERS AND SOIL (I, III)

The transport and removal of enteric viruses and faecal indicators in SFs was studied in a pilot-scale experiment (III) and drinking water outbreak I (I). In general, the removals of bacterial indicators were higher than those of enteric viruses and coliphages (Table 7).

SF-2 and SF-3+P were the most efficient pilot systems for removing microbes, and the numbers usually decreased to below LOD. In pilot SF-1, the average log_{10} removals were lower than achieved with SF-2 and SF-3+P, except for with adenoviruses, which were removed 0.3 log_{10} more efficiently in SF-1 than in SF-3+P (p = 0.017, n = 8). Pilot SF-3 was clearly the most inefficient system for removing microbes.
**Table 7. Log_{10} removals of enteric viruses and faecal indicators in sand filters (SFs)**

<table>
<thead>
<tr>
<th></th>
<th>SF-1*</th>
<th>SF-2</th>
<th>SF-3</th>
<th>SF-3+P</th>
<th>SF in outbreak</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Norovirus GI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Removal</td>
<td>1.6±0.9</td>
<td>2.2±1.0</td>
<td>0.6±0.6</td>
<td>2.2±0.8</td>
<td>nd</td>
</tr>
<tr>
<td>Range</td>
<td>0.3→3.5</td>
<td>&gt;0.3→3.5 (3.5)</td>
<td>0.0→2.0</td>
<td>&gt;1.2→3.6 (na)</td>
<td></td>
</tr>
<tr>
<td><strong>Norovirus GII</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>Removal</td>
<td>3.3±0.7</td>
<td>3.5±0.8</td>
<td>0.8±0.7</td>
<td>3.2±0.9</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.8→4.2</td>
<td>&gt;1.8→5.0 (2.2)</td>
<td>0.2→2.4</td>
<td>&gt;1.9→4.1 (3.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Adenovirus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nd</td>
</tr>
<tr>
<td>Removal</td>
<td>3.0±0.5</td>
<td>3.2±0.4</td>
<td>0.9±0.7</td>
<td>2.8±0.3</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.6→3.5</td>
<td>2.3→3.8</td>
<td>0.1→2.8</td>
<td>2.2→3.1</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;6.2</td>
</tr>
<tr>
<td>Removal</td>
<td>4.3±0.8</td>
<td>5.0±0.7</td>
<td>1.4±0.7</td>
<td>5.4±0.7</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>3.2→6.4</td>
<td>3.7→6.9</td>
<td>0.4→2.6</td>
<td>4.4→6.4</td>
<td></td>
</tr>
<tr>
<td><strong>Intestinal enterococci</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>Removal</td>
<td>5.3±0.8</td>
<td>5.4±0.7</td>
<td>1.4±0.5</td>
<td>5.1±0.4</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>3.7→6.5</td>
<td>4.2→6.9</td>
<td>0.5→2.3</td>
<td>&gt;4.7→6.0 (4.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Clostridia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Removal</td>
<td>4.6±0.5</td>
<td>4.6±0.5</td>
<td>0.9±0.6</td>
<td>4.5±0.4</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>&gt;3.6→5.9 (na)</td>
<td>&gt;3.6→5.9 (na)</td>
<td>0.0→2.4</td>
<td>&gt;3.6→5.1 (na)</td>
<td></td>
</tr>
<tr>
<td><strong>Somatic coliphages</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nd**</td>
</tr>
<tr>
<td>Removal</td>
<td>3.3±1.5</td>
<td>4.3±1.3</td>
<td>0.6±0.6</td>
<td>5.2±1.2</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.4→6.3</td>
<td>1.7→6.6</td>
<td>0.0→2.1</td>
<td>2.4→6.5</td>
<td></td>
</tr>
<tr>
<td><strong>F-specific coliphages</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nd</td>
</tr>
<tr>
<td>Removal</td>
<td>4.1±1.3</td>
<td>5.2±0.8</td>
<td>0.7±0.6</td>
<td>5.3±1.3</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.9→6.7</td>
<td>2.5→6.7</td>
<td>0.0→2.2</td>
<td>2.0→6.7</td>
<td></td>
</tr>
</tbody>
</table>

*nd = not detected; na = not available

*For the pilot SFs (SF-1-SF-3+P), the average removal and range (min-max) are shown, while the minimum quantitative value of the range (if observed) is shown in brackets.

**Somatic coliphages were only found in the collection tank of the OWTS.

Statistically significant differences between the removals of viruses were noted in the pilot SF-1. In SF-1, norovirus GII was removed 1.8 log_{10} more efficiently than norovirus GI (p = 0.068, n = 4), and F-specific coliphages were removed at about one log_{10} unit better than somatic coliphages in SF-1 (p = 0.001, n = 16) and SF-2 (p = 0.005, n = 10).

The effect of season on the removal of noro- and adenoviruses was noted in the pilot SF-1 and SF-3. In both SFs, the removal was lower during winter, and in SF-3 the reduction in the removal of adenoviruses by 0.7 log_{10} was statistically significant (p = 0.016). Moreover, in SF-1 somatic coliphages were removed 1.7 log_{10} (p = 0.01) less in winter and F-specific coliphages 1.4 log_{10} (p = 0.016) less in winter. In SF-3, the removals of other microbes, except for clostridia, were also reduced during winter. The performance of SF-2 was more stable than either SF-1 or SF-3 during winter. In SF-2, the winter removal rates for adenoviruses, norovirus GI and norovirus GII were 0.6 (p = 0.042), 1.3 (p = 0.017) and 0.6 log_{10} (p = 0.069) higher than in SF-1, respectively, and 2.9 (p = 0.012), 2.2 (p = 0.017) and 3.5 log_{10} (p = 0.017)
higher than in SF-3, respectively. Moreover, in SF-1 and SF-2 the removals of *E. coli* and intestinal enterococci were more stable throughout the year and did not decrease during winter. Neither under- nor overloading appeared to have a clear effect on the treatment efficiency of the SFs.

In SF-1, a statistically significant positive correlation was noted between the removal of norovirus G1 and *E. coli* (*r* = 0.89, *p* = 0.007, *n* = 7). In SF-2, no statistically significant correlations were noted between the removals of enteric viruses and indicator microbes. In SF-3, bacteria were removed similarly as a group, while on the other hand, the removals of enteric viruses and coliphages were correlated (*r* = 0.50–0.72). In addition, the removal of clostridia correlated with the removal of norovirus GII in SF-3 (*r* = 0.56, *p* < 0.05, *n* = 18).

In the drinking water outbreak study, the removals of norovirus GII and clostridia were comparable with the removals of pilot SF-3, whereas *E. coli* and intestinal enterococci showed higher removals comparable with pilot SF-1, SF-2 and SF-3+P (Table 7). The additional removal through soil between the collection tank of the OWTS and the groundwater well was 2.0 log_{10} for norovirus GII. For *C. perfringens* this additional removal was >3.5 log_{10}. For other indicator bacteria, only small additional removals were noted, partly due to proximity of the detection limit of the methods. For example, *E. coli* was already below LOD in the effluent sample taken from the collection tank.

### 5.3 PERSISTENCE OF ENTERIC VIRUSES AND INDICATOR MICROBES IN WATER

The persistence of noro- and adenovirus genome was assessed using laboratory experiments (I, IV, V) and the studies performed during the outbreak investigations (I).

#### 5.3.1 Persistence of norovirus in wastewater (I, IV)

The laboratory study (IV) with two spiked norovirus strains (GII_A and GII_B) and indigenous norovirus GII (GII_ind) showed that the persistence of noroviruses in wastewater was highest at 3 °C and lowest at 36 °C, except for GII_A, which showed the highest long-term persistence at 21 °C (Figure 3). Differences in persistence were noted between the strains, and GII_ind was statistically the most persistent at all temperatures. At 3 °C, GII_ind, GII_A and GII_B showed 0.8, 3.1 and 2.8 log_{10} reductions, respectively, during the 140-day study. The difference in removals between GII_A and GII_B was not statistically significant at 3 °C. At 21 °C, the persistence of all three strains differed statistically from each other, showing 1.3, 2.6 and 4.2 log_{10} reductions for GII_ind, GII_A and GII_B, respectively. At 36 °C, the numbers of all strains decayed below LOD and the reductions of GII_A and GII_B were not statistically different from each other during the first 40 days (p =
Modelling of the decay curves showed that log-linear decay was observed only at 36 °C. At lower temperatures, non-linear models produced the best fit for the data (Table 8 and 9).

In the long-term persistence study carried out with outbreak samples at 4 °C (I), the norovirus genome was detected in wastewater for up to 1262 days, with an average reduction rate of 0.0022 ± 0.0007 log\(_{10}\)/d.

### 5.3.2 Persistence of microbes in drinking water and distribution network biofilms (I, IV, V)

The laboratory study (IV) with the spiked norovirus strains (GII_A and GII_B) showed that the persistence of the virus genome in drinking water was highest at 3 °C and lowest at 36 °C (Figure 3, Table 9). At 3 °C, no reduction was observed during the one-year study, and no statistical difference in persistence between the two norovirus strains was noted. At 21 °C, both strains were also detected throughout the whole one-year study period, and the persistence was comparable during the first 80 days (log\(_{10}\) reduction 0.2 and 0.3, respectively, p = 0.059). Subsequently, GII_A persisted better achieving 1.8 and 3.3 log\(_{10}\) reductions, respectively (p = 0.001). Similarly, the persistence of GII_A and GII_B was comparable during the first 20 days (log\(_{10}\) reduction 0.5 and 0.6, respectively, p = 0.028) at 36 °C. After 20 days, GII_A clearly persisted better and was detectable throughout the whole study period compared to GII_B (p < 0.001), which was not detected after 160 days at 36 °C. Log-linear decay was observed only at 36 °C. At 21 °C, non-linear Weibull decay and the double Weibull decay models were applied to obtain the best fit for GII_A and GII_B, respectively (Table 8 and 9).

The drinking water outbreak studies (I) also showed the high persistence of viral genomes in water. In outbreak I, norovirus GII was detected for at least 16 days in a groundwater well, showing a 0.4 log\(_{10}\) reduction. However, the groundwater well might have been contaminated for up to about two months prior to the sampling if calculated from the onset of the illness cases (20 June–23 August 2011). In outbreak II, noro- and adenoviruses were detected in the contaminated groundwater well for at least 108 days. During this period, only about 2.6 and 1.2 log\(_{10}\) reductions for norovirus and adenovirus, respectively, were observed in the well. At the same time, *E. coli*, coliforms and intestinal enterococci were not detected in the well after the first samples (11 days later), and over 2.6, 2.8 and 0.3 log\(_{10}\) reductions, respectively, were noted. For comparison, norovirus GII and adenovirus reductions in the well were 2.0 and 0.5 log\(_{10}\), respectively, after 11 days. *C. perfringens* was not detected in the first samples, but after increasing the analysed volume from 100 mL to 1000 mL, *C. perfringens* was occasionally detected in subsequent well samples for at least 108 days.

In the long-term persistence study carried out with outbreak samples at 4 °C (I), the norovirus genome was detected in drinking water (containing both
groundwater well and tap water samples) for up to 1277 days, with an average reduction rate of 0.0017 ± 0.0003 log₁₀/d. Similarly, the adenovirus genome was detected for up to 1343 days in drinking water, indicating an average reduction rate of 0.0014 ± 0.0007 log₁₀/d.

The persistence of adenovirus in a laboratory-scale distribution network was studied in non-decontaminated control pipelines (V). In two separate experiments (PAA and chlorine), adenovirus was detected in both water and biofilm samples for up to four days following contamination. In comparison, *E. coli* was detected in water for three days and in biofilm occasionally for up to four days. The numbers of adenovirus decreased by 2.9 ± 1.4 log₁₀ in water after the first day of contamination. Within the same time, *E. coli* numbers declined by 6.7 ± 1.0 log₁₀. The final reduction of adenovirus was 2.4 and 4.4 log₁₀ in water after the three-day (PAA experiment) and four-day (chlorine experiment) experiments, respectively. During the same period of time, *E. coli* was reduced by 6.9, and by over 9.8 log₁₀ in water.
Figure 3. Persistence of the norovirus genome in drinking water (DW) and wastewater (WW) at different temperatures: (A) norovirus GII_A, (B) norovirus GII_B and (C) norovirus GII_ind (only in wastewater). The identified curves represent the modelled decay of best fit. The error bars show the standard deviation for duplicate extractions. The below LOQ results are shown with an asterisk (*), but not fitted into the curves. DW samples were collected for 365 days and WW samples for 140 days.
Table 8. Summary of the persistence results using the first-order log-linear and/or non-linear decay models: RMSE = root mean sum of squared error; DW = drinking water, WW = wastewater

<table>
<thead>
<tr>
<th>T</th>
<th>Water</th>
<th>Virus</th>
<th>$k_{\text{max}}$</th>
<th>R²</th>
<th>RMSE</th>
<th>Log-Linear Model</th>
<th>Non-Linear Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R²</td>
<td>RMSE</td>
</tr>
<tr>
<td>3 °C</td>
<td>DW</td>
<td>GII_A</td>
<td>N/A</td>
<td>0.957</td>
<td>0.30</td>
<td>2.46 ± 0.20</td>
<td>57.06 ± 2.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GII_B</td>
<td>N/A</td>
<td>0.986</td>
<td>0.14</td>
<td>2.63 ± 0.56</td>
<td>50.06 ± 1.94</td>
</tr>
<tr>
<td>WW</td>
<td></td>
<td>GII_A</td>
<td>0.06 ± 0.001</td>
<td>0.957</td>
<td>0.30</td>
<td>2.46 ± 0.20</td>
<td>57.06 ± 2.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GII_B</td>
<td>0.05 ± 0.002</td>
<td>0.986</td>
<td>0.14</td>
<td>2.63 ± 0.56</td>
<td>50.06 ± 1.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GII ind</td>
<td>0.02 ± 0.003</td>
<td>0.801</td>
<td>0.20</td>
<td>58.85 ± 3.64</td>
<td>0.23 ± 0.17</td>
</tr>
<tr>
<td>21 °C</td>
<td>DW</td>
<td>GII_A</td>
<td>0.01 ± 0.001</td>
<td>0.885</td>
<td>0.22</td>
<td>298.2 ± 8.6</td>
<td>2.63 ± 0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GII_B</td>
<td>0.02 ± 0.002</td>
<td>0.962</td>
<td>0.28</td>
<td>2.20 ± 0.20</td>
<td>131.6 ± 10.3</td>
</tr>
<tr>
<td>WW</td>
<td></td>
<td>GII_A</td>
<td>0.04 ± 0.010</td>
<td>0.577</td>
<td>0.80</td>
<td>9.42 ± 0.30</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GII_B</td>
<td>0.06 ± 0.020</td>
<td>0.544</td>
<td>1.35</td>
<td>4.71 ± 0.73</td>
<td>0.59 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GII ind</td>
<td>0.02 ± 0.005</td>
<td>0.636</td>
<td>0.31</td>
<td>8.40 ± 2.25</td>
<td>0.30 ± 0.09</td>
</tr>
<tr>
<td>36 °C</td>
<td>DW</td>
<td>GII_A</td>
<td>0.04 ± 0.003</td>
<td>0.967</td>
<td>0.31</td>
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<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GII_B</td>
<td>0.07 ± 0.004</td>
<td>0.988</td>
<td>0.06</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>WW</td>
<td></td>
<td>GII_A</td>
<td>0.21 ± 0.010</td>
<td>0.996</td>
<td>0.11</td>
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<td>N/A</td>
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<tr>
<td></td>
<td></td>
<td>GII_B</td>
<td>0.18 ± 0.010</td>
<td>0.982</td>
<td>0.19</td>
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<td>N/A</td>
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<tr>
<td></td>
<td></td>
<td>GII ind</td>
<td>0.05 ± 0.010</td>
<td>0.880</td>
<td>0.07</td>
<td>N/A</td>
<td>N/A</td>
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</tbody>
</table>

N/A = not applicable
5.3.3 Effect of the matrix on persistence (IV)

A comparison of the decay rates obtained from the laboratory study showed that GII_A and GII_B persisted better in drinking water compared to wastewater at each temperature (Table 9). These differences were statistically significant at 21 °C and 36 °C.

Table 9. Decay rates of the norovirus genome at different temperatures and under different water matrices: T90 and T99.99 values (days) for log-linear decay, and TFL and T4L (time required to achieve 1 and 4 log₁₀ reduction, respectively) values (days) for non-linear decay are presented: DW = drinking water, WW = wastewater

<table>
<thead>
<tr>
<th>T</th>
<th>Water</th>
<th>Virus</th>
<th>Best Fitting Model</th>
<th>Log-Linear</th>
<th>Non-Linear</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T90</td>
<td>T99.99</td>
<td>TFL</td>
</tr>
<tr>
<td>3 °C</td>
<td>DW</td>
<td>GII_A</td>
<td>N/A</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_A</td>
<td>N/A</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_A</td>
<td>Double Weibull</td>
<td>38</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_B</td>
<td>Double Weibull</td>
<td>45</td>
<td>179</td>
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<td></td>
<td>WW</td>
<td>GII_ind</td>
<td>Log-linear shoulder tail</td>
<td>115</td>
<td>461</td>
</tr>
<tr>
<td>21 °C</td>
<td>DW</td>
<td>GII_A</td>
<td>Weibull</td>
<td>230</td>
<td>921</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_A</td>
<td>Log-linear shoulder tail</td>
<td>58</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_B</td>
<td>Log-linear shoulder tail</td>
<td>38</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_ind</td>
<td>Log-linear shoulder tail</td>
<td>115</td>
<td>461</td>
</tr>
<tr>
<td>36 °C</td>
<td>DW</td>
<td>GII_A</td>
<td>Log-linear</td>
<td>58</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_A</td>
<td>Log-linear</td>
<td>33</td>
<td>132</td>
</tr>
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<td>WW</td>
<td>GII_B</td>
<td>Log-linear</td>
<td>11</td>
<td>44</td>
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<tr>
<td></td>
<td>WW</td>
<td>GII_ind</td>
<td>Log-linear</td>
<td>13</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>GII_ind</td>
<td>Log-linear</td>
<td>46</td>
<td>184</td>
</tr>
</tbody>
</table>

N/A=not applicable; Na=not achieved
5.4 DECONTAMINATION OF A DRINKING WATER DISTRIBUTION NETWORK (I, V)

The effect of the decontamination measures on the occurrence of enteric viruses and indicator microbes in a drinking water distribution network was studied in drinking water outbreak II (I) and a laboratory-scale pipeline system (V). In outbreak II, noro- and adenoviruses were detected in the drinking water distribution network for at least 59 days from the time of notification of the contamination (19 days from the start of the continuous chlorination), showing 2.8 and 1.7 average log_{10} reductions, respectively. This result suggests that adenoviruses are more persistent than noroviruses in a chlorinated distribution network (p = 0.028). Eventually, noro- and adenoviruses were reduced to below LOD after 108 days (68 days from the start of the continuous chlorination), achieving average reductions of >3.3 log_{10} (p = 0.027) and >3.2 (p = 0.025) log_{10}, respectively.

E. coli, coliform bacteria and intestinal enterococci were removed effectively from the chlorinated distribution network during waterborne outbreak II. They were not detected in the tap water samples 11 days after the time of notification of the contamination. During these 11 days (3 June–14 June 2011), E. coli was reduced by >3 log_{10} in tap water. At the same time, norovirus GII and adenoviruses were reduced by 1.5 and 1.0 log_{10}, respectively. C. perfringens was occasionally detected at low numbers in the tap water samples for at least 122 days, but not after 131 days.

In the laboratory-scale pipeline study (V), PAA and chlorine effectively removed adenoviruses from the system. With PAA, the outlet water was decontaminated within 24 h (>3.3 log_{10} reduction after the start of decontamination and >5.3 log_{10} total reduction), and with chlorine it took from 24 to 48 h to decontaminate the water (>4.0 log_{10} reduction after the start of decontamination and >5.5 log_{10} total reduction). In the biofilm samples, adenoviruses were not detected after 24 h and 1 h from the start of decontamination with PAA and chlorine, respectively. E. coli was no longer detectable in the water after 1 h of decontamination (PAA and chlorine), except for one sample in the chlorine experiment after 72 h. The total decrease of E. coli in the water was >8.4 log_{10} in the PAA experiment (>2.9 log_{10} after the start of decontamination) and >9.8 log_{10} in the chlorine experiment (>2.3 log_{10} after the start of decontamination). In biofilms, E. coli was decontaminated within 1 h in the PAA experiment and within 24 h in the chlorine experiment.
6 DISCUSSION

6.1 OCCURRENCE OF ENTERIC VIRUSES IN FINNISH WATER ENVIRONMENTS (I–III)

In Finland, treated wastewater effluents are commonly discharged into surface waters, usually rivers or lakes. An awareness of the potential occurrence of pathogenic microbes in these water environments is important in order to assess the possible health risks to humans, e.g. during recreational activities. In this study, norovirus GI, norovirus GII and adenoviruses were found in 94.7%, 100% and 100%, respectively, of 19 influent samples collected from municipal WWTP during a one-year period. The high prevalence of enteric viruses in a medium- to large-sized municipal WWTP has been commonly observed in many studies worldwide (Nordgren et al., 2009; Hewitt et al., 2011; Hata et al., 2013; Kitajima et al., 2014; Victoria et al., 2014). Due to inefficient wastewater treatment, effluents often contain considerable numbers of enteric viruses, as also noted in a recent study, where norovirus GI, norovirus GII and adenoviruses were detected in 85.7%, 83.9% and 91.1% of the 56 effluent samples collected from seven municipal WWTPs (Perkola et al., unpublished). In Finland, few studies have described the presence of enteric viruses in wastewater and surface water under the influence of wastewater (Hörman et al., 2004; Maunula et al., 2012; Hokajärvi et al., 2013).

The stable occurrence of adenoviruses in wastewater throughout the year, as noted in this study, supports their use as markers of human wastewater pollution (Pina et al., 1998; Bofill-Mas et al., 2006; Katayama et al., 2008; Schindwein et al., 2010). Although noroviruses were also detected throughout the year in wastewater, their numbers varied, being highest in winter, as also stated previously (Haramoto et al., 2006; Nordgren et al., 2009). Seasonal peaks observed in the occurrence of noroviruses suggest typical local epidemics among the population. The common occurrence of enteric viruses in municipal WWTPs throughout the year poses a constant health risk through the use of receiving waters. In addition, the increased health risk must be taken into account during, e.g. the malfunction of the wastewater treatment systems or sewage pipe breakages.

In small-scale systems, like private OWTSs, the occurrence of pathogenic viruses is occasional and may vary from non-detected to very high numbers, since enteric viruses are only present in the stools of an infected person. In this study, norovirus GII was detected in high numbers in the influent and effluent of the OWTS causing the waterborne outbreak, whereas adenoviruses were not detected. Noteworthy, due to high number of viruses excreted in faeces, it is possible that even one infected person may challenge the treatment capacity of a small system and lead to contamination of the groundwater and/or surrounding surface water. In addition, a
recent one-year field study showed a high prevalence of noro- and adenoviruses in the effluents of seven private SF-based OWTSs (Martikainen et al., 2018). Martikainen et al. (2018) found that adenoviruses were detected in the effluents of all seven systems (with 39.6% of the samples being positive), and norovirus GII was detected in six of seven systems (with 72.9% of the samples being positive), suggesting that these systems may act as virus reservoirs. However, studies describing the outbreaks associated with OWTSs are still rare (Anderson et al., 2003; Borchardt et al., 2011; Gunnarsdottir et al., 2013; Jack et al., 2013) and may well be under-reported. One reason for this may be that the health problems related to the water quality of the households using private systems remains within the family or small community. The outbreak caused by the OWTS described in this study might also have remained hidden without the high number of visitors to the property. Therefore, public awareness of the risks associated with OWTSs should be increased.

High numbers of noroviruses were detected in groundwater and tap water samples in drinking water outbreaks I and II. Both outbreaks were clearly caused by wastewater contamination of the groundwater well. These outbreaks are good representatives of typical Finnish drinking water outbreaks that occur in small vulnerable groundwater supplies, which do not treat the water (Zacheus & Miettinen, 2011; Klove et al., 2017).

On the other hand, only 25% of the water samples collected during seven bathing water outbreaks were positive for noro- and/or adenoviruses. The small number of positive samples may be partly explained by the challenges related to the timing of the sampling period. In natural surface waters, the decay of the viruses due to environmental stress (water temperature >25 °C and UV light) may occur faster compared to, e.g. groundwater (Bae & Schwab, 2008). In addition, virus sedimentation may have played a larger role since the restrictions against bathing were already set. Most of the bathing water outbreaks occurred in small lakes, suggesting that the increased number of users due to a long heatwave (FMI, 2014) exceeded the self-cleaning capacity of the beach. In these outbreaks, where no external contamination source could be detected, the investigations suggested that the source of the contamination were the beach users themselves.

6.2 TRANSPORT AND REMOVAL OF ENTERIC VIRUSES IN SAND FILTERS AND SOIL (I, III)

The transport and removal of viruses in soil is affected by a variety of physicochemical and biological factors, and the numbers of viruses are decreased by die-off, physical straining or by adsorption into soil surfaces (Schijven & Hassanizadeh, 2000; Stevik et al., 2004; Pedley et al., 2006; Lusk et al., 2017). An OWTS based on soil infiltration takes advantage of this removal. In this study, the transport of viruses was assessed by their capability to pass through soil in SFs and
measured by log$_{10}$ removals. Both the pilot-scale study (III) and outbreak study (I) revealed that viruses can transport through SFs, and significant differences in removal results between the systems were noted.

During a one-year pilot study, the log$_{10}$ removal of enteric viruses ranged from 0.0 to over 5.0, depending on the SF, season and virus. In the waterborne outbreak caused by an OWTS, the log$_{10}$ removal of norovirus GII in SF was 1.1. This produced an effluent that still contained remarkably high numbers of noroviruses (1 400 GC/mL), which managed to break into the groundwater. It was calculated that, in theory, it would have required from 1.8 to over 4.0 log$_{10}$ removal of noroviruses in the SF (assuming that the additional removal after SF was 2.0 log$_{10}$) to reduce the probability of occurrence of the outbreak. Then, the groundwater would have contained noroviruses at less than the infectious dose for daily per capita water consumption (18–2 800 GC/L) (Teunis et al., 2008; WHO, 2011; Atmar et al., 2014). Based on our pilot study and previous studies (Olson et al., 2005), it might have been possible to achieve the required removal by using the available SF-based systems that have shown the best microbial removal efficiencies.

The log$_{10}$ removals of viruses in the best pilot SFs may be regarded as high compared to those achieved in conventional municipal WWTPs, where virus removal shows a high rate of variation and is typically up to 2–3 log$_{10}$ (Koivunen et al., 2003; Wen et al., 2009; Nordgren et al., 2009; Naughton & Rousselot, 2017). The pilot SFs also performed well compared to different OWTSs based on sand filtration or other principles (summarised by Olson et al., 2005). Moreover, in this study many of the effluent results for enteric viruses were below LOD, thus the actual removal efficiencies were underestimated. The wastewater used in the pilot SFs originated from a relatively large WWTP and may be expected to provide more stable enteric virus numbers than wastewater produced by one family or a small community (Hewitt et al., 2011). The SF of an OWTS might be occasionally loaded with exceptionally high numbers of pathogenic viruses, as noted in the outbreak study, which may result in higher penetration of these viruses through the systems, representing a realistic threat to receiving waters.

Even though no effect of under- or overloading was observed in the pilot SFs, the environmental conditions preceding the outbreak caused by an OWTS, including a long dry season (0.6 mm within 13 days) followed by a high precipitation period (47.1 mm within 7 days) (FMI, 2017), may have favoured the transport of viruses through soil. This suggestion is supported by previous studies showing an association between waterborne outbreaks and heavy rainfall events (Curriero et al., 2001; Fong et al., 2007; Vantarakis et al., 2011; Mellou et al., 2014; Wallender et al., 2014) and high precipitation with enhanced virus transport into groundwater (Bradbury et al., 2013; Gotkowitz et al., 2016). The mechanism behind this may be partly explained by the increased saturation state, which favours transport, but also the low ionic strength of rainfall water may cause bound viruses to desorb from the soil particles (Schijven & Hassanizadeh, 2000; Nicosia et al.,
In addition, increased precipitation may cause a rise in the groundwater table and thus reduce the vertical separation distance between the infiltrative surface and the groundwater responsible for virus removal.

Seasonal functioning of SFs is important in cold temperate climates, since the microbial purification efficiency of OWTSs may be reduced during winter (Pundsack et al., 2001; Olson et al., 2005; study III). This may be partly explained by the lower amount of biofilms required for optimal purification, and it still remains to be determined how the biofilms of SFs can survive throughout the winter. Other factors that can enhance virus transport during winter may be related to low temperature, which favours virus survival as well as may reduce the antagonistic effect of indigenous microorganisms (John & Rose, 2005; Motz et al., 2012; Lusk et al., 2017).

The results suggest that norovirus GII was removed more efficiently than GI in the pilot SFs. Similarly, da Silva et al. (2007), Haramoto et al. (2006) and Nordgren et al. (2009) presented higher removals for norovirus GI than GII during wastewater treatment. The difference may be attributable to differences in the charge and hydrophobicity of the surface of the virus, which are affected by environmental characteristics such as pH and ionic strength (Schijven & Hassanizadeh, 2000). In addition, adenoviruses seemed to be more resistant than noroviruses to alkaline conditions (pH 12.3), which were present in SF-3+P. This may be partly due to the nature of the virus genome, adenoviruses having a more stable dsDNA genome compared to the ssRNA genomes of noroviruses.

In Finland, municipalities have set the minimum setback distances between an OWTS and the drinking water supply. Typically, a 30–100 m distance is required between the OWTS and drinking water well, and a 0.5–2 m vertical protective soil layer between the OWTS and the groundwater table. Similarly, most states in the USA have adopted a setback distance of 30.5 m between drinking water wells and septic systems (DeBorde et al., 1999), and a vertical separation distance of 30–45 cm between the bottom of the drain field and the seasonal high groundwater table (USEPA, 2002; Karathanasis et al., 2006). In our study, the setback distance of 45 m was not enough to prevent groundwater contamination and a drinking water outbreak. The exact thickness of the groundwater protective soil layer was not known, but the system was constructed according to local regulations (a minimum setback distance of 30–50 m and a vertical separation distance of 1 m). Other studies have also demonstrated the potential for the rapid and extensive movement of viruses ranging from days to weeks during tens to hundreds of metres of distances between an OWTS and groundwater well (Scandura & Sobsey, 1997; Borchardt et al., 2007; Borchardt et al., 2011; Bradbury et al., 2013). For instance, Borchardt et al. (2011) found that noroviruses likely traveled a distance of 188 m from an OWTS to an adjacent private groundwater well in 15 days in a 35-m thick vadose zone of fractured aquifer. A recent study calculated the safe setback distances at a range of 39–144 m in sand aquifers, 66–289 m in gravel aquifers and 1–2.5 km in coarse
gravel aquifers (Blaschke et al., 2016). Thus, the results strongly suggest that a rapid pathogen transport through an aquifer is highly site-specific and that current construction practices do not necessarily prevent viral waterborne outbreaks. Overall, these studies underline the importance of proper site-specific planning, construction and placement of the OWTS for their successful and safe operation.

### 6.3 PERSISTENCE OF ENTERIC VIRUSES IN WATER (I, IV, V)

The persistence of viruses plays an important role in water safety assessments. The management of contamination cases as well as specific modelling and risk assessment scenarios related to, e.g. the transport and fate of viruses in a water environment, requires information regarding their persistence and survival. In this study, the long persistence of virus genomes in water environments was noted. Noro- and adenovirus genomes were detected in the outbreak water samples stored at 4 °C for up to 1 277 and 1 343 days, respectively. In addition, no reduction in norovirus genome numbers was observed in drinking water at 3 °C over the course of the one-year laboratory study. These results are in line with previous studies presenting the long persistence of enteric viruses in water (Charles et al., 2009; Ogorzaly et al., 2010; Seitz et al., 2011).

This study clearly demonstrated the effect of temperature, water matrix and norovirus strain on genome persistence. Noroviruses persisted longer in cold temperatures in both drinking and wastewater, which is a consistent result with previous studies showing the temperature dependency of norovirus genomes with respect to persistence (Bae & Schwab, 2008; Ngazoa et al., 2008; Skraber et al., 2009; Liu et al., 2012). In Finland, the temperature of groundwater, which follows the annual average air temperature, ranges from 3 °C to 7 °C (Arola, 2015). This temperature range may be considered ideal for the persistence of viruses. The long persistence of the norovirus genome, especially at lower temperatures, may increase the possibility of viruses reaching groundwater as well as cause prolonged outbreak management cases, as shown in our drinking water outbreak study.

On the other hand, in surface waters, where the temperature range is higher and depends on the season (<4 °C in winter and >20 °C in summer in Finland), the persistence of viruses may be lower during summer. During the summer, other factors, such as the UV light, also negatively affects the persistence of viruses in surface waters. In bathing water outbreak II, noro- and adenoviruses were detected in the water on at least six days, but fewer than 12 days.

In addition to temperature, the water matrix was found to have a significant role in the persistence of the norovirus genome in laboratory study (IV). Norovirus decay appeared to occur faster in wastewater than in drinking water. This finding regarding better persistence of noroviruses in clean water is consistent with previous studies (Bae & Schwab, 2008; Ngazoa et al., 2008; Bertrand et al., 2012). This is probably due to the higher presence of organic matter and indigenous
microorganisms in wastewater, which may have negative effects on norovirus persistence (John & Rose, 2005; Gerba, 2007; Murphy, 2017; Yates, 2017). However, in the long-term persistence study carried out with outbreak samples at 4 °C (I), the effect of the matrix on the persistence of the norovirus GII genome was not evident. The long-term persistence of noro- and adenoviruses in wastewater is also supported by a field study, where these viruses were detected over several seasons within the same OWTS effluents (Martikainen et al., 2018).

Differences were also noted between the persistence of different norovirus strains and even between the same genotype in different inoculums. These differences may be attributable to several factors, such as the genotype, age and status of the virus, as well as other contents originating from the stool, such as antiviral compounds and enzymes (nucleases and proteases). It has been suggested that norovirus GI genotypes are more frequently involved in food- or waterborne outbreaks than GII, which could imply that GI is more stable in the environment (Lysen et al., 2009; Butot et al., 2009; Perez-Sautu et al., 2012). This finding is partly supported by the high prevalence of norovirus GI in investigated bathing water outbreaks (II). However, controversial results have also been presented (Verhaelen et al., 2012), and more studies are needed to reveal the impact of the virus strain on persistence.

The decay rates of the noroviruses used in this study were comparable or lower than those presented in previous studies (Bae & Schwab, 2008; Skraber et al., 2009; Liu et al., 2012). This may be explained by differences in experimental conditions, such as the test water properties as well as the studied norovirus strain and its status, but also by the length of the experiment. In this study, non-linear decay was characteristic of norovirus genome degradation at lower temperatures. This suggests that the commonly applied simple first-order, log-linear regression model (Bae & Schwab, 2008; Skraber et al., 2009; Liu et al., 2012) may not produce the best fit, but sufficient follow-up time as well as non-linear modelling is required to reliably assess the decay of viruses in water. Indeed, a tailing effect was commonly noted in the non-linear decay curves in wastewater and may explain the noted long-term persistence of norovirus in stored outbreak samples. Previous studies have also used non-linear modelling successfully to describe the persistence of viruses (Charles et al., 2009; Verhaelen et al., 2012; Bozkurt et al., 2014; Kim et al., 2017).

Our laboratory-scale pipeline study (V) showed that adenoviruses remained detectable in both the water and biofilms for at least four days after the contamination if a decontaminant had not been added. Other studies have also noted the long persistence of enteric viruses in drinking and wastewater biofilms (Storey & Ashbolt, 2003; Skraber et al., 2005; Lehtola et al., 2007). These findings underline the importance of active cleaning measures.

In the risk assessments related to control and management of outbreaks, molecular methods are commonly used for norovirus detection without the
knowledge of the infectious state of the virus (ISO/TS, 2013). Due to the methodological limitation, the long-term persistence results do not necessarily reliably describe the health risk for consumers. However, noroviruses have been shown to remain infectious in groundwater for at least 61 days in a human volunteer study (Seitz et al., 2011). Moreover, human adenovirus 41 has remained infectious in both drinking water and surface water after 70 days of incubation at 4 °C and 20 °C (Prevost et al., 2016), and human adenovirus 2 in groundwater for at least 364 days at 12 °C (Charles et al., 2009). Therefore, particularly in drinking water it may be reasonable to determine that water containing even traces of the virus genome is unsuitable for human consumption. On the other hand, results based on the molecular detection of noroviruses in other environmental samples, such as surface water, should be interpreted with care and in the context of available epidemiological or clinical information (Boxman et al., 2011; Lopman et al., 2012).

6.4 DECONTAMINATION OF DISTRIBUTION NETWORK (I, V)

Due to the long persistence of viruses in water, active cleaning measures are necessary for contamination events. However, a contaminated distribution network may be challenging to clean. Even though the laboratory-scale pipeline system was quickly decontaminated off adenoviruses with PAA or chlorine (within 24 h and 48 h in the biofilm and water samples, respectively), the practice in the field is more challenging. Many factors, such as the age of the network, biofilm richness, possible dead-ends in the network, initial extent of the contamination, presence of the contamination source, decontaminant demand in the network, availability of trained personnel and delays in starting the decontamination may hamper decontamination practices. Due to these factors, in the drinking water outbreak study it took 108 days from the notification of contamination to ascertain the removal of noro- and adenoviruses from the distribution network. Indeed, viruses were still detected 19 days after the start of the continuous chlorination. Similarly, Miettinen et al. (2012) found that even enhanced chlorination was unable to efficiently clean the contaminated distribution network in a large waterborne outbreak, where viruses were detected for over two months (Laine et al., 2011).

The biofilms covering a pipe’s interior may play a central role in the success of the decontamination measures in a drinking water distribution system (Quignon et al., 1997; Storey & Ashbolt, 2003; Långmark et al., 2005; Skraber et al., 2005; Lehtola et al., 2007; Miettinen et al., 2012). Our laboratory study showed that viruses were bound in biofilms and were constantly shed into the water in small amounts. Thus, careful and sufficiently long cleaning of the pipes is required, even though the contamination source can be eliminated.
6.5 VALUE OF INDICATOR MICROBES (I–III, V)

The high variety of possible pathogens present in water makes the use of indicator microbes economically reasonable. The routine uses of water quality indicators may, in the best case, reveal a water contamination in time and prevent a waterborne outbreak. However, usually patients are the first sign of water contamination with pathogens. There are numerous examples of outbreaks where indicators have been absent or within the required level, but enteric viruses have been detected (Bosch et al., 1991; Maunula et al., 2005; Martinelli et al., 2007; Blanco et al., 2017; Parkkali et al., 2017). In this study, *E. coli* was able to indicate groundwater contamination in two drinking water outbreaks and intestinal enterococci was detected in outbreak II. In addition, *C. perfringens* was detected after increasing the analysed volume from 100 mL to 1 000 mL. These findings may be partly explained by the massive contamination of the groundwater noted in these outbreaks. On the other hand, coliphages (analysed only in outbreak I) were not detected in the contaminated groundwater samples. In the bathing water outbreaks, the FIB threshold for management actions was exceeded only in one of eight outbreaks. For this outbreak, a clear external contamination source was identified, as 2 000–3 000 m$^3$ of raw wastewater had overflowed near the bathing site. In the other outbreaks, the levels of FIB were low and the bathing water quality was classified as excellent according to the European Union’s Bathing Water Directive (EU, 2006). This finding is supported by a Dutch study showing a lack of correlation between high compliance with European bathing water legislation and the occurrence of outbreaks (Schets et al., 2011).

Both the pilot-scale study and the outbreak study revealed that viruses can transport through the SFs of OWTSs more efficiently than the commonly used indicator bacteria. No consistent correlations in transport were noted between the indicators and enteric viruses, except in SF-3, where coliphages were removed similarly to the enteric viruses. In the outbreak study, a similar removal was noted with norovirus GII and *C. perfringens* in the SF. This observation is partly supported by the positive correlation noted between the removals of norovirus GII and clostridia in pilot SF-3. However, in the outbreak study *C. perfringens* showed higher additional removal between an OWTS and groundwater well compared to norovirus GII, and it was not detected in the groundwater well.

Decontamination measures performed in the drinking water outbreak study were effective against *E. coli*, coliform bacteria and intestinal enterococci. These indicators were efficiently reduced from both well and tap water most probably due to excessive chlorination and the flushing measures performed after the first sampling. Similarly, in the laboratory-scale pipeline study the counts of *E. coli* declined efficiently in water and biofilm even without decontamination, as has also been shown in a previous study (Lehtola et al., 2007). However, *C. perfringens* proved to be persistent, and in outbreak study the tap water still contained small
amounts of *C. perfringens* 82 days after the start of continuous chlorination. The long persistence of *C. perfringens* in water has also been noted earlier (Medema, 1997).

Our study supports the consensus that traditional FIB may be inadequate to describe the occurrence and fate of enteric viruses in water (Leclerc et al., 2001; Fong & Lipp, 2005; Harwood et al., 2005; Boehm et al., 2009; Jovanović Galović et al., 2016). Even though correlations can be found in influent, different transport properties and the survival characteristics of indicators and enteric viruses hinder further correlations. Nevertheless, the use of indicator microbes, especially *E. coli*, in routine water quality analysis for the detection of drinking water contamination is reasonable. In addition, *C. perfringens*, if present, may be a good candidate for verifying the safety of a drinking water distribution system after decontamination. However, indicator analyses from larger volumes of water may be needed, as demonstrated in our study with *C. perfringens*. Overall, the value of indicator microbes in the prediction of enteric viruses seems to be case-specific and may require massive contamination. Therefore, direct monitoring of pathogenic viruses may be required to assess the health risks related to water.

### 6.6 FUTURE RESEARCH NEEDS

In Finland, contamination of groundwater sources poses a greatest risk for water safety (Zacheus & Miettinen, 2011; Klove et al., 2017). Particularly, the small groundwater plants producing drinking water without any additional treatment are vulnerable to contamination. OWTSs located close to a groundwater well providing the drinking water are one of the largest threats to the microbiological contamination of groundwater (Scandura & Sobsey, 1997; Beller et al., 1997; Borchardt et al., 2003; Anderson et al., 2003; Borchardt et al., 2011; Bremer & Harter, 2012; Gunnarsdottir et al., 2013; Jack et al., 2013; Pitkänen et al., 2015). OWTSs are not especially planned for microbial removal, but rather for the removal of solids and nutrients. More studies are needed for a better understanding of the transport of enteric viruses in soil and aquifer environments. In particular, the complex spatial and temporal variations in factors affecting virus transport and their fate in situ need more careful examination. This knowledge is needed to direct the current construction practices toward more efficient OWTSs in regards to virus removal.

In addition to the physicochemical and microbiological properties of soil, the processes that may enhance virus transport, such as high precipitation events (Bradbury et al., 2013; Gotkowitz et al., 2016), need careful investigation. In the future, extreme weather events, including heavy rainfalls, are expected to increase due to climate change, and this may cause more groundwater contaminations. A better understanding of virus transport in soil and groundwater will also be needed regarding ageing water infrastructure. For example, leakages in an old distribution network and sewerage systems may challenge water safety more often in the future. Overall, the increased knowledge can be used to establish safe site-specific
setback distances in order to minimise the risk of groundwater contamination. In addition, this information would be useful for the proper management of contamination cases.

More efficient OWTSs are needed in vulnerable locations, where safe setback distances may be impossible to achieve with current systems. In these locations, closed septic tanks can be used. However, the tertiary treatment of wastewater or disinfection might also help to achieve the treatment requirements (Naidoo & Olaniran, 2014). More studies are needed to assess the applicability of tertiary treatment systems tailored also for small-scale systems or private households.

More studies are also needed for assessing the risks related to the increased usage of small inland bathing sites vulnerable to contamination. As noted in this study, heatwaves will most probably increase the recreational use of surface waters. This will pose an increasing infection risk to beach users, since enteric viruses may end up in water not only through wastewater discharge but also through an individual’s excretion during usage of the beach (Gerba, 2000). Predicted changes in the climate may increase the occurrence of heatwaves and thus the risk of bathing water outbreaks.

New methods for the detection of viruses from environmental samples are constantly being developed and the need for more efficient methods continues in the future. Especially water samples that putatively contain small numbers of viruses, such as groundwater and drinking water, need to be analysed using high volumes of water. More efficient concentration methods, such as DEUF (Kearns et al., 2008; Leskinen et al., 2009; Smith & Hill, 2009; Francy et al., 2013; Rhodes et al., 2016), will help to improve the recognition of water contamination cases. Increased knowledge about the microbial pollution of groundwater and surface water is also a prerequisite for better preparedness and risk estimations. Improved methodology and standardisation is also important in order to enable reliable comparisons of the studies.

Metagenomic sequencing has been recognised as a promising analytical tool because it detects all viruses in a single protocol (Nieuwenhuijse & Koopmans, 2017). With this method, new and emerging viruses and variants can also be detected. Nevertheless, several issues related to sample preparation, bioinformatic tools and general knowledge of the virome must be addressed before metagenomic sequencing can be applied in environmental surveillance and outbreak investigations (Nieuwenhuijse & Koopmans, 2017).

In this study, enteric viruses were detected with molecular methods based on the genome detection of a virus. Therefore, the results may not reliably describe the infectious risks related to positive virus findings. Despite recent progress in the cultivation of noroviruses (Jones et al., 2015; Ettayebi et al., 2016), currently no reliable and feasible test is available for estimating the loss of norovirus infectivity (Knight et al., 2013; Knight et al., 2016). However, the results of this study emphasise the need for and importance of a practical and reliable infectivity assay.
for noroviruses to reveal the actual infectious risk related to long-term genome persistence. In the future, before comprehensive infectivity tests are available, it might be reasonable to include a test with, e.g. PMA, to obtain a better estimate of the associated risk, especially in outbreak and contamination situations where decontamination practices are performed (Fuster et al., 2016).
7 CONCLUSIONS

Based on the results, the main conclusions of this thesis are as follows:

- Municipal wastewater contains enteric viruses (noro- and adenoviruses) throughout the year, posing a constant threat to the microbial quality of receiving waters. The numbers of adenoviruses were stable in wastewater, which supports their use as a marker of human wastewater contamination.
- Noroviruses were found as the main causative agents of two drinking water and eight bathing water outbreaks that occurred in Finland.
- Different OWTSs vary greatly in their microbial treatment efficiencies, and current construction practices do not necessarily prevent groundwater contamination and viral waterborne outbreaks. Therefore, careful site-specific planning, construction and placement of the treatment systems is necessary for their successful and safe operation in order to protect and maintain water safety.
- Noro- and adenoviruses showed high persistence in water. Virus genomes were detected for at least 3.5 years in outbreak samples stored at 4 °C. In the laboratory study, the persistence of noroviruses was dependent on temperature, water matrix and virus strain. Overall, a low temperature favoured persistence, while persistence was higher in drinking water compared to wastewater and indigenous strains persisted better than spiked noroviruses in wastewater.
- The long persistence of enteric viruses in water indicates that allowing sufficient time to pass is not the proper way to manage water-related problems. Instead, prompt and active control measures are needed. For the decontamination of a drinking water distribution network, chlorine and PAA are efficient decontaminants. Prolonged cleaning measures may be needed after massive contaminations in situ.
- Traditional FIB (E. coli and intestinal enterococci) can reveal water contamination, especially during massive contamination episodes. However, their occurrence and fate in water environments does not correlate consistently with enteric viruses. In addition, their validity in assessing the success of control measures, such as decontamination of a distribution network, is not recommended. Due to high degree of variation in physicochemical and biological factors that affect the fate of different microbes in waterborne outbreaks, pathogenic enteric viruses need to be examined separately.

In summary, this study increases the knowledge of the factors contributing to the success of enteric viruses at spreading through water and explains their superior role as causative agents of waterborne outbreaks. Moreover, this study provides valuable input data for the quantitative microbial risk assessment (QMRA).
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Waterborne outbreaks are a constant threat to public health worldwide. The most important waterborne pathogens include enteric viruses, such as noroviruses. This thesis provides new information regarding the occurrence, transport, persistence and control of enteric viruses in water environments. Furthermore, the thesis assesses the suitability of commonly used indicator microbes to describe the water quality as well as the occurrence and fate of enteric viruses in water environments.