
Assessing the impact of wastewater treatment plant effluent on the norovirus contamination of shellfisheries



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Declaration

I, John Flannery, declare that the results presented are to the best of my knowledge correct. This thesis represents my own original work, except where specifically stated in the text and acknowledgements. The contribution of other authors to chapters 5, 6 and 7 are acknowledged in the publications shown in Appendix A. Any quotation or paraphrase from the published or unpublished work of another person has been duly acknowledged in this work which I present for examination. This work has not been previously accepted for a higher degree either at NUI Galway or elsewhere.

Signed:

Date:

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List of abbreviations

AdV	<i>Adenovirus</i>
AEN	<i>Adsorption-elution using electronegative membrane filters</i>
AEP	<i>Adsorption-elution using electropositive membrane filters</i>
AstV	<i>Astrovirus</i>
BOD	<i>Biochemical Oxygen Demand</i>
CaCV	<i>Canine calicivirus</i>
C_q	<i>Quantification cycle</i>
C_t	<i>Cycle threshold</i>
D₉₀	<i>UV dose required to reduce concentrations by 1 log</i>
EFSA	<i>European Food Safety Authority</i>
EM	<i>Electron Microscopy</i>
EU	<i>European Union</i>
EV	<i>Enterovirus</i>
FCV	<i>Feline calicivirus</i>
FRNA	<i>F-specific RNA (bacteriophage)</i>
FSAI	<i>Food Safety Authority of Ireland</i>
HAV	<i>Hepatitis A virus</i>
HBGA	<i>Histological Blood Group Antigen</i>
HEV	<i>Hepatitis E virus</i>
ICC-RT-qPCR	<i>Integrated cell culture-RT-qPCR</i>
(t)LOD	<i>Theoretical Limit of Detection</i>
LOQ	<i>Theoretical Limit of Quantification</i>
MuNoV	<i>Murine norovirus</i>
NoV	<i>Norovirus</i>
PCR	<i>Polymerase Chain Reaction</i>
PEG	<i>Polyethylene glycol</i>
PV	<i>Poliovirus</i>
ReoV	<i>Reovirus</i>
RoV	<i>Rotavirus</i>
RT-PCR	<i>Reverse Transcription Polymerase Chain Reaction</i>
RT-qPCR	<i>Reverse Transcription Quantitative Polymerase Chain Reaction</i>
S₉₀	<i>Fluence required to reduce concentrations by 1 log</i>
SaV	<i>SaPVirus</i>
SFPA	<i>Sea Fisheries Protection Authority</i>
SRSV	<i>Small Round Structured Virus</i>
T₉₀	<i>Time required to reduce concentrations by 1 log</i>
TCID₅₀	<i>Median tissue culture infectious dose</i>
UWWTD	<i>Urban Wastewater Treatment Directive</i>
VTEC	<i>Verocytotoxigenic Escherichia coli</i>
WFD	<i>Water Framework Directive</i>

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Abstract

Norovirus (NoV) is the most common cause of viral gastroenteritis in the developed world. The discharge of Wastewater treatment plant (WWTP) effluent to the marine environment has implications for the virus quality of shellfish harvested that can accumulate NoV from contaminated water. This research project aimed to investigate NoV concentrations in WWTPs, the subsequent NoV contamination of oysters and the inactivation kinetics for NoV in the marine environment.

Concentrations of NoV were determined in weekly samples of wastewater at different stages of treatment (influent, post primary and post secondary). NoV concentrations were also determined in weekly samples of oysters placed adjacent to the WWTP outfall. NoV was detected year-round and concentrations peaked during the winter months that were correlated with concentrations in oysters. In a second WWTP providing UV disinfection, the reduction of NoV was similar to FRNA bacteriophage GA using RT-qPCR. However, the reduction of infectious FRNA bacteriophage GA was significantly greater than that obtained using the RT-qPCR assay. A significantly higher percentage of infectious virus was present in combined sewer overflow effluent than in UV disinfected effluent.

The inactivation kinetics of NoV and FRNA bacteriophage GA in was investigated in a lab setting. The reduction in detectable viruses was not significantly different between summer and winter for NoV GI and NoV GII. However, using FRNA bacteriophage GA the RT-qPCR assay significantly overestimated the survival of infectious virus. Overall, the findings from this thesis demonstrate that RT-qPCR is an unsuitable method to determine the reduction of infectious NoV that occurs during wastewater treatment and in the marine environment.

Chapter 1. Introduction

1.1 General Introduction

Shellfish provide a nutritionally healthy food that is enjoyed worldwide. One of the major concerns for shellfish management agencies is the ability of bivalve molluscs to accumulate pathogenic viruses arising from human or animal faecal material from the waters in which they are grown (Lees, 2000). The consumption of sewage-contaminated shellfish has been responsible for numerous outbreaks of illness caused by NoV (Bellou et al., 2013). As demands for production of shellfish are increasing (Browne et al., 2008), the onus on producers to provide a safe and ready to eat product is of increasing importance.

The microbiological quality of aquatic environments and shellfish is assessed using bacterial indicator organisms such as *E. coli* (Jofre, 2007). However, bacterial indicator organisms have been shown to poorly reflect enteric viruses through wastewater treatment, in the marine environment and persistence in shellfish (Doré and Lees, 1995; Flannery et al., 2012; Mocé-Llivina et al., 2002; Sinton et al., 2002). Although wastewater treatment plants (WWTPs) provide an initial mitigating system for protecting shellfisheries from faecal contamination, outbreaks of shellfish-associated NoV illness continue to occur throughout Europe (Bellou et al., 2013).

Few studies investigating the survival of NoV during WWTPs exist as no cell culture method is available for NoV (daSilva et al., 2007, Nordgren et al., 2009). In addition, no studies exist concerning the inactivation kinetics of NoV in under sunlight and temperature conditions in the marine environment. The absence of such data presents a challenge to water and shellfish management agencies engaged in controlling the risk posed by NoV contamination in shellfisheries.

1.2 Wastewater-associated pathogenic microorganisms

A diverse microbiological flora comprising bacterial, protozoan and viral pathogens is present in wastewater (Ottoson et al., 2006a). Wastewater has long been considered a source of microbial pathogens given the fact that at any one time, disease and infection are endemic in the population. Since the waterborne transmission of cholera was first demonstrated in 1854 (Snow, 1855) strategies have existed to protect public health from drinking water contamination (Bitton, 2005). However, despite these strategies, waterborne illnesses continue to occur (Reynolds et al., 2008) especially in developing countries (Lee and Schwab, 2005)

1.2.1 Bacterial pathogens

Many pathogenic bacteria are present in human faeces, thus sewage-contaminated waters can contain high concentrations of bacteria. The development of wastewater treatment technologies in the twentieth century has been attributed to the decline in bacterial-associated waterborne illness in the developed world (Leclerc *et al.*, 2002). However, limitations in wastewater treatment processes may lead to the transmission of pathogenic bacteria through recreational use or the consumption of contaminated shellfish. Table 1.1 lists a number of important bacterial pathogens implicated in waterborne and foodborne illness.

Table 1.1 Bacterial pathogens present in municipal wastewater

Genus	Organism	Possible symptoms of illness	Implicated in water or food borne illness
<i>Escherichia</i>	<i>E. coli</i> VTEC serogroups	Abdominal cramping, severe bloody diarrhoea, sometimes leading to haemolytic uremic syndrome, renal failure and death	Both, mainly foodborne
<i>Shigella</i>	Various species	Causes “shigellosis” or bacillary dysentery. Fever, abdominal cramps, chills, vomiting and diarrhoea containing blood and mucus	Both, mainly foodborne
<i>Listeria</i>	<i>L. monocytogenes</i>	Fever, chills, headache, backache, sometimes abdominal pain and diarrhoea. Sometimes meningitis or abortion in pregnant women	Foodborne
<i>Campylobacter</i>	Various species	Fever, nausea, abdominal pains, bloody diarrhoea and vomiting and sometimes Guillan-Barré syndrome	Both, mainly food borne
<i>Yersinia</i>	<i>Y. pestis</i>	Fever, abdominal pain and bloody diarrhoea and polyarthrititis	Both
<i>Salmonella</i>	<i>S. enterica</i> (various serogroups)	Stomach pain, diarrhoea, nausea, chills, fever, and headache	Both, mainly foodborne
	<i>S. Typhi</i> / <i>Paratyphi</i>	Typhoid fever	
<i>Vibrio</i>	<i>V. vulnificus</i>	Septicaemia or gastroenteritis	Both, mainly waterborne
	<i>V. parahaemolyticus</i>	Profuse diarrhoea and vomiting resulting in the loss of fluids	
	<i>V. cholera</i>	which can cause death	

Adapted from (Bitton, 2005)

Although many pathogenic bacteria are found in wastewater, bacterial illness associated with shellfish consumption appears to constitute a minimal public health hazard in Europe (Lees, 2000). The implementation of various national monitoring programmes in developed countries has significantly reduced incidences of bacterial illness. Where such monitoring programmes exist shellfish-mediated bacterial illness is generally associated with illegally harvested shellfish from contaminated areas or inadequate post harvest treatment processes.

1.2.2 Protozoan pathogens

Protozoa can survive outside their host for long periods of time as they can form cysts (*Giardia* Spp.) or oocysts (*Cryptosporidium* Spp.) when placed under adverse environmental conditions (Baldursson and Karanis, 2011). These cysts are resistant to various processes used in water and wastewater treatment and this is reflected by their frequent implication in waterborne illness (Baldursson and Karanis, 2011). It is likely that protozoa released in wastewater effluents can be accumulated by shellfish from the surrounding waters (Robertson, 2007), however, they are an uncommon cause of reported shellfish-borne illnesses (Yoder et al., 2010).

Table 1.2 Protozoan pathogens present in municipal wastewater

Organism	Associated disease	Major reservoir
<i>Giardia lamblia</i>	Giardiasis	Human and animal feces
<i>Entamoeba histolytica</i>	Amoebic dysentery	Human feces
<i>Acanthamoeba castellanii</i>	Amoebic meningoencephalitis	Soil and water
<i>Naehleria gruberi</i>	Amoebic meningoencephalitis	Soil and water
<i>Balantidium coli</i>	Dysentery/intestinal ulcers	Human faeces
<i>Cryptosporidium</i>	Profuse and watery diarrhea; weight loss; nausea; low-grade fever	Human and animal faeces
<i>Cyclospora</i>	Watery diarrhea alternating with constipation	Faeces, contaminated fruits and vegetables

Adapted from (Bitton, 2005)

1.2.3 Viral pathogens

Improved sanitation practices in the developed world in the early 20th century coincided with reports of a disease known as infantile paralysis. The virus responsible for this disease was poliovirus (PV) and was contracted through the consumption of water impacted by faecal waste. PV infection in the early months of life causes asymptomatic infection and confers life-long immunity thus prior to developments in sanitation, the majority of the population had immunity to poliovirus and reports of illness were rare. The subsequent improvements in sanitation lead to persons contracting PV at later stages of life, when the virus can infect nerve tissues and cause acute flaccid paralysis or poliomyelitis. The development of vaccines against PV in the mid-20th century has led to the eradication of polio throughout the developed world.

While progress was being made towards PV eradication through vaccination, the field of environmental virology gained further attention following a large hepatitis outbreak that occurred in India in 1955. The cause of this outbreak was drinking water that had been contaminated by a nearby WWTP with Hepatitis E virus (HEV) (Dennis and Wolman, 1959). Although the WWTP provided chlorine disinfection, some 30,000 cases of hepatitis occurred amongst the population. It was clear that sanitation treatments, as practiced at that time, were not sufficient to remove viruses from wastewater and that the threat to public health still existed (Bosch, 1998).

Enteric viruses are typically released in large numbers (up to 10^{11} g⁻¹) in the stools of infected persons (Gerba, 2007), therefore direct or indirect faecal contamination of surface, ground and marine waters are the main source of human viruses in the environment. In rural settings, run-off from agricultural practices may account for a significant portion of viruses detected in ground water (Fong and Lipp, 2005). In urban settings, intense periods of rainfall can overwhelm WWTPs that results in the discharge of partially or untreated wastewaters directly into a receiving body (Flannery et al., 2013a; Fong and Lipp, 2005; Le Guyader et al., 2008). In addition, bathers in streams or lakes may release large numbers of enteric viruses into water bodies (Kay et al., 1994; Rose et al., 1987). Table 1.3 lists a number of enteric viruses that are of importance to water management agencies.

While many different enteric viruses are present in wastewater, epidemiological studies have shown that few have been implicated in shellfish vectored illnesses (Le Guyader et al., 2008). For instance, outbreaks of illness attributed to NoV and HAV have been frequently linked to the consumption of shellfish, whereas shellfish-related outbreaks of HEV, astrovirus (AstV), enterovirus (EV), adenovirus (AdV), aichi virus and rotavirus (RV) illness are less frequently reported (Formiga-Cruz et al., 2003; Le Guyader et al., 2000, 2009; Lees, 2000; Myrmel et al., 2004). It remains that the most frequent viral illness associated with the consumption of shellfish is NoV gastroenteritis.

Table 1.3 Viral pathogens present in municipal wastewater

Family	Genus	Type species	Size (genome composition)	Water or food- borne	Possible associated disease
<i>Picornaviridae</i>	Enterovirus	Poliovirus	28 nm (ssRNA)	Both	Paralysis, meningitis, poliomyelitis.
		Coxsackieviruses			Paralysis, common cold, diarrhoea
Echovirus		Hand-foot-and mouth disease			
EV 68 to 71		Acute haemorrhagic conjunctivitis			
	Hepatovirus	Hepatitis A virus		Both	Infectious hepatitis
<i>Reoviridae</i>	Reovirus	Human reoviruses	70 nm (dsRNA)	Mostly water	Respiratory and Gastrointestinal illnesses
	Rotavirus	Human rotaviruses			Gastroenteritis
<i>Caliciviridae</i>	Norovirus	Norwalk virus	34 nm (ssRNA)	Both	Gastroenteritis
	Sapovirus	Sapporovirus			Gastroenteritis
<i>Hepeviridae</i>	Hepatitis E virus	Hepatitis E virus	34 nm (ssRNA)	Mainly water	Infectious hepatitis
<i>Astroviridae</i>	Astrovirus	Human astroviruses	28 nm (ssRNA)	Both	Gastroenteritis
<i>Coronaviridae</i>	Coronavirus	Human Coronaviruses	140nm (ssRNA)		Enterocolitis, severe acute respiratory syndrome (SARS)
<i>Adenoviridae</i>	Mastadenovirus	Human enteric adenoviruses	100 nm (dsDNA)	Water	Respiratory infection, conjunctivitis and gastroenteritis

Adapted from (Abdel-Moety et al., 2008; Carter, 2005; Grabow, 2007)

1.3 Norovirus

In 1979, the family *Caliciviridae* was recognised in the Third Report of the International Committee on Taxonomy of Viruses. The family name was derived from the cup-like (calyx, which means cup in Greek) appearance of the virion under negative stain electron microscopy (EM). The first human calicivirus to be identified using EM was Norwalk virus following an outbreak of viral gastroenteritis at a primary school in Norwalk, Ohio, USA in 1968 (Kaipikian et al., 1972). In the following years further morphologically similar, although antigenically-distinct viruses were identified as causes of outbreaks of viral gastroenteritis. These viruses were termed "small round structured viruses" (SRSVs) and were generally named after location of the outbreak (Glass et al., 2009). These SRSVs proved difficult to study as cell culture was unsuccessful in propagating the virus and EM detection was not always possible (Newell et al., 2010). Some immunological reagents became available through human volunteer studies and were used in conjunction with EM with limited success at this time (Thornhill et al., 1977). During subsequent years, it was found that SRSVs caused a significant number of human and veterinary diseases (Table 1.4) (Clarke and Lambden, 2001).

Table 1.4 Genera within the *Caliciviridae*

Family	Genus	Type species
<i>Caliciviridae</i>	Vesivirus	Swine vesicular exanthema virus
	Lagovirus	Rabbit haemorrhagic disease virus
	Norovirus (NoV)	Norwalk virus
	SaPVirus (SaV)	Sapporo virus
	Nebovirus	Newbury-1 virus

The two genera of the *Caliciviridae* that cause human illness are NoV and SaV, however, SaV outbreak reports are far less common and account for only a few outbreaks a year (Rodríguez-Lázaro et al., 2011).

1.3.1 Epidemiology of NoV

NoV infections demonstrate a seasonal distribution with increasing levels of infection peaking in the winter months (Mounts et al., 2000). This distribution is so pronounced that acute gastroenteritis caused by NoV was originally described as “the winter vomiting bug” although it has been detected year-round in environmental samples (Bresee et al., 2002; Flannery et al., 2012).

Figure 1.1 Weekly laboratory reports of NoV in England and Wales, 2009 to 2013

Source: www.hpa.org.uk/hpr/infections/enteric.htm#ntrcfvr

Transmission of NoV is via the faecal-oral route either directly through contaminated food or water or via aerosolized droplet nuclei (Widdowson et al., 2005). Food may be contaminated by both symptomatic and asymptomatic food-handlers (Ozawa et al., 2007) or by contaminated waters used to wash fruits, vegetables and salads (Koopmans and Duizer, 2004). NoV illness is usually of self-limiting duration lasting two to three days however some infections in the elderly and immune compromised individuals have been associated with serious complications and death (Karst, 2010; Mattner et al., 2006; Schwartz et al., 2011). The virus is shed in large numbers in the vomit and stool of infected individuals, most commonly during the symptomatic period; however, NoV can continue to be shed long after recovery (Siebenga et al., 2008).

The clinical presentations of NoV were described in 1982 to help distinguish outbreaks attributed to the virus from other agents of gastrointestinal illness (Kaplan et al., 1982). The criteria specified were (i) vomiting occurring in more than half of affected persons (ii) mean incubation period of twenty four to forty eight hours (iii) mean duration of illness lasting twelve to sixty hours and (iv) the absence of a bacterial pathogen in stool cultures. In the absence of suitable detection methods for NoV gastroenteritis, such criteria were traditionally used to determine “classic human calicivirus” infections. Recently, these criteria have been reassessed and found to provide an accurate determination of NoV illness (Turcios et al., 2006).

An outbreak can be defined by two or more infections originating from a single point source. The first reported shellfish-associated NoV outbreak occurred in the winter of 1976/77 in the UK following the consumption of sewage-contaminated cockles (Appleton and Pereira, 1977). Since that time, NoV infections have been recognised in many outbreaks of acute gastroenteritis occurring in all age groups throughout the world particularly in schools, hospitals, cruise ships, restaurants and residential homes (Lopman et al., 2003; McCarthy et al., 2000; Siebenga et al., 2008; Wikswo et al., 2011).

In the USA, it is estimated that over 5,400,000 people become ill from NoV infections each year resulting in approximately 15,000 hospitalisations with 149 deaths (Hall et al., 2011). During the period 2009-2010 in the USA, NoV was identified as the leading cause of gastroenteritis outbreaks (n=1,908) causing and 69,145 illnesses, 1,093 hospitalizations, and 125 (86%) deaths (Hall et al., 2013). In Ireland, out of all bacterial, viral or protozoan gastroenteritis outbreaks (n=3841) in 2011, NoV was the causative agent in 2621 outbreaks (HPSC, 2011). Since July 2011 86.7 % of NoV-confirmed outbreaks were associated with GII-4 strains in the UK (Public Health England, 2013). However, unlike person-to-person outbreaks, numerous NoV genotypes have been responsible for shellfish-associated outbreaks (Le Guyader et al., 2012) as shellfish have been shown to accumulate a wide variety of genotypes (Rajko-Nenow et al., 2013, 2012).

NoV is considered to have a low infectious dose with an estimate placed at as few as 18 virus particles in clinical trials (Teunis et al., 2008). However, in a review of oyster-

related outbreaks, the median infectious dose (ID_{50}) estimated for NoV GI and GII ranged between 1.6 and 7.51 genome copies per oyster consumed (Thebault et al., 2013). The long-term excretion (up to two weeks post-infection) of NoV is a likely contributor to the high numbers of secondary cases observed in outbreaks (Kirkwood and Streitberg, 2008; Koopmans and Duizer, 2004; Rockx et al., 2002).

Despite the high frequency of NoV illness, immunity is as yet poorly understood (Glass et al., 2009). Early NoV challenge studies have demonstrated that infection can elicit both short and long-term immunities (Johnson et al., 1990; Parrino et al., 1977) and that individuals can remain susceptible and/or become infected multiple times during their lives (Jaykus et al., 1994). While there may be many antigenically and genetically distinct strains of NoV circulating in the community at any time, evidence indicates that following infection with a particular strain, cross-protection does not occur against other strains (Donaldson et al., 2010). This lack of broad-spectrum immunity may be explained by the high mutation rate of the NoV genome, as often occurs within RNA viruses (Tan and Jiang, 2005). Specific histological blood group antigens (HBGAs) have been identified as receptors for the attachment of different NoV strains to mucosal cell surfaces (Harrington et al., 2004; Hutson et al., 2004).

1.3.2 Detection of NoV in environmental samples

For many years EM was the only technique available to detect viruses that did not produce cytopathic effect (CPE) such as NoV. However, EM is time-consuming and laborious and is most suited to clinical sample analysis where viral concentrations are great enough for detection (Hedberg and Osterholm, 1993). The culture system for NoV demonstrated by Straub et al., (2007,2011) has not been reproduced by other workers (Papafragkou et al., 2013). As concentrations of enteric viruses such as NoV in environmental samples may be too low to be detected using EM, a concentration procedure is often used prior to the detection method (Wyn-Jones, 2007). The most commonly used concentration procedures for virus detection in environmental waters are outlined in detail in Chapter 3 of this thesis.

In recent years, molecular-based techniques have become the most widely utilised method of detection for NoV as they can be highly specific and sensitive. Initially, nested RT-PCR methods were used to detect NoV in environmental samples (Henshilwood et al., 2003; Wyn-Jones et al., 2000), however, the method did not allow for quantification and was time consuming. The recent development of RT-qPCR methods, has allowed for the better detection of NoV in environmental samples (Doré et al., 2010; Flannery et al., 2012; Lowther, 2011).

1.3.2.1 Standardisation of molecular methods for NoV

With the increased use of RT-qPCR methods to detect viruses in environmental samples, the opportunity to develop a standardised method for NoV was recognised by the scientific community. In 2004, CEN, the European Committee for Normalisation, established a working group (CEN/TC 275/WG 6/TAG 4) on “detection of viruses in food” with the specific aim of developing a horizontal method for NoV and HAV in a range of foodstuffs, including shellfish. A consensus was reached on a number of important aspects of method standardisation such as the use of RT-qPCR, the extraction method for isolation of viral RNA and the use of controls (positive/negative RNA extraction and internal positive control for PCR inhibition). In 2013, the International Organisation for Standardisation (ISO) published the finalised method for the horizontal detection of NoV and HAV in foodstuffs (Anonymous, 2013).

1.3.3 RT-qPCR overestimation of infectious virus concentrations

While RT-qPCR is the most specific technique to detect RNA viruses in environmental samples, it has been demonstrated that this method can overestimate the concentration of infectious viruses in these samples (Nuanualsuwan and Cliver, 2002; Pecson et al., 2009; Rajko-Nenow et al., 2013). The reasons behind this overestimation is due to the persistence of genomes of inactivated viruses that remain either partially or fully intact that may be still detected (Nuanualsuwan et al., 2002; Wong et al., 2007). Virus survival relates to the viruses ability to infect its host while persistence relates to the

ability to detect the virus. For this reason, molecular methods can be said to provide an estimation of the total viral concentration, while plaque assays will provide an estimation of the infectious virus concentration (Flannery et al., 2013a).

The overestimation of infectious viruses using RT-qPCR throughout a WWTP and under simulated environmental conditions is shown in Chapter 5 and Chapter 6 of this thesis, respectively. The discrepancy between infectious (determined using cell culture) and total (determined using PCR, RT-PCR or RT-qPCR) has been noted for a number of viruses in various settings (Flannery et al., 2013a, 2013b; He and Jiang, 2005; Hewitt et al., 2011). One method that has been developed to selectively detect infective RNA viruses is integrated cell culture (ICC) RT-PCR. Li et al., used cell culture in conjunction with RT-qPCR to show that thermal inactivation of RV cannot be detected using RT-qPCR and that this method overestimates infectious RV concentrations (Li et al., 2010). However, this method includes a culture-based step that is not available for many viruses such as NoV. In the absence of a cell culture system for the target virus, various techniques have been employed to reduce the potential overestimation provided by RT-qPCR detection methods (Li et al., 2012).

Other approaches have been based on the rationale that an intact and infectious capsid will bind selectively to its receptor or to an antibody whereas a noninfectious virus will not (Li et al., 2012). Dancho et al., (2012) investigated the use of porcine gastric mucin conjugated to magnetic beads prior to RT-qPCR for NoV. Using a stock of NoV GI.1, the authors found that an increase in temperature or UV light dose caused a decrease in the binding of NoV and the subsequent detection of NoV using RT-qPCR. While this method allowed for differentiation of infectious NoV from total NoV, the variety of genotypes present in environmental samples (Rajko-Nenow et al., 2013) means that this approach is generally unsuitable for use in environmental detection. An alternative approach was investigated by Wolf et al., (2009) who used a long-range RT-PCR to detect genomic damage in NoV. Under increasing dosages of UV, the authors found no decrease in RT-qPCR signal using a standard RT-qPCR assay whereas a significant decrease in qPCR amplification occurred when the RT reaction was primed at the poly-adenylated 3' region

of the genome. No definitive approach to differentiate infectious from non-infectious virus has been determined.

1.4 Urban Wastewater treatment

Municipal wastewater contains organic compounds such as proteins, carbohydrates, fats and nutrients from human waste (Gray, 2004). The main purpose of wastewater treatment is physical removal of solids and the reduction of the Biochemical Oxygen Demand (BOD). Wastewater treatment processes may involve gravimetric settling as in the case of primary treatment systems or biologically-assisted settling as in the case of secondary treatment systems. The secondary treatment systems are designed to reduce the BOD as microorganisms are used to consume biological matter in the wastewater. A further objective of wastewater treatment is to remove or reduce levels of pathogenic microorganisms. With the exception of disinfection processes, wastewater treatment is not directed towards the removal of microbial pathogens. Removal of microorganisms is achieved through a number of physical, chemical and biological processes. In general, bacteria are reduced to a greater extent during wastewater treatment than enteric viruses (Flannery et al., 2012,2013; Payment et al., 2001; Zhang and Farahbakhsh, 2007).

1.4.1 Primary wastewater treatment

Primary treatment methods involve the use of disintegrators that remove large debris that could damage machinery and settlement tanks that separate and remove large particles through gravity. The solid material that has settled is referred to as primary sludge with the overflow being brought to the next stage of treatment (Gray, 2004). The primary sludge may be applied to land following a de-watering process such as centrifugation and heat treatment. Directive 86/278/ EEC exists to regulate the application of sludge to land exist however, heavy metals are the only parameters stipulated and no microbiological provisions exist (Directive, 1986).

1.4.1.1 Microbiological reduction during primary treatment

As primary treatment processes involve short settling and retention times, bacteria and viruses are not removed by settling processes unless they are associated with large particulates (Payment et al., 2001). It has been shown that the reduction of total and faecal coliforms achieved during primary wastewater treatment ranges between 0.19 and 0.50 log (Aulicino et al., 1996; Flannery et al., 2012; George et al., 2002; Lucena et al., 2004; Payment et al., 2001). However, primary treatment, when used in conjunction with coagulant addition (a process used to enhance phosphorous removal), has been shown to achieve log reductions of 0.76 and 0.82 for total and faecal coliforms, respectively (Zhang and Farahbakhsh, 2007).

Similar to bacterial indicator organisms, bacteriophage have been reported to undergo little reduction during primary treatment. The reduction of somatic coliphage, FRNA bacteriophage and *Bacteroides fragilis* phages ranged between 0.3 and 0.5 log during primary treatment in a number of different treatment plants (Lucena et al., 2004). In Chapter 4 of this thesis, FRNA bacteriophage were found to be reduced by 0.32 log during primary treatment (Flannery et al., 2012). The addition of ferrous chloride to primary treatment has been shown to achieve a greater reduction of somatic and FRNA bacteriophage during primary treatment that ranged between 0.3-1.9 log (Zhang and Farahbakhsh, 2007). A number of early studies investigating the reduction of EV during primary treatment found viral reductions between 0.11 and 0.77 log (Payment et al., 1986; Rao et al., 1981). Similar reductions for EV were found in a number of subsequent studies (Aulicino et al., 1996; Payment et al., 2001; Weatherley, 1999).

Currently, limited information is available regarding the reduction of NoV throughout primary treatment processes. Nordgren et al, found that NoV was reduced by 0.7 log during primary treatment in an activated sludge treatment plant (Nordgren et al., 2009). In Chapter 4 of this thesis, NoV GI and GII was found to be reduced by 0.13 and 0.14 log, respectively during primary treatment (Flannery et al., 2012). In general, however, counts of indicator bacteria and viruses are not significantly reduced by primary settlement (Flannery et al., 2012; George et al., 2002; Kay et al., 2008). Therefore, the majority of viruses

remain in the effluent after primary treatment. In addition, it has been suggested that disintegration of solids may actually release some viruses into the aqueous environment (Bitton, 2005). This may be due to the fact that the enteric viruses present may be tightly bound to or within faecal material which are released during the screening process.

1.4.2 Secondary wastewater treatment

Secondary treatment is an aerobic, biological process that metabolises and flocculates colloidal and dissolved organic material. Following primary treatment, the effluent is exposed to a heterogeneous flora of microorganisms (biomass) that reduces the BOD in the presence of nutrients (provided by wastewater) and oxygen. There are two main types of systems used in secondary wastewater treatment processes; fixed-film or suspended-growth systems (Bitton, 2005). Fixed-film growth system treatment processes includes trickling/percolating filters and rotating biological contactors where the biomass grows on solid media and the effluent is passed over the surface of the biomass. In suspended-growth systems such as activated sludge, the biomass is mixed with wastewater and can generally be operated in a smaller space than fixed-film systems that treat an equivalent volume of water. The characteristics of fixed-film and suspended-growth treatment processes are outlined in Table 1.5. During secondary wastewater treatment processes, bacteria can be removed through grazing by ciliated protozoa, natural inactivation and through adsorption to or within sludge flocs (Gray, 2004; Omura et al., 1989).

Table 1.5 Characteristics of secondary wastewater treatment processes

Treatment process	Mechanism of treatment	Main advantages	Main disadvantages
Trickling/percolating filters	Primary effluent is distributed by a revolving sprinkler suspended over a bed of porous material that microbes colonise to reduce BOD	Cheap and can effectively reduce BOD	Treatment efficiency reduced during winter months
Rotating biological contactors	Microorganisms that reduce BOD are attached to rotating support disks partially submerged in primary effluent	Requires a small footprint	Limited surface area of media
Fluidised bed reactor/ biological aerated filtration	Wastewater is injected into the base of the reactor at that contains filter media allowing for a high concentration of biomass	Requires less space, low capital costs	Greater running costs than other secondary treatment methods
Conventional activated sludge	Primary effluent is combined with a floc of sludge particles undergoes aeration which causes the generation of a large biomass that reduces the BOD of the wastewater	Provides good BOD removal. Low installation costs	Operating costs can be high
Membrane bioreactor	Involves an activated sludge bioreactor in conjunction with membrane filtration.	Produces high quality of effluent	Increased cost of building and operating
Sequence batch reactor	Similar to activated sludge however, all stages of treatment are carried out in one chamber known as a fill and draw process	Requires less space than activated sludge systems	A finite amount of wastewater can be treated
Oxidation ponds	Wastewater is discharged to large water tight ponds (often in a series) whereby heterotrophic bacteria and algae reduce the BOD over a long retention time	Cheap to construct and operate	Long retention times and limited to warm climates

Adapted from (Bitton 2005; Gray 2004)

1.4.2.1 Microbiological reduction during secondary treatment

The exact process for virus reduction throughout wastewater treatments is unclear however, the physical removal of viruses via activated sludge may account for 95% of the reduction (Carter, 2005). It is now believed that the primary removal method for viruses is by adsorption onto sludge flocs with predation having a negligible effect on virus concentration in the liquid phase (Gray, 2004). As it has been shown that the majority of viruses in wastewater are solids-associated (Bitton, 1980; Omura et al., 1989), the ability of activated sludge to remove viruses is related to the technologies' capacity to remove solids (Gerba et al., 1978). This may explain the increased viral reduction efficiency provided by activated sludge systems over tricking/percolating filtration systems (Bitton, 2005; Gray, 2004; Morris, 1984; Ward, 1982).

The majority of studies concerning virus reduction during secondary treatment have mainly involved the investigation of the activated sludge process as it is the most widely used method of secondary treatment. Bacterial indicator organisms such as faecal coliforms, total coliforms and *E. coli* have been shown to be reduced by 1.17-3.23 log during secondary treatment (Carducci et al., 2008; Flannery et al., 2012; Ottoson et al., 2006a; Zhang and Farahbakhsh, 2007). Initial investigations of viral reduction during secondary treatment systems were carried out using culturable viruses such as EV and removal efficiencies of 90% and 98% were found for poliovirus type 1 and coxsackievirus type A9, respectively (Rao et al., 1977; Varma et al., 1974). These early studies noted that bacteria can be more efficiently removed than viruses and that differences in viral removal rates between WWTPs utilising the same secondary processes (Bitton, 1980). In addition to intra-works differences, it has also been suggested that removal efficiencies of viruses may be species dependent (Gray, 2004).

Table 1.6 lists the log reductions reported for viruses and bacteria. However, it should be stated that results presented in the literature may not be directly comparable to one another as different detection methods have been used throughout. Virus detection in environmental settings has been hindered by the low levels of virus present in these

samples so a concentration procedure is often required (Ikner et al., 2012; Wyn-Jones, 2007). In addition, not all viruses can be cultured in-vitro (such as NoV) and those that can may not always produce a cytopathic effect (Wong et al., 2007). Furthermore, (Teunis *et al.*, 2005) have statistically demonstrated that plaque assays dependent on cytopathic effect may underestimate virus concentrations since it is not always possible to resolve plaques caused by more than one virus.

Table 1.6 Microbial reductions during secondary wastewater treatment processes

Microorganisms	WWTP process	Detection method	Log reduction	Reference
AstV	Biological treatment	qPCR	2.00	(Le Cann et al., 2004)
HAV	Activated sludge (pilot scale)	Cell culture	5.90	(Arraj et al., 2005)
PV		Cell culture	6.60	
RoV		Cell culture	9.20	
Somatic bacteriophage		Plaque assay	2.30	
FRNA bacteriophage		Plaque assay	3.20	
FRNA bacteriophage	Activated sludge	Plaque assay	1.70	(Lodder and de Roda Husman, 2005)
Somatic coliphage		Plaque assay	1.20	
EV		Cell culture	1.42	
ReoV		Cell culture	1.22	
RV		Cell culture	1.22	
NoV		RT-PCR	0.57	
NoV		RT-PCR	0.90	
EV	Activated sludge	RT-PCR	1.30	(Ottoson et al., 2006a)
EV		RT-PCR	1.67	
NoV		RT-PCR	0.95	
<i>E. coli</i>		Plate count	3.23	
FRNA bacteriophage		Plaque assay	3.47	
Somatic coliphage	Plaque assay	2.32		

Continued overleaf

Table 1.6 continued

Microorganisms	WWTP process	Detection method	Log reduction	Reference
Total coliforms	Activated sludge	Membrane filtration	1.9	(Zhang and Farahbakhsh, 2007)
Faecal coliforms		Plaque assay	2.3	
Somatic bacteriophage		Plaque assay	1.5-2.3	
FRNA bacteriophage		Plaque assay	0.8-2.2	
NoV	Activated sludge	RT-qPCR	0.9 0	(Nordgren et al., 2009)
AdV	Activated sludge	q PCR	1.77	(Fong et al., 2010)
AdV	Activated sludge	qPCR	0.69	(Prado et al., 2011)
NoV GII		RT-qPCR	0.21	
RV	Activated sludge	ICC-RT-qPCR	2.00-2.83	(Li et al., 2011)
<i>E. coli</i>	Activated sludge	MPN	1.49	(Flannery et al., 2012)
FRNA bacteriophage		Plaque assay	2.13	
NoV GI		RT-qPCR	0.80	
NoV GII		RT-qPCR	0.92	
NoV GI	Activated sludge		1.65	(Hata et al., 2013)
NoV GII			1.81	
SaV			3.28	
AstV			2.42	
FRNA phage GII			2.50	
<i>E. coli</i>	Activated sludge	MPN	1.50	(Flannery et al., 2013a)
FRNA bacteriophage		Plaque assay	1.45	
FRNA bacteriophage		Plaque assay	1.69	
FRNA bacteriophage		RT-qPCR	0.61	
NoV GI		RT-qPCR	0.45	
NoV GII		RT-qPCR	0.41	

1.4.3 Disinfection technologies for wastewater treatment

Where the intended receiving water is considered particularly vulnerable to microbial pollution, secondary treated wastewater may be further treated using a disinfection process. Table 1.7 shows the disinfection technologies available for wastewater treatment. Chlorination was the earliest disinfection process used; however as it has been shown to generate carcinogens, other technologies such as UV disinfection or microfiltration are becoming increasingly used (Bitton, 2005).

Table 1.7 Wastewater disinfection processes

Process	Mode of action	Advantages	Disadvantages	Persists	By-products
Chlorine	Oxidises cell/capsid components	Effectively reduces pathogens and is economical	Generates carcinogens, de-chlorination on discharge	Yes	THMs, singlet oxygen
Ozone	Similar to chlorine	pH independent and is more efficient than free chlorine for spores and viruses	High operational costs.	No	Can form H ₂ O ₂
UV	Nucleic acids absorb UV, forming covalent bonds that prevent replication	Less expensive to install and operate than chemical treatment facilities	Turbidity of water must be low	No	Several active oxygen species
Micro-filtration	Physical separation depending on pore size (<100 nm used)	Eliminate microbes without chemically modifying water	Very high capital and operation costs	No	No

Sourced from (Morato *et al.*, 2003)

As viruses lack metabolic activity, the capsid and/or nucleic acid are the primary targets for disinfection (Bitton, 2005). The viral capsid protects the viral nucleic acid from the surrounding environment and destruction of the capsid results in the release of nucleic acid. Different disinfectants used in wastewater treatment have different effects on viruses, for instance; ozone is the only disinfectant that can modify the structure of lipids which may affect enveloped viruses more than non-enveloped viruses such as NoV (Morato *et al.*, 2003). UV light, when absorbed by nucleic acids, causes cross-linking and dimerisation of the aromatic pyrimidine nucleotide bases cytosine, uracil and thymine (Figure 1.2). The wavelengths of light responsible for the majority of nucleic acid damage (termed germicidal wavelengths) occur around 260 nm and are exploited for use in disinfection UV technology (Bitton, 2005).

Figure 1.2 UV light dimerisation of thymine bases in DNA

Source (Pierce and Benjamin, 2005)

1.4.3.1 Microbiological reduction during wastewater disinfection

Disinfection of final effluents to remove pathogenic microorganisms is becoming an increasingly used practice in Europe. Although organisms differ in their susceptibility to disinfection processes (Meng and Gerba, 1996), environmental factors such as the organic matter concentration, pH or temperature can also affect disinfection (Bitton, 2005). The efficiency of chemical disinfectants reduces over time due to consumption through reactions with organic or chemical matter present in the water (Gardner and Peel, 1991).

In general, the effectiveness of disinfection depends on the quality of the secondary treated effluent as effluents with a high turbidity may be treated less successfully since solid matter can shield organisms, especially from UV light (Templeton et al., 2008) and organic material can consume available chemical disinfectants (Black et al., 2009). In general, short contact times, low disinfectant doses and high flow rates all mitigate against effective disinfection. While chlorination is effective in reducing viruses in drinking water, a number of studies have shown that chlorination of wastewater does not reduce viruses (Carducci et al., 2008; Petrinca et al., 2009; Prado et al., 2011; Tyrrell et al., 1995). The limited reduction of viruses may be due to solid-aggregated viruses in the secondary treated effluent (Prado et al., 2011).

In bench-scale studies, Blatchley et al., found that UV irradiation was consistently more effective as a virucide than chlorination/dechlorination under the conditions of application used (Blatchley III *et al.*, 2007). UV disinfection is becoming increasingly used as issues regarding the generation of carcinogens from the chlorination of wastewater exist; however, lack of standardisation in determining UV dosage is one difficulty in the design of such systems. The collimated beam technique of Qualls and Johnson (1983) has been applied extensively to determine the UV dosages required to achieve inactivation for a number of microbial pathogens and indicator organisms. Figure 1.3 shows the D_{90} values for a number of viruses determined using the collimated beam technique and illustrates the differences between and within virus families.

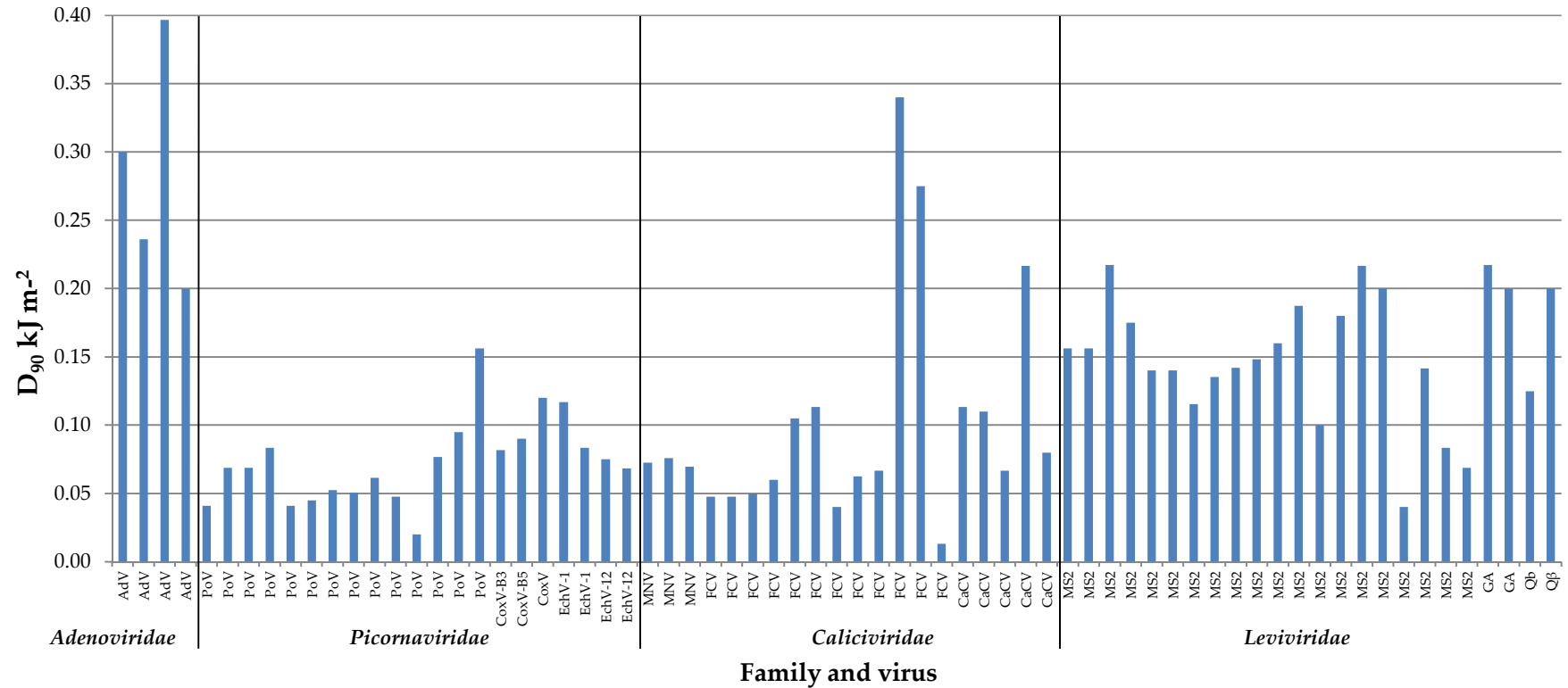


Figure 1.3 D₉₀ values for viruses in bench-scale UV inactivation experiments

Sources: (Bowker et al., 2011; Crance et al., 1998; de Roda Husman et al., 2004; Duizer et al., 2004; Gerba et al., 2002; Lee et al., 2008; Meng and Gerba, 1996; Nuanualsuwan et al., 2002; Park et al., 2011; Simonet and Gantzer, 2006; Thurston-Enriquez et al., 2003; Tree et al., 2005, 1997)

1.5 Urban WasteWater Treatment Directive

The Urban Waste Water Treatment Directive (UWWTD) (Directive 91/271/EEC) came into effect on 30 June 1993 and aimed to address the pollution threat posed by urban and industrial wastewater. The Directive outlines that member states are obliged to set up WWTPs to collect wastewater from agglomerations of varying size. The degree of wastewater treatment depends on the size and the location of the discharge. Wastewater discharges from WWTPs serving a population equivalent (P.E.) of between 10,000 and 150,000 P.E. in coastal areas and those serving a P.E. of between 2,000 and 10,000 P.E. in estuarine waters, are required to receive primary and secondary treatment prior to discharge. The obligation to set up treatment is not limited to agglomerations of more than 2,000 P.E. and the directive states that discharges from smaller agglomerations must be subject to an appropriate treatment whenever a collecting network exists.

The UWWTD outlines minimum requirements for primary and secondary treatment in terms of BOD and suspended solids. However, no requirements for tertiary treatment are stipulated and there are no explicit requirements for microbial load reduction prior to discharge in receiving waters. The continuous discharge of untreated wastewater to the marine environment is not allowed under the directive. Combined sewer overflows (CSOs) which typically occur following periods of increased rainfall, are considered within the directive. Again, no specific requirements for microbial treatment are specified; instead, the Member States have the authority to determine suitable strategies to limit pollution from CSOs.

1.6 Shellfish Waters Directive

The Shellfish Waters Directive (SWD) 79/923/EEC, was adopted in 1979 to protect and improve the quality of coastal or brackish waters where shellfish grow and to ensure high quality of directly edible shellfish products harvested from a particular area. As such, the directive prescribes the minimum quality criteria which must be met and also specifies the minimum sampling frequency and reference methods of analyses which must be used. The SWD is not intended by itself to protect the safety of shellfish for consumption as this

element is controlled by the member states competent authorities such as regulation (EC) No. 854/2004 that sets out the official controls for live bivalve molluscs from classified production areas. The directive will be repealed in 2013 by the Water Framework Directive.

1.7 Water Framework Directive

In response to the increasing threat of pollution and the increasing demand from the public for cleaner rivers, lakes and beaches, EU passed the Water Framework Directive (Directive 2000/60/EC) (WFD) on 22 December 2000. This Directive establishes a framework for the protection of all waters bodies including rivers, lakes, estuaries, coastal waters and groundwater and their dependent wildlife under one legislative document. The WFD is intended to replace a number of other water quality directives (such as the Shellfish Waters Directive 79/923/EEC) and will incorporate a number of others (including the UWWTD 91/271 /EEC). The WFD commits European Union member states to achieve “good” status of all water bodies by 2015.

In the WFD, ecological quality is divided into five classes (high, good, moderate, poor and bad) and is derived from measurements of biological, hydro morphological and physicochemical elements. The main aim of the WFD is to maintain or improve the existing ecological quality of water bodies. Under the WFD, at least mandatory standards need to be met for protected areas such as shellfish growing waters, bathing waters, freshwater fish designations, nitrate vulnerable zones and areas designated as sensitive under the UWWTD. Some concern has arisen since the WFD was adopted as the lack of a specific microbiological standard may potentially place shellfish growing waters at risk when the Shellfish Waters Directive is repealed in 2013. As yet it is unclear how this omission will be addressed to safeguard shellfish harvesting areas.

1.8 Survival of enteric viruses in the marine environment

The transmission of viral diseases depends partly on the ability of the virus to survive for sufficient time in the environment in order to infect a human host. Determining the survival of viruses in the marine environment is critical to assess the risks posed by

such pathogens when discharged into receiving waters. It is generally understood that enteric viruses persist for longer periods than non-enteric viruses in the environment and this may reflect their frequent implication in waterborne illnesses (Gerba, 2007). Microorganisms, when discharged into marine environments, must endure a number of different challenges to ensure transmission to their host organisms. The factors most-likely to influence the survival of viruses in the marine environment are shown in Table 1.8.

Table 1.8 Factors affecting virus survival in the marine environment

Environmental factor	Contribution
Temperature	Possibly the most important factor; viruses survive longer at lower temperatures
Solar radiation	UV light can damage nucleic acids, preventing replication
pH	Most viruses are stable at the pH values of most natural waters
Salinity	May protect viruses through aggregation
Organic matter and suspended solids	The presence of solids arising naturally or from wastewater prolongs virus survival
Predation	Aquatic microflora may be antagonistic

Adapted from (Gerba, 2007).

1.8.1 Effect of pH on viruses

In general, viruses are stable at pH values typically recorded for most natural waters (pH 5-9) with enteric viruses being more stable at a pH between 3 and 5 (Gerba, 2007). It has been shown that enteric viruses can tolerate pH levels beyond those found in mammalian systems (Gerba and Goyal, 1982) and this may explain their survival in environmental settings and implication in illness. However, as pH resistance can differ between virus families and also between genera within a family, this may have implications on identifying a suitable viral indicator organism. For instance, Papafragkou compared the pH inactivation profiles for murine norovirus (MuNoV) and FCV and found

that MuNoV is more stable at pH ranging between 2-10, whereas FCV undergoes inactivation at pH <3 and pH >9 (Papafragkou, 2006).

1.8.2 Effect of water salinity

Bacterial resistance to changes in salinity is dependent on various osmoregulatory processes (Darcan et al., 2003); however, the viral capsid has no such mechanisms. Despite this, viruses are generally more resistant to salinity gradients than bacteria (Sinton et al., 1994). When investigating the inactivation rates of bacteria and viruses in seawater, it was found that faecal coliforms, enterococci, *E. coli* and somatic coliphage were reduced more rapidly than FRNA bacteriophage (Sinton et al., 2002). It has been shown that for HAV and PV, increasing the salinity in synthetic seawater caused no significant differences in inactivation rate as measured by infectivity (Gantzer et al., 1998). It has been shown that salinity can have an effect on the aggregation of viruses to particulate matter (Gerba and Schaubberger, 1975; Young and Sharp, 1977).

1.8.3 Effect of microbial predation

Lab-scale studies on virus survival have indicated that naturally occurring bacteria can have an effect on viruses (Gerba, 2007) and that eliminating bacteria and higher forms of life increases viral survival (Gerba, 2005). It seems that the effects of autochthonous and bacterial-derived anti-viral compounds are more than biological processes such as predation (Bitton, 2005). However, it is unclear whether these virucidal compounds continue to have an effect on viruses when introduced into the marine environment.

1.8.4 Effect of temperature

A significant factor influencing the survival of viruses in aquatic environments is temperature (Fong and Lipp, 2005; Nasser and Oman, 1999; Yates et al., 1985). A number of studies have reported that PV, HAV somatic bacteriophage and FRNA bacteriophage are more stable at lower temperatures using infectivity assays (Biziagos et al., 1988; Chung and Sobsey, 1993). Although viruses are affected by differing temperatures, it has been

suggested that temperature fluctuations are insignificant when wastewater is discharged into coastal waters (Fong and Lipp, 2005) and implies that temperature-mediated viral reduction upon discharge that occurs is negligible. An investigation into the survival of NoV in groundwater found that NoV remains infectious for up to 62 days when stored at room temperature and will remain detectable for over three years (Seitz et al., 2011).

1.8.5 Effect of solar radiation

The electromagnetic spectrum consists of the entire range of frequencies and wavelengths at which energy, produced by the sun, travels. The amount of radiant energy that is received at one astronomical unit (i.e. the distance from the sun to the earth) on a planar surface is 1361 Wm^2 and is termed the solar constant. Nitrogen and oxygen in the atmosphere absorb short-wave ultraviolet radiation up to 280 nm whereby they undergo photo-dissociation and ozone is produced. As only a small percentage of UV reaches the surface of the Earth, the majority of solar radiation is comprised of wavelengths greater than 290 nm (Coulson, 2012; Kay et al., 2005) as shown in Figure 1.4.

Figure 1.4 The solar spectrum.

Source: http://commons.wikimedia.org/wiki/File:Solar_Spectrum.png

After temperature, UV radiation (from sunlight) is cited as the most common factor leading to the inactivation of viruses (Fong and Lipp, 2005). However, the wavelengths of light responsible for the majority of nucleic acid damage that are exploited for use in disinfection technologies, are not detected terrestrially. Thus the reduction of microbes is mainly due to wavelengths greater than 290 nm (Kay *et al.*, 2005). The contribution that the many wavelengths of sunlight make to the inactivation of pathogenic microorganisms is a function of latitude, the stage of the solar cycle, depth and cloud cover (Kay *et al.*, 2005).

As terrestrial sunlight contains a number of irradiance wavelengths ranging from UVB (293–315 nm), UVA (315–400 nm), visible light (400–800 nm) and infra-red (>800 nm) it has been demonstrated that not all wavelengths contribute to inactivation. For instance, it has been reported that half of the entire damaging spectrum of solar radiation occurs at wavelengths below 370 nm, a further quarter between 370 and 400 nm and the remainder of lethality occurring between 400 and 500 nm (Gameson and Gould, 1985). Studies carried out by Sinton *et al.* have found that approximately half of the inactivation of enterococci and FRNA bacteriophage when exposed to sunlight occurs below wavelengths of 360 nm and that that inactivation kinetics of enterococci at wavelengths greater than 550 nm was similar to inactivation under dark conditions (Sinton *et al.*, 2002, 1994).

Solar radiation can be an important contributor in reducing the infectivity of marine bacteriophage and viruses in seawater (Flannery *et al.*, 2013b; Noble *et al.*, 2004; Sinton *et al.*, 2002, 1999). Viruses are considered to be more resilient than bacteria because of their low molecular weight *i.e.*, they provide a lower target density for disinfection than bacteria (Fong and Lipp, 2005). In addition, double-stranded (ds) DNA containing viruses are more resistant to UV radiation than ssRNA containing viruses because an undamaged DNA strand may serve as a template for repair by host enzymes (Gerba *et al.*, 2002; Thurston-Enriquez *et al.*, 2003). Investigations into viral inactivation have typically relied on germicidal UV-C wavelengths; therefore, extrapolation to reflect their survival under typical terrestrial sunlight conditions may be erroneous. Few studies have investigated the inactivation of viruses caused by sunlight (Table 1.9).

Table 1.9 Sunlight inactivation of viruses

Virus	Matrix	Sunlight source	Temperature (°C) or environmental condition	Assay	S ₉₀ (MJ/m ²) cm	Reference
FRNA phage	unfiltered effluent	Natural sunlight	Not specified	Plaque	4.00	(Davies-Colley et al., 1994)
	filtered effluent			Plaque	6.00	
	PBS			Plaque	6.00	
	Seawater	Natural	14-20	plaque	13.70	(Sinton et al., 1999)
	Seawater	sunlight	08-10	plaque	22.80	
	River water+WSP	Natural sunlight	16	plaque	32.80	(Sinton et al., 2002)
	River water+ RS		16	plaque	45.70	
	River water+WSP		12	plaque	30.90	
	River water+RS		12	plaque	30.80	
	PV	Deionised H ₂ O	Solar simulated	25	plaque	2.85
40				plaque	5.56	
40				plaque	4.08	
40				plaque	3.40	
NoV GI	Seawater	Solar simulated	10	RT-qPCR	18.07	(Flannery et al., 2013b)
NoV GI	Seawater		17	RT-qPCR	18.18	
NoV GII	Seawater		10	RT-qPCR	16.92	
NoV GII	Seawater		17	RT-qPCR	17.30	
Phage GA	Seawater		10	RT-qPCR	15.73	
	Seawater		17	RT-qPCR	13.96	
	Seawater		10	Plaque	0.86	
	Seawater		17	Plaque	0.20	

WSP; Waste stabilisation pond effluent, RS; raw sewage

1.9 Indicators for the microbiological quality of water and shellfish

The enumeration of pathogens in environmental samples can be difficult and expensive; therefore, indicators of faecal pollution have been used in water and shellfish regulations. Ideally, faecal indicator organisms should occur naturally in high numbers in the faeces of humans and other warm-blooded animals, but should not be naturally present in the environment (Griffin, 2001; Jofre, 2007).

The most widely used indicators of faecal contamination are coliforms which comprise a heterogeneous group of bacteria originating mainly from faeces. They have been considered to be good indicators of faecal contamination as they have similar inactivation kinetics as the pathogenic microorganisms that they are used to indicate (Bitton, 2005). *E. coli* is considered to be the most reliable faecal indicator in comparison with other coliform groups (Edberg et al., 2000). Enterococci (or faecal streptococci) are considered to be an alternative to faecal coliforms as they are more tolerant to environmental stresses (Davies-Colley et al., 1994; Mascher et al., 2003; Sinton et al., 1994) and concentrations in bathing waters water have been reported to correlate with health risks (Kay et al., 1994). However, bacterial indicator organisms have been shown to be more susceptible to environmental stresses than bacteriophage (Sinton et al., 2002, 1994). For these reasons, bacteriophage are considered to be more suitable as indicator organisms for enteric viruses in environmental settings (Doré et al., 2000; Grabow, 2001; Havelaar et al., 1993).

Bacteriophage were recognised as being present in the gastrointestinal tract of humans in the early 1900s and their use to indicate faecal bacteria subsequently developed to be used as indicators for enteric viruses (Leclerc et al., 2000). When monitoring for enteric viruses in environmental waters, the presence of faecally-derived bacteriophage is considered to be more appropriate than specific analysis for enteric pathogens (Grabow, 2001). This is due to the fact that many different pathogenic enteric viruses can be encompassed by the presence of bacteriophage (Grabow, 2001).

Bacteriophage that are specific for a particular cellular receptor common to a group of bacteria are termed somatic bacteriophage (Harper and Kinchington, 1998).

Bacteriophage of the intestinal bacterium *E. coli* (sometimes termed coliphages) have been shown to be valid indexes of enteric viral pollution as they are present in wastewater and other faecally contaminated waters at concentrations equal to or greater than pathogenic enteric viruses (Grabow, 2001). Bacteriophage that infect the human-intestinal bacteria *Bacteroides fragilis* HSP40, have been correlated with indicate human faecal pollution (Puig et al., 2000). Bacteriophage that require the expression of fertility fimbriae (F pili) on their host for successful infection are termed male-specific bacteriophage and belong to the families *Inoviridae* and *Leviviridae* (Cole et al., 2003).

1.9.1 F-specific RNA bacteriophage

Fertility or F-pili are typically expressed from a plasmid and are used by bacteria for the exchange of genetic material from one F+ or male/donor bacterium to an F- or female recipient bacterium during conjugation (Novotny and Lavin, 1971). The expression of F pili is regulated by several factors such as the presence of the F plasmid within the bacterium and temperature. F pili have been shown to be maximally expressed between 37-42°C with expression non-existent at 25°C and below (Woody and Cliver, 1995). F-specific bacteriophage of the family *Inoviridae* are filamentous DNA bacteriophage approximately 760-1950 nm in length and approximately 6 nm wide. Their use as indicators of faecal contamination has been widely disputed as they are not as well characterised as the *Leviviridae* and do not have the morphology or environmental stability of the pathogens they are intended to indicate (Figure 1.5) (Grabow, 2001).

Figure 1.5 TEM of enteric viruses and bacteriophage.

Viruses shown are HAV (A), NoV (B), PV (C), RV (D), Levivirus (E) and Inovirus (F). The scale bar represents 50 nm.

F-specific RNA (FRNA) bacteriophage of the family *Leviviridae* are icosahedral viruses of 21-30 nm diameter (Jofre, 2007) and have attracted interest as useful viral indexes because their morphology and survival characteristics resemble those of non-enveloped RNA human enteric viruses (Grabow, 2001). They have also been used to indicate the presence of enteric viruses in a number of matrices such as ground and surface waters (Leclerc et al., 2000; Sundram et al., 2006), and monitoring shellfish beds (Doré et al., 2000; Flannery et al., 2009) and in evaluating wastewater treatment efficiencies (Flannery et al., 2012; Havelaar and Hogeboom, 1984; Tree et al., 1997).

The family *Leviviridae* is divided into two genera; *Levivirus* and *Allolevivirus*. The *Leviviridae* contain a +sense ssRNA genome that encodes four genes; an assembly protein, a capsid protein, a lysis protein, and a replicase (RNA dependent-RNA polymerase) protein (Figure 1.6). The *Levivirus* genus contain a specific lysis protein whereas in the *Allolevivirus* genus, the A2 protein, present in the read-through protein, functions as a lysis protein (Young et al., 2000). Each FRNA bacteriophage virion contains 180 copies of the capsid or coat protein, 1 copy of the genome, 1 copy of the assembly protein, and, in the

Allolevivirus genus, 15 copies of the read-through protein (Friedman et al., 2009; Van Regenmortel et al., 2000).

Figure 1.6 *Leviviridae* virion and genome organisation

Source: <http://viralzone.expasy.org>

The genus *Levivirus* is subdivided into genogroups I and II, and *Allolevivirus* is subdivided into genogroups III and IV. The main difference between *Levivirus* MS2 and GA is a 60-nts deletion in the 3' UTR while the difference between *Allolevivirus* Q β and SP is a 90-nts deletion in the assembly protein (Van Regenmortel et al., 2000). FRNA bacteriophage groups MS2 and SP have been associated with animal faecal sources, while GA and Q β have been associated with human-derived wastes. For this reason, FRNA bacteriophage have been investigated as a microbial source tracking tool (Beekwilder et al., 1996; Furuse et al., 1981; Hsu et al., 1995; Sundram et al., 2006).

Initial investigations of the potential to track the source of faecal pollution involved the use of phage antisera that showed a predominance of GA and Q β in wastewater from human sources (Furuse et al., 1981). However, the method was found to be tedious and time consuming, and antisera were not readily available (Hsu et al., 1995). Further investigation showed that the phage serogroups could be assigned into respective genogroups through the use of hybridisation with oligonucleotide probes (Hsu et al., 1995).

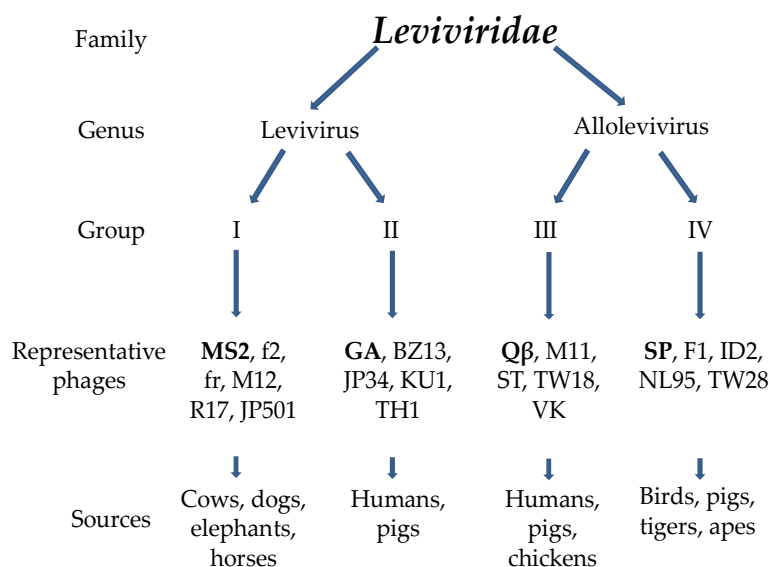


Figure 1.7 *Leviviridae* genera and subgroups

Genotyping of FRNA bacteriophage using oligonucleotide probes have been used to identify the source of faecal contamination in surface waters (Beekwilder et al., 1996). Similar to the findings of Furuse *et al.*, (1981) FRNA bacteriophage GA and Q β have been reported to be present in surface water impacted by human wastewater while the other genogroups are associated with a variety of animal sources (Figure 1.7) (Brion et al., 2002; Cole et al., 2003; Sundram et al., 2006). As the detection of FRNA bacteriophage using oligonucleotide probes is fastidious and labour intensive, a number of RT-PCR (Vinjé et al., 2004) and real-time RT-PCR methods have been developed to allow for the rapid detection of FRNA bacteriophage (Dryden et al., 2006; O'Connell et al., 2006; Ogorzaly and Gantzer, 2006). Wolf *et al.*, designed a broadly reactive multiplex real-time RT-qPCR to detect FRNA bacteriophage genogroups in shellfish and river water using hydrolysis probes (Wolf et al., 2008). The authors designed probes specific for GA and Q β using seven and three FRNA bacteriophage strains, respectively and the assay was found to have 100% homology with FRNA bacteriophage reference strains.

1.10 Bivalve molluscan shellfish

Bivalve molluscs belong to the class Bivalvia, and are so named by referring to the two valves of the shell, in which the soft bodied animal lives. Members of this class include many edible shellfish such as the pacific oyster (*Crassostrea gigas*) and the common blue mussel (*Mytilus edulis*). Bivalve molluscs feed on plankton, microorganisms and organic matter suspended in the water that enters the mouth and is filtered to the stomach via ciliary action. The stomach is embedded within the digestive gland known as the hepatopancreas where the food particles are digested. The accumulation of viruses in shellfish tissues has been shown to be concentrated in the hepatopancreas (McLeod et al., 2009). More recent investigations have shown that NoV bioaccumulation in oyster digestive tissues occurs through specific binding to carbohydrates of the histo-blood group, that are similar to the human histo-blood group antigens (HBGAs) (Le Guyader et al., 2012; Le Guyader et al., 2006). Bivalve molluscs can filter a large volumes of water when feeding, for instance, *C. gigas* can filter 30 litres of seawater per hour (Diederich, 2006). Thus, bivalve molluscs can bioaccumulate viruses to levels much higher than those found in surrounding waters (Richards, 2001). Oysters are particularly at risk of transmitting human enteric viruses because they are frequently eaten raw or only lightly cooked (Lees, 2000).

1.10.1 Microbiological risks associated with shellfish consumption

In 1894, two cases of typhoid fever were recorded in Connecticut, U.S.A. following consumption of shellfish harvested from faecally contaminated coastal waters (Rippey, 1994). Bivalve shellfish were subsequently identified as the causative agents acting as vectors for the transmission of enteric disease. Following this outbreak, typhoid fever associated with the consumption of oysters and clams in the United States and Europe continued to occur. It was not until legislation for the sanitary quality of water and shellfish, in addition to improved WWTPs, that the number of cases gradually diminished, with the final cases of shellfish-associated typhoid occurring in the 1950s (Rippey, 1994).

Although bacteria were well established as causing illness in faecally-contaminated shellfish, viruses were infrequently identified as aetiological agents of illness due to

inadequate diagnostic technology (Lees, 2000). However, in 1955, an outbreak of infectious hepatitis affected 119 people in Sweden as a result of consuming clams that were harvested in proximity to a sewage outfall in Stockholm (Roos, 1956). A subsequent outbreak of infectious hepatitis involving 540 people that consumed clams from the same harvesting area (Roos, 1956), confirmed the role of shellfish in transmitting viral infections. Since that report, numerous outbreaks of virus illness associated with the consumption of shellfish have been recorded (Bellou et al., 2013; Lees, 2000; Potasman et al., 2002).

The largest ever recorded outbreak of foodborne viral illness occurred in Shanghai, China in 1988 when almost 300,000 cases of infectious hepatitis caused by HAV were reported following the consumption of contaminated clams (Halliday et al., 1991). In the 1990s, NoV was implicated as the primary cause of infectious illness associated with shellfish consumption (CDC, 2001). In 1993, a large multi-state outbreak of oyster associated NoV-gastroenteritis occurred in the United States with 186,000 recorded infections (Berg et al., 2000). In Europe, an outbreak of NoV gastroenteritis associated with the consumption of oysters was reported in Scandinavia where approximately 350 cases were reported in Denmark alone (Christensen et al., 1998). Despite the implementation of bacterial controls on shellfish consumption, outbreaks of NoV gastroenteritis have continued to occur (Table 1.10).

Table 1.10 Outbreaks of shellfish-borne viral illness

Shellfish	Country	No of cases	Virus	Reference
Oysters	Australia	467	HAV	(Conaty et al., 2000)
Oysters	USA	171	NoV	(Shieh et al., 2000)
Clams	Spain	183	HAV	(Shanchez et al., 2002)
Clams	USA	5	NoV	(Kingsley et al., 2002)
Oysters	France	14	NoV	(Le Guyader et al., 2003)
Mussels	Italy	103	NoV	(Prato et al., 2004)
Oysters	France	127	NoV	(F. Le Guyader et al., 2006)
	Italy	202		
Oysters	USA	39	HAV	(Shieh et al., 2007)
Oysters	Australia	83	NoV	Webby et al., 2007)
Oysters	France	37	NoV	(Le Guyader et al., 2008)
Oysters	France	23	NoV	(Le Guyader et al., 2010)
Oysters	Ireland and UK	70	NoV	(Doré et al., 2010)
Oysters	UK	11	NoV	(Baker et al., 2010)
Oysters	UK	287	NoV	(Lowther et al., 2012)
Oysters	Canada	36	NoV	(McIntyre et al., 2012)

Human enteric viruses such as NoV and HAV are the most common aetiological agents of human illness transmitted by bivalve molluscan shellfish (Bellou et al., 2013; Formiga-Cruz et al., 2002; Lees, 2000; Richards, 2001). NoV is the main cause of shellfish-borne illness, being responsible for 83.7% of outbreaks with HAV being responsible for 12.8% of outbreaks (Bellou et al., 2013).

1.10.2 European legislation to control microbial risks associated with shellfish

The original Shellfish Waters Directive (79/923/EEC) did not in itself aim to ensure the protection of public health. Instead, the objective of the Shellfish Hygiene Directive 1991 (91/494/EEC) was to guard the quality of shellfish harvesting waters by laying down guidelines for the production and marketing of live bivalve molluscs. On 1st January 2006,

Directive (91/492/EEC) was replaced by new European legislation consisting of five regulations, which together are commonly termed the “Hygiene Package”. These Regulations set out the statutory requirements in relation to food hygiene and premises and three regulations relate to bivalve mollusc production. Regulation 852/2004 states that the responsibility for food safety lies with the food business operator. In addition, a Hazard Analysis Critical Control Point system must be put in place and systems to allow for traceability of food products is required. Regulation EC No. 853/2004 is similar to Directive 91/492/EEC and specifies the requirements for foods of animal origin for industry. Regulation EC No 854/2004 concerns the organisation and application of official controls for products of animal origin by competent authorities in member states.

In Ireland, the Sea Fisheries Protection Authority (SFPA) is the competent authority for the Classification of Live Bivalve Mollusc Production Areas. Shellfish harvesting areas from which live bivalve molluscs are harvested for human consumption are classified as being Class A, B or C depending on the quality of the waters from which they are taken (Table 1.11). The aquaculture of bivalve molluscs is licensed in harvest areas based on the *E. coli* content of shellfish farmed within the harvesting area that are classified based on the *E. coli* results of monthly samples.

Table 1.11 Classification of shellfish harvesting areas.

Category	Microbiological Standard	Treatment required
A	<230 <i>E. coli</i> 100 g ⁻¹	May go direct for human consumption
B	<4,600 <i>E. coli</i> 100 g ⁻¹	Must be depurated, heat treated or relayed to meet class A requirements
C	<60,000 <i>E. coli</i> 100 g ⁻¹	Relay for 2 months to meet class A or B requirements-may also undergo heat-treatment.
Prohibited	>60,000 <i>E. coli</i> 100 g ⁻¹	Harvesting prohibited

Source (Anonymous, 2004)

1.10.3 Post harvest strategies to control microbial risks in shellfish

While heat-treatment (cooking) is effective in reducing microbial concentrations in shellfish, it is not suitable for species traditionally consumed raw or lightly cooked. Other pre-harvest control strategies rely on extending the natural filter-feeding process of the animal in clean seawater in order to purge microbial contaminants (Lees, 2000). Relaying is a practice where shellfish harvested from polluted areas are re-laid to microbiologically-clean environments, allowing shellfish to naturally cleanse themselves of microorganisms (Richards, 1988). Another strategy is depuration where contaminated shellfish are placed in tanks containing disinfected seawater under specific conditions over a period of two or three days (Doré et al., 2003; Jaykus et al., 1994). Depuration is used extensively around the world and has been successful in virtually eliminating bacterial illnesses associated with shellfish consumption where practised (Lees, 2000). While depuration of oysters is not efficient in removing viruses (Doré and Lees, 1995; Loisy et al., 2005), Doré et al. found that depuration efficiency can be improved when performed at conditions similar to those for optimum growth of shellfish (Doré et al., 2003). Such practices have been used successfully in the management of an oyster-related NoV outbreak (Doré et al., 2010).

1.11 Scope of this thesis

The main objective of this study was to investigate the survival of NoV during WWTPs and its accumulation in oysters. A secondary objective was to determine the likely reduction of NoV when discharged into the marine environment under conditions representative of summer and winter in Ireland.

Chapter 2 involved the development of an RT-qPCR assay for FRNA bacteriophage GA which would be used in conjunction with a plaque assay to provide more information on the likely survival of infectious NoV in environmental samples. An assessment of the methods precision and accuracy was carried out on wastewater and oyster samples.

At the outset of this research project, no standardised procedure was available for the concentrations of NoV in wastewater. **Chapter 3** describes the evaluation of a virus concentration method to detect NoV in wastewater samples. From an initial comparison of

three concentration methods, further assessment of an adsorption elution procedure was made. The analytical recovery achieved by the method was carried out using synthetic wastewater that was seeded NoV and FRNA bacteriophage GA.

Chapter 4 investigated the concentrations of NoV in wastewater effluent of a WWTP providing secondary treatment and the concentrations detected in oysters harvested nearby over a year-long period. In addition, the log reductions achieved by the WWTP for *E. coli*, FRNA bacteriophage and NoV were calculated.

Since the reduction of infectious NoV is difficult to determine using RT-qPCR, **Chapter 5** investigated concentrations of FRNA bacteriophage using a plaque assay and an RT-qPCR assay. The WWTP investigated discharged UV disinfected effluent and CSO effluent thus the ratio of infectious to total FRNA bacteriophage was calculated for each effluent type.

Chapter 6 aimed to determine the survival of NoV in the marine environment under temperature and sunlight conditions representative of those found in Ireland. The T_{90} and S_{90} values for NoV was calculated and compared with those of FRNA bacteriophage GA using an RT-qPCR assay and a plaque assay.

The principal conclusions from this research project, along with future recommendations are outlined in **Chapter 7**.

1.12 References

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Chapter 2. Materials and methods

2.1 Wastewater samples

Wastewater samples from two WWTPs were used to determine the most prevalent FRNA bacteriophage group present and the most appropriate virus concentration procedure. WWTP 1 treated wastewater from a population equivalent (PE) of 91,600 and received a daily incoming volume of wastewater of 45,000 m³. WWTP1 treated wastewater with a conventional activated sludge system. WWTP2 treated wastewater from a PE of 10,000 and received an average daily volume of incoming wastewater of 3,000 m³. WWTP2 treated wastewater in a sequenced batch reactor. Preliminary treatment at both plants provided screening and grit removal. One-litre samples of wastewater from both WWTPs were collected in polyethylene bottles that were transported under chilled (<15 °C) temperatures to the laboratory within 24 h of collection.

2.2 Preparation of synthetic sewage (SYNTHESES)

A synthetic wastewater (SYNTHESES) was prepared according to Aiyuk et al., (Aiyuk and Verstraete, 2004) (Table 2.1). The SYNTHESES was stored for a maximum of 1 month under chilled and dark conditions (<5°C).

Table 2.1 Characteristics and composition of SYNTHESES

Characteristics	Concentration (mg l ⁻¹)
COD total	500 ± 50
COD soluble	170 ± 40
TKN	49 ± 8
NH ₄	27 ± 7
PO ₃	21 ± 2
Suspended solids	200 ± 50
Alkalinity	423 ± 41

2.3 Virus extraction from oyster samples

Oyster (*Crassostrea gigas*) samples were collected as part of a routine bacteriological monitoring programme at the Marine Institute. All samples were received into the laboratory chilled below 15°C and within 24 h of sampling. Virus extraction procedures were based on those described in ISO15216 (Anonymous, 2013). Ten oysters were shucked and the hepatopancreas (HP) was dissected from each oyster and finely chopped. Two grams of oyster HP were weighed, and 2 ml of 100 µg ml⁻¹ proteinase K solution (30 U mg⁻¹; Sigma-Aldrich) was added to the oyster hepatopancreas. The sample was then incubated at 37°C with shaking at 150 rpm for 1 h followed by incubation at 60°C for 15 min. The sample was then centrifuged at 3,000 × g for 5 min, and the supernatant was retained for RNA extraction. The oyster extracts were either stored at 4°C prior to RNA extraction within 24 h or stored at -80°C prior to RNA extraction within 1 month.

2.4 Preparation of FRNA bacteriophage stocks and NoV GII positive faecal material

FRNA bacteriophage MS2, GA and Qβ were kindly provided by the European Union reference laboratory for monitoring bacteriological and viral contamination of bivalve molluscs, Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK. FRNA bacteriophage SP was isolated from a mixed culture of FRNA bacteriophage. All bacteriophage genotypes were confirmed using oligonucleotide probes as described in section 2.9. To generate working stocks of each FRNA bacteriophage genogroup, 1 ml of an FRNA bacteriophage genogroup was added to an exponentially growing culture of *S. Typhimurium* WG49 as described in ISO 10705-1 (Anonymous, 1995). Following incubation, 2 ml chloroform was added and the flask was stored overnight at 5°C. The following day, 10% glycerol stocks of bacteriophage was prepared and stored at -80°C to be used within six months. Following preparation, each FRNA bacteriophage working stock was assigned to its respective genogroup using the oligonucleotide probe hybridisation procedure. Faecal material, positive for NoV GII by RT-qPCR, was received frozen into the laboratory. Upon defrosting, the faecal material (1 g) was diluted 1:10 using PBS and divided into 1 ml aliquots prior to storage at -80°C.

2.5 Concentration of viruses using PEG precipitation

The concentration of viruses from wastewater using PEG precipitation was carried out as described by da Silva et al., (2007). Forty millilitres of wastewater from WWTP1 (influent, primary treated or final effluent) was placed in a 50 ml tube to which was added 10 ml of a 50% PEG 6000 solution (Sigma). The tube was placed on a rocking platform for 15-20 h and was incubated at 4°C. The sample was centrifuged at 1500 × g for 1.5 h following which; the supernatant was decanted to waste. The pellet was resuspended in 1 ml of sterile molecular biology grade water and 1 ml of Proteinase K solution (100 µg ml⁻¹; Sigma) and incubated at 56°C for 30 min. The sample was stored at -20°C prior to RNA extraction.

2.6 Concentration of viruses using an AEN procedure

A conventional filter adsorption-elution method was used for the concentration of viruses and was based on published methods (APHA, 2005; Katayama et al., 2002). Forty millilitres of wastewater or SYNTHES sample was used for the concentration procedure. 2.5M MgCl₂ (Sigma) was added to the samples and then was passed through an AP20 pre-filter filter (Millipore) that was placed on top of a type HA negatively charged membrane filter (0.45 µm pore size; Millipore). Membrane filters were rinsed with 15 ml of 0.14 M NaCl and were then placed in 4 ml glycine-NaOH 1% beef extract pH 9.5 buffer. Viruses were eluted from the membrane by shaking at 500 rpm for 20 minutes. The eluate was transferred to an Amicon® Ultra-4 centrifugal filter unit (Millipore) and neutralised using 100 µl HCl. Concentration of the eluates was performed at 4000 × g for 10 minutes. The filter walls were then rinsed using 550 µl of molecular biology grade water and transferred to a 2 ml microcentrifuge tube. The wastewater concentrates were stored at -20°C for less than 1 month prior to RNA extraction.

2.7 Concentration of viruses using an AEP procedure

The concentration of viruses using electropositive membrane filters was carried out as published described (APHA, 2005). Briefly, 40 ml of wastewater or SYNTHES was passed through a positively charged 0.45 μm pore size membrane (Zetapore 0.45SP, 47mm discs). Membrane filters were rinsed with 15 ml of 0.14 M NaCl and were then placed in 4 ml glycine-NaOH 1% beef extract pH 9.5 buffer. The elution of viruses and concentration was carried out as described in section 3.2.2.

2.8 Enumeration of FRNA bacteriophage in oysters and wastewater

Briefly, 1-ml volumes of wastewater or oyster homogenate (Section 2.2.7) and 1 ml of host culture ($>10^6$ CFU ml⁻¹) were added to 2.5 ml of molten 1% tryptone-yeast extract-glucose agar held at 45°C. This was poured onto 2% tryptone-yeast extract-glucose agar plates and incubated overnight at 37°C. FRNA bacteriophage plaques were identified as small (< 6 mm diameter) opaque plaques and were counted. The results were expressed as the number of PFU 100 ml⁻¹ or 100 g⁻¹ for all plaques on each plate.

2.9 Oligonucleotide probe hybridisation to detect FRNA bacteriophage genogroups

FRNA bacteriophage genogroups were identified as described by Sundram et al. (2006) with some modifications. For characterising the FRNA bacteriophage genogroups in the working stocks, wastewater and oyster samples, characteristic plaques were individually transferred to a nitrocellulose positively charged membrane (Amersham) using a wooden applicator stick. Membranes were immersed in denaturing solution (0.05 M NaOH, 0.15 M NaCl) for 1 minute and were placed in neutralising solution (0.1 M sodium acetate) for five minutes. Membranes were allowed to air dry and were placed in an oven at 80°C for 45 minutes. Following this, membranes were allowed to equilibrate in DIG Easy Hyb solution (Roche) for 30 minutes at 37°C and were then immersed in DIG Easy Hyb containing 5 pM ml⁻¹ oligonucleotide probe (Eurogentec) (Table 2.2) for 60 minutes at 37°C. Membranes were washed twice in wash buffer 1 containing $0.2 \times \text{SSC}$

and 0.1% SDS for 10 min. Membranes were placed in DIG blocking buffer (Roche) for 1 hour. Membranes were placed in fresh blocking buffer containing 1 μ l anti-digoxigenin-AP Fab fragments for 45 minutes. The membranes were then placed in detection buffer (Roche) containing the detection substrate (Roche) and were allowed to develop in the dark for three hours. FRNA bacteriophage plaques positive for the respective genogroups were identified as dark blue-purple areas on the membrane and were expressed as GA or Q β PFU 100 ml⁻¹ or 100 g⁻¹ for wastewater and shellfish samples, respectively.

Table 2.2 Sequences of oligonucleotide probes used for probe hybridisation

Probe sequence	Target bacteriophage (genogroup)	Genbank accession no.
5'- AATCGTTCAGGAAGTGAGATTCAAAACC- 3'	GA (II)	NC_001426
5'-AGCCAGAGATTACCAGCAGTAGC-3'	Q β (III)	AY099114

Source (Havelaar et al., 1990)

2.10 Virus RNA extraction procedure

As per the standardised method for the detection of NoV in foodstuffs (Anonymous, 2013), viral RNA was extracted using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux) following the manufacturer's instructions. Five-hundred microlitres of wastewater concentrate, FRNA bacteriophage stock or shellfish extract was used for each extraction and viral RNA was eluted into 100 μ l of elution buffer. The eluted RNA was stored at -80°C until analysis using real-time RT-qPCR was undertaken (<1 month). A single water aliquot was processed alongside wastewater samples as an RNA negative extraction control.

2.11 Preparation of double stranded (ds) DNA-plasmid standards for FRNA bacteriophage GA

Fragments carrying the target sequence for FRNA bacteriophage GA were produced by amplification of target RNA using the real-time RT-qPCR protocol as described in section 2.8. Products from six reactions were then combined of which 50 μl was purified using Wizard 8V PCR purification system (Promega). This was followed by an A-tailing procedure performed on 5 μl of the PCR amplicon with a final extension for 10 minutes at 70° C. Amplicons were purified with QIAquick purification kit (Qiagen) and inserted into the pGEM-T Easy vector (Figure 2.1) (Promega) transformed into JM109 competent cells (Promega). The GA plasmid concentration was 12.4 ng μl^{-1} with an A_{260}/A_{280} of 1.8 as determined using a Nanodrop spectrophotometer. The mass of an individual plasmid molecule was calculated by multiplying the plasmid length in base pairs (bp) by 607.4 (the molecular weight of an average base pair) and dividing by the Avogadro constant (6.02×10^{23}). The number of bp in the plasmid is 3126 bp, therefore, the mass of the plasmid is 3.15×10^{-18} g. The concentration of plasmid was divided by the mass in g of a single plasmid molecule to calculate the concentration of plasmid DNA in copies μl^{-1} . Working stocks of 10^5 copies μl^{-1} were prepared for the plasmid and stored at -20°C.



Figure 2.1 pGEM-T Easy vector used for generation of dsDNA standard

2.12 Detection of NoV GII and FRNA bacteriophage GA by RT-qPCR

RT-qPCR analysis was carried out using an Applied Biosystems AB7500 instrument using the RNA Ultrasense one-step qRT-PCR system (Invitrogen). The reaction was carried out in 20 μ l in a single tube, containing 1 \times reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 \times μ l Rox and 1.25 μ l of enzyme. Primers and probes for all targets are listed in Table 2.3. In addition, 2.5 μ l of Taqman Exogenous internal positive control (IPC) (Applied Biosystems) reaction mix and 0.5 μ l of IPC template DNA were added to control for PCR inhibition for FRNA bacteriophage GA.

Table 2.3 Sequences of primers and probes used in this study

Name	Sequence (5'-3')	Sense	Reference
FRNA bacteriophage GA			
Forward	TCTATGTATGGATCGCACTCG	+	(Wolf et al., 2008)
Reverse	GTAGGCAAGTCCATCAAAGTC	-	(Wolf et al., 2008)
Probe	TGCTGTCCGATTTACGTCTATCTTCA	+	(Wolf et al., 2008)
NoV GI			
Forward (QNIF4)	CGCTGGATGCGNTTCCAT	+	(da Silva et al., 2007)
Reverse (NV1LCR)	CCTTAGACGCCATCATCATTTAC	-	(Svraka et al., 2007)
Probe (NV1LCpr)	TGGACAGGAGAYCGCRATCT	+	(Svraka et al., 2007)
NoV GII			
Forward (QNIF2)	ATGTTCAAGRTGGATGAGRTTCTCWGA	+	(Loisy et al., 2005)
Reverse (COG2R)	TCGACGCCATCTTCATTCACA	-	(Kageyama et al., 2003)
Probe (QNIFS)	AGCACGTGGGAGGGCGATCG	+	(Loisy et al., 2005)

Duplicate 5 μ l aliquots of sample RNA was added to adjacent wells of a 96-well optical reaction plate in addition to no template controls. For NoV analysis, the plate was incubated at 55°C for 60 minutes and 95°C for 5 minutes followed by 45 cycles of 95°C for 15 s, 60°C for 1 min, and 65°C for 1 min. Reaction conditions for the FRNA bacteriophage

GA RT-qPCR assay were similar to those used by Wolf et. al, (2008) and involved an initial incubation at 55°C for one hour followed by 95°C for 5 min and then 45 cycles of 95°C for 15 seconds and 58°C for 1 min. For quantification, dsDNA standards of 10^5 to 10^1 genome copies μl^{-1} for each target were assayed in duplicate on the same PCR plate used for sample analysis. Each sample Cq value was compared with the standard curve Cq values and ascribed a concentration (genome copies. μl^{-1}). Wastewater and oyster samples with amplification efficiency greater than 25% were accepted for inclusion in this study. The theoretical LOD (tLOD) of both assays was 1 genome copy per duplicate RT-qPCR reaction and equated to 20 detectable virus genome copies 100 g^{-1} for oyster samples and 25 detectable virus genome copies 100 ml^{-1} for wastewater samples.

Wastewater and oyster samples with amplification efficiency greater than 25% were accepted for inclusion in this study as described in Chapter 4 of this thesis. For FRNA bacteriophage GA, PCR efficiency was controlled for using Exogenous Internal Positive Control reagents. The theoretical LOD (tLOD) of both assays was 1 genome copy per duplicate RT-qPCR reaction and equated to 25 detectable virus genome copies 100 ml^{-1} for wastewater samples.

*Chapter 3. Selection and validation of a one-step
real-time reverse-transcription quantitative PCR
assay to detect FRNA bacteriophage GA in
wastewater and oysters*

Abstract

In this study, a previously published broadly-reactive multiplex RT-PCR assay for FRNA bacteriophage (Wolf et al., 2008) was adapted to quantify FRNA bacteriophage concentrations in wastewater and oysters. FRNA bacteriophage genogroup GA was found to be the dominant genogroup in wastewater, thus an RT-qPCR for FRNA bacteriophage GA was adapted and validated for use in wastewater and oysters. The primers and probe for FRNA bacteriophage GA were found to be highly specific when compared against other enteric viruses likely to be present in wastewater. Double stranded (ds) DNA plasmids containing the target region were generated for quantification and the RT-qPCR assay demonstrated amplification efficiency of 0.92. The precision of the RT-qPCR was evaluated through inter and intra-assay variability and the mean % coefficient of variance was 5.90 and 9.53% for oyster and wastewater samples, respectively. The accuracy of the RT-qPCR was evaluated through recovery of FRNA bacteriophage GA from spiked oyster hepatopancreas (HP) and SYNTHES (a synthetic wastewater). The accuracy of the RT-qPCR ranged between 88-115% and 73-116% for oyster HP and SYNTHES samples, respectively. The performance of the RT-qPCR during this validation demonstrated that the method is appropriate for use in quantifying FRNA bacteriophage GA concentrations in wastewater and oysters. The method was used throughout this research project to compare the concentrations of infectious and total FRNA bacteriophage GA reduction during wastewater treatment and in the marine environment to those of total NoV.

3.1 Introduction

The survival kinetics of NoV in WWTPs and the marine environment are poorly understood. FRNA Bacteriophage of the family *Leviviridae* are icosahedral viruses of 26 nm diameter that are proposed as suitable viral surrogates because their morphology and survival characteristics resemble those of non-enveloped RNA human enteric viruses such as NoV (Grabow, 2001). They have been used to indicate the presence of enteric viruses in a number of matrices such as ground and surface waters (Leclerc et al., 2000; Sundram et al., 2006), for monitoring shellfish beds (Doré et al., 2000; Flannery et al., 2009) and for evaluating wastewater treatment efficiencies (Flannery et al., 2012; Havelaar and Hogeboom, 1984; Tree et al., 1997). In particular, FRNA bacteriophage GA and QB have been reported to be present in surface water impacted by human wastewater and are attractive target organisms to indicate the presence of human enteric viruses (Brion et al., 2002; Cole et al., 2003; Sundram et al., 2006). A number of RT-PCR (Vinjé et al., 2004) and real-time RT-PCR methods have been developed to allow for the rapid detection of FRNA bacteriophage (Dryden et al., 2006; O'Connell et al., 2006; Ogorzaly and Gantzer, 2006). Wolf et al., designed a broadly reactive multiplex real-time RT-qPCR to detect FRNA bacteriophage genogroups in shellfish and river water using hydrolysis probes (Wolf et al., 2008). The authors designed primers and probes targeting the human-specific FRNA bacteriophage GA and QB. The primer/probe sets designed were found to be specific for their target organisms when challenged with a number of enteric viral and bacterial pathogens. The assay was found to demonstrate a high level of sensitivity and allowed for quantification of FRNA bacteriophage in shellfish and water samples.

While an RT-qPCR method for FRNA bacteriophage has the potential for microbial source tracking (Wolf et al., 2010), when used in conjunction with the plaque assay for FRNA bacteriophage, it may also prove useful to indicate the likely survival of enteric viruses. Currently, it is not possible to culture norovirus (Papafraqkou et al., 2013) and molecular methods such as RT-qPCR provide the only practical approach for detection in shellfish and water samples. However, RT-qPCR detection does not allow for differentiation between infectious and non-

infectious viruses (Nuanualsuwan et al., 2002; Pecson et al., 2011) and thus it is difficult to interpret the infectious risk associated with concentrations of NoV detected in environmental samples. Thus the comparison of an RT-qPCR assay with a plaque assay for FRNA bacteriophage may yield important information of the extent of the overestimation of infectious NoV as judged by RT-qPCR in environmental samples. Assessing the survival of NoV in WWTPs and the marine environment was the main objective of this research project, thus the selection and validation of an RT-qPCR for FRNA bacteriophage was required.

The multiplex RT-PCR assay designed by Wolf et al., was a semi-quantitative, and thus was unsuitable for use in comparing NoV and FRNA bacteriophage GA concentrations. FRNA bacteriophage GA and Q β are the dominant genogroups in wastewater and thus an assessment of which of these genogroups were the more prevalent genogroup was required prior to RT-qPCR assay development. This evaluation would be carried out using the standardised plaque assay and a previously described genotyping procedure for FRNA bacteriophage (Sundram et al., 2006) on wastewater samples. The multiplex RT-qPCR assay required modification to an RT-qPCR assay for the most prevalent genogroup identified in wastewater. In addition, it is desirable for the assay to be compatible with ISO15216-1 that outlines the standardised one-step RT-qPCR procedure for the detection of NoV in foodstuffs (Anonymous, 2013). The NoV assay is a monoplex one-step RT-qPCR and involves the use of magnetic silica as an RNA extraction procedure and the use of a one-step RT-qPCR system that has been specifically designed for the detection of low concentrations of viruses. To allow for quantification, dsDNA plasmids which carry the specific target sequence were required as they are considered to be a more accurate and reliable standard than standards based on viable counts of viruses (Wolf et al., 2008).

To have confidence in the results generated using the newly adapted monoplex RT-qPCR assay for FRNA bacteriophage, a validation of the procedure was required. The criteria that are evaluated to determine whether an assay is fit for purpose are outlined below:

Specificity

An assay must be specific for its target organism and should not detect other organisms likely to be present in the same sample matrices. The specificity of a PCR assay should be evaluated by challenging the assay with organisms similar to the target organism.

Linearity

The linearity of a PCR assay is determined by its ability to induce a response (for qPCR that is the C_q value) that is directly proportional to the concentration of the target. The demonstration of a linear relationship can be evaluated by calculation of a regression line and determining the R² value.

Efficiency

The efficiency of RT-qPCR is based on the assumption that each cycle will yield a doubling of target sequence. Therefore, linear regression analysis of the C_q values obtained for 10-fold dilutions of target will yield a slope of -3.33. Using the formula $\text{Efficiency} = (10^{-1/\text{slope}}) - 1$ (Ramakers et al., 2003), a 100% efficient PCR will give a value of 1.00.

Limit of detection and limit of quantitation

The limit of detection (LOD) can be defined as the smallest concentration of target that can be reliably detected or differentiated from the background for a particular matrix (National Association of Testing Authorities, Australia, 2012). The LOD can be represented by the theoretical LOD (tLOD) that is the lowest concentration of target that in theory can be reliably detected (Figure 3.1) (Anonymous, 2013). The limit of quantification (LOQ) is defined as the smallest concentration of target that can be reliably detected and quantified with acceptable accuracy and precision (Anonymous, 2013; U.S. Department of Health and Human Services, 2001).

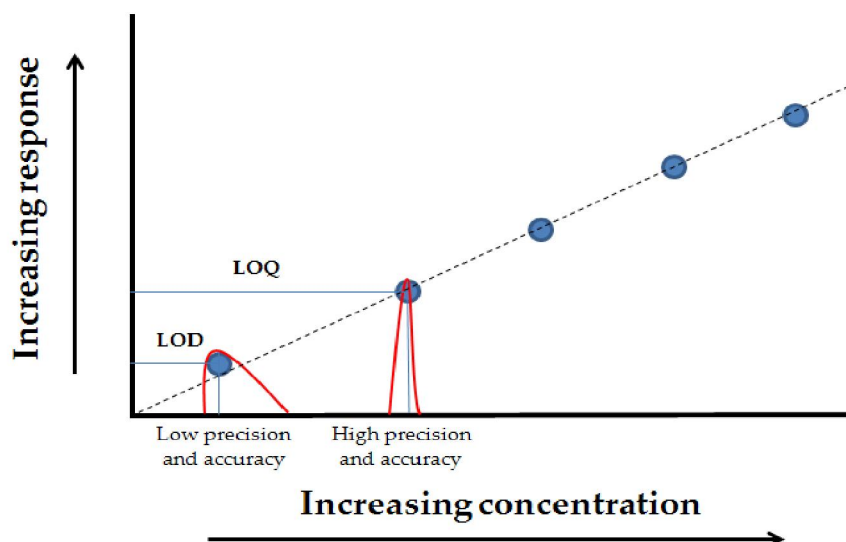


Figure 3.1 shows the LOD and LOQ for an analytical method with respect to accuracy and precision.

Precision

The precision of an analytical method describes the closeness of individual measures of a qPCR assay when applied repeatedly on multiple aliquots of a single homogeneous sample. Precision is therefore a measure of the spread of repeated measurements and provides no indication of how close those results are to the true value (this is termed accuracy) as shown in Figure 3.2

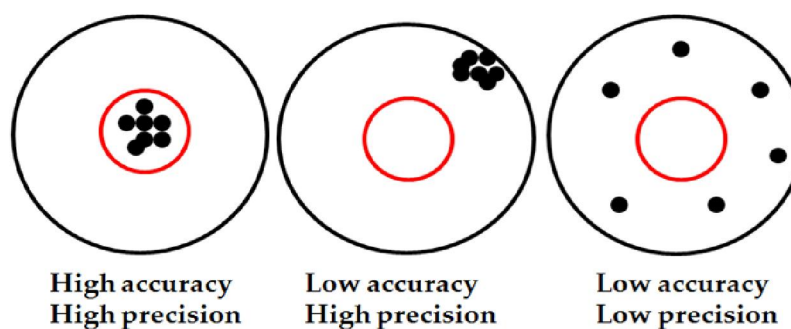


Figure 3.2 Accuracy and precision

Precision should be assessed on samples containing a range of concentrations of target and the %CV should not exceed 15% (U.S. Department of Health and Human Services, 2001). Precision can be further subdivided into intra-assay variability (variability through repeated analyses of one sample by one analyst) and inter assay variability (analysis of the same sample by two analysts).

The standard deviation, percent coefficient of variation (%CV) and confidence interval should be reported for each type of precision investigated.

Accuracy

Accuracy is the term used to describe the closeness of mean results obtained by the method to the true value or concentration of the analyte and therefore includes the effect of both precision and trueness (expressed in the form of bias) (National Association of Testing Authorities, Australia, 2012) (Figure 3.3). Trueness or bias, describes the closeness of agreement between the average value for replicate measurements and the reference concentration.

Figure 3.3 Accuracy as a measure of trueness and precision

Source: http://en.wikipedia.org/wiki/File:Accuracy_and_precision.svg

Accuracy is usually determined by replicate analysis of samples that contain known concentrations of the target and should be measured using a minimum of three concentrations. The deviation of the mean value of the replicate measurements from the true value serves as the measure of accuracy and should be within 15% of the actual value (U.S. Department of Health and Human Services, 2001).

Considering the above criteria, this chapter outlines the selection and validation of an RT-qPCR assay for FRNA bacteriophage GA for use in shellfish and wastewater analysis that will feature in Chapter 5 and Chapter 6 of this thesis.

3.2 Materials and methods

3.2.1 Determination of most prevalent FRNA bacteriophage in wastewater

To determine the most prevalent genogroup present in wastewater, effluent from a WWTP treating a population equivalent of 91,600 and providing secondary treatment was analysed for FRNA bacteriophage using a standard plaque assay ISO10705-1 (Anonymous, 1995) To determine the most common human-specific FRNA bacteriophage in wastewater and thus the most appropriate target to develop a monoplex RT-qPCR assay, FRNA bacteriophage genogroups were characterised in wastewater samples. A total of 10 wastewater samples collected over a period of five weeks were screened for FRNA bacteriophage GA and QB using the plaque assay and oligonucleotide probe hybridisation.

3.2.2 Comparison of FRNA bacteriophage concentrations in oysters using RT-qPCR and plaque assay

To allow for comparison of using the RT-qPCR and plaque assay in oysters, concentrations of FRNA bacteriophage GA (genome copies g⁻¹ HP) were adjusted to genome copies per 100 g whole flesh. The concentrations expressed per gram HP were divided by the mass ratio of HP to total flesh times 100 to yield concentrations per 100 g total flesh as described below.

$$\frac{\text{Concn. (genome copies g}^{-1}\text{)HP} \times \text{weight of HP (g) in 10 oysters}}{\text{weight of whole flesh (g) of 10 oysters}} \times 100$$

3.2.3 Approach to validation of RT-qPCR assay

To allow for validation of the RT-qPCR assay, the following parameters were investigated;

3.2.3.1 Specificity of primers and probes used in RT-qPCR assay

To determine the specificity of the primers and probes used to detect FRNA bacteriophage GA, RNA from four cultures of FRNA bacteriophage (GA, Q β , MS2 and SP), NoV (GI and GII) and HAV was analysed. The RT-qPCR assay was performed on individual aliquots of viral RNA and on a mixture of all viral RNA.

3.2.3.2 Determination of linearity of RT-qPCR assay

An RT-qPCR should allow for a linear detection of the target virus over a wide range of concentrations to be suitable for application in environmental samples. To assess the linearity of the RT-qPCR assay over an extended range, the original FRNA bacteriophage GA plasmid preparation used for the construction of the standard curve was serially diluted to cover a range of 1×10^8 to 1×10^1 genome copies ul^{-1} .

3.2.3.3 Determination of efficiency of RT-qPCR assay

An assessment of the efficiency of the RT-qPCR was carried out on 10 individual dsDNA standard curves (range of 1×10^8 to 1×10^1 copies ul^{-1}) used for the quantification of FRNA bacteriophage GA in 5 wastewater and 5 oyster samples. Using the formula $\text{Efficiency} = (10^{-1/\text{slope}}) - 1$ (Ramakers et al., 2003), the mean efficiency was calculated.

3.2.3.4 Determination of precision of the RT-qPCR assay

Two analysts were required to determine the inter-assay variability of the RT-qPCR assay. SYNTHES (500 ml) was spiked with a culture of FRNA bacteriophage GA and was divided into two aliquots of 250 ml. The spiked SYNTHES was then processed by each analyst five times using the virus concentration procedure followed by the RT-qPCR. To assess the intra-assay variability, three wastewater samples and three environmentally contaminated oyster samples were analysed using the RT-qPCR for FRNA bacteriophage GA. Analysis was carried out five times for each oyster and wastewater sample.

3.2.3.5 Determination of accuracy of the RT-qPCR assay

In the absence of certified reference materials for FRNA bacteriophage GA, an estimate of the accuracy of the assay was initially determined by comparison with the standardised plaque assay (ISO10705) with oligonucleotide probe

hybridisation. Initially, an assessment of RT-qPCR accuracy was determined by analysing an exponentially growing culture of FRNA bacteriophage GA using the RT-qPCR alongside the standard plaque assay. Results from the plaque assay were assumed to represent the reference value. During the log phase of the growth cycle, 1 ml of culture was taken and serially diluted. Dilutions were analysed in duplicate immediately using the RT-qPCR and plaque assay and carried out on three separate occasions. The accuracy of the RT-qPCR in environmental samples was then assessed. Naturally contaminated oyster and wastewater samples (10 each) were analysed using the plaque assay and genotype analysis, and the RT-qPCR assay.

It has been reported that RT-qPCR assays can detect non-infectious viruses and thus may not accurately reflect concentrations determined using plaque assay. Therefore, further determination of accuracy was carried out on the basis of virus recovery data from GA spiked oyster and wastewater samples. Five 2-g aliquots of HP from a batch of non-contaminated (FRNA bacteriophage GA-free) oysters were spiked with varying concentrations (to yield 10^2 - 10^7 genome copies g^{-1}) of FRNA bacteriophage GA. As it was not possible to obtain FRNA bacteriophage GA-free wastewater samples, a synthetic wastewater (SYNTHESES), which correctly reflects the chemical composition of municipal wastewater, was spiked with varying concentrations (to yield 10^2 - 10^8 genome copies 100 ml^{-1}) of FRNA bacteriophage GA. The assessment of accuracy was carried out on 3 separate occasions for oyster and wastewater samples.

3.3 Results

3.3.1 Determination of most prevalent FRNA bacteriophage in wastewater

Table 3.1 shows the distribution of FRNA bacteriophage GA and QB in wastewater samples. FRNA bacteriophage GA and QB was identified in 77% and 9% of all plaques, respectively. From these results, the decision was made to validate the RT-qPCR for FRNA bacteriophage GA.

Table 3.1 Distribution of FRNA bacteriophage GA and Q β in wastewater using oligonucleotide probe hybridisation

	Total FRNA bacteriophage		FRNA bacteriophage GA		FRNA bacteriophage III	
	Plaques	PFU 100 ml ⁻¹	Plaques (%)	PFU 100 ml ⁻¹	Plaques (%)	PFU 100 ml ⁻¹
Influent	68	1.50 × 10 ⁵	58 (85)	1.28 × 10 ⁵	2 (2)	3.00 10 ³
	8	1.87 × 10 ⁴	4 (50)	9.35 × 10 ³	2 (25)	4.67 10 ³
	56	1.18 × 10 ⁴	44 (78)	9.27 × 10 ³	8 (14)	1.65 10 ²
	37	9.25 × 10 ³	30 (81)	7.50 × 10 ³	4 (10)	9.25 10 ²
	28	6.00 × 10 ⁴	21 (75)	4.50 × 10 ⁴	3 (10)	6.00 10 ³
Effluent	2	4.00 × 10 ²	2 (100)	4.00 × 10 ²	0 (0)	0.00 10 ⁰
	61	1.43 × 10 ³	24 (39)	5.63 × 10 ²	13 (21)	3.00 10 ²
	6	1.62 × 10 ¹	6 (100)	1.62 × 10 ¹	0 (0)	0.00 10 ⁰
	10	2.37 × 10 ¹	9 (90)	2.13 × 10 ¹	0 (0)	0.00 10 ⁰
	142	3.23 × 10 ⁴	104 (73)	2.37 × 10 ⁴	11 (7)	2.26 10 ³
Mean	N.A	N.A	77.1	N.A	8.9	N.A

N.A.: not applicable

3.3.2 Specificity of GA primers and probes

The primers and probes used in the RT-qPCR assays for FRNA bacteriophage GA (GA) were specific for their target RNA (Table 3.2). The primers did not cross react with any other viral RNA amplification of non-target RNA did not occur.

Table 3.2 Specificity of the RT-qPCR assay for FRNA bacteriophage GA.

	Mean Cq value							
	GA	Q β	MS2	SP	NoV GI	NoV GII	HAV	Mixed
GII	18.36	Undet.	Undet.	Undet.	Undet.	Undet.	Undet.	19.01

3.3.3 Range of linearity of the RT-qPCR assay

The linear dynamic range of the RT-qPCR assay was initially assessed using an 8- \log_{10} dilution series of plasmid dsDNA. The assay demonstrated linear detection across the entire dilution range (10^1 to 10^8 genome copies ul^{-1}) with an R^2 value of 0.999 (Figure 3.4).

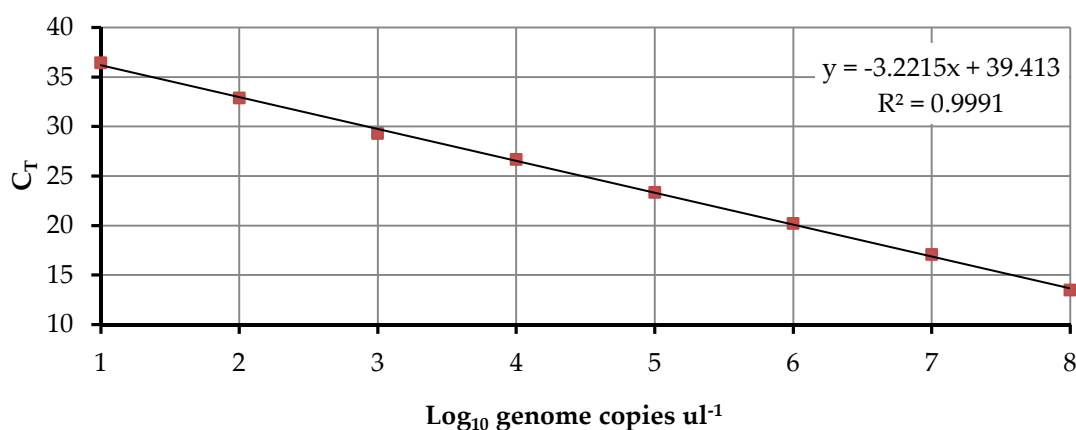


Figure 3.4 Linearity of RT-qPCR on serial dilution of GA plasmid preparation

2.3.4 Efficiency of RT-qPCR assay

Tables 3.3 and 3.4 shows the C_q values from the 10 RT-qPCR assays. The mean efficiency value of the ten RT-qPCR runs was 0.92 (Table 2.5). R² values for the FRNA bacteriophage GA dsDNA standard curves ranged between 0.98 and 0.99.

Table 3.3 Slope and intercept values of dsDNA standard curves

	Mean value (Range)	SD	%CV
R ²	0.99 (0.98 to 1.00)	0.01	0.66
Slope	-3.54 (-3.28 to -3.77)	0.17	4.71
Intercept	18.89 (18.10 to 19.75)	0.56	2.96
Efficiency	0.92 (0.85 to 1.01)	0.06	6.45

Table 3.4 dsDNA standard curve Cq values.

Copies μl^{-1}	RT-qPCR assay number										Mean	SD	% CV
	1	2	3	4	5	6	7	8	9	10			
	FRNA bacteriophage GA dsDNA Cq Values												
10^5	22.01	22.14	22.97	23.12	22.86	22.97	22.15	22.09	22.51	22.40	22.52	0.42	1.88
10^4	25.70	25.63	26.50	26.38	27.05	26.90	25.75	25.56	25.70	25.80	26.10	0.56	2.15
10^3	28.98	29.08	29.65	30.06	29.20	29.05	29.09	28.84	29.42	29.50	29.29	0.37	1.26
10^2	32.22	32.12	33.03	33.95	33.47	33.54	32.28	33.10	32.25	33.83	32.98	0.71	2.16
10^1	35.71	36.98	37.12	36.37	37.00	38.54	36.93	37.01	35.65	36.98	36.83	0.82	2.21

3.3.4 LOD and LOQ of RT-qPCR assay

The tLOD of the RT-qPCR assay was considered to be 1 genome copy per 10 μ l. By taking into account the original sample homogenate volume, the RNA sample extraction volume and the final RNA elution, the tLOD equates to 20 genome copies g^{-1} HP and 25 genome copies 100 ml^{-1} wastewater. In the absence of a study to evaluate the LOQ, the lowest concentration of plasmid DNA (10^1 genome copies μl^{-1}) was assumed to provide a conservative estimate of the LOQ which would equate to 2×10^2 genome copies g^{-1} HP and 2.5×10^2 genome copies 100 ml^{-1} wastewater.

3.3.5 Precision of RT-qPCR

Results of the assessment of inter-assay variability in spiked SYNTHES are presented in Table 3.5. The results achieved by each analyst showed a %CV of less than 5%, and using a two sample t-test no significant difference was found to exist between concentrations ($P = 0.75$).

Table 3.5 Inter-assay repeatability of the RT-qPCR assay in SYNTHES

	Log ₁₀ genome copies 100 ml^{-1}	
	Analyst 1	Analyst 2
Sample 1	8.41	8.53
Sample 2	9.05	8.80
Sample 3	8.89	9.02
Sample 4	8.54	8.44
Sample 5	9.08	9.33
Mean	8.79	8.82
SD	0.30	0.36
% CV	3.45	4.12

The results from the assessment of intra-assay variability of the RT-qPCR assay are presented in Table 3.6. In environmentally contaminated oyster and wastewater samples, the %CV was less than 10%.

Table 3.6 Intra-assay repeatability of the RT-qPCR assay on oyster and wastewater samples

Sample	Log ₁₀ genome copies g ⁻¹ oyster HP			Log ₁₀ genome copies 100 ml ⁻¹ wastewater		
	1	2	3	1	2	3
Replicate 1	3.28	4.32	4.03	5.54	6.38	5.57
Replicate 2	3.59	4.17	3.55	5.44	6.09	5.30
Replicate 3	3.88	4.23	3.38	5.40	6.16	5.86
Replicate 4	3.93	4.04	3.73	5.99	6.87	6.17
Replicate 5	3.81	4.11	3.98	5.80	6.99	6.07
Mean	3.70	4.18	3.73	5.63	6.50	5.79
SD	0.27	0.11	0.27	0.25	0.41	0.36
% CV	7.22	2.59	7.42	4.48	6.31	6.20

3.3.6 Accuracy of the RT-qPCR assay

3.3.6.1 Accuracy of RT-qPCR (cultured FRNA bacteriophage GA)

The assessment of accuracy was carried out using log-diluted cultures of FRNA bacteriophage GA three separate occasions. Mean concentrations of FRNA bacteriophage GA are presented in Table 3.7. The mean R^2 value of three replicates for the RT-qPCR assay was 0.997 and showed comparable results with those of the plaque assay.

Table 3.7 RT-qPCR and plaque assay concentrations in exponentially growing cultures of FRNA bacteriophage GA

Dilution	RT-qPCR			Plaque assay		
	Log ₁₀ genome copies ml ⁻¹			Log ₁₀ PFU ml ⁻¹		
	Culture 1	Culture 2	Culture 3	Culture 1	Culture 2	Culture 3
Neat	4.31	4.72	4.29	4.20	4.29	4.35
10-1	3.31	3.70	3.35	3.45	2.94	3.16
10-2	2.50	2.48	2.41	2.45	2.08	2.21
10-3	1.61	1.78	1.60	1.30	1.18	1.52

Using a student's t-test, concentrations determined using the RT-qPCR were not significantly different to those obtained using the plaque assay ($P=0.21$).

3.3.6.2 Accuracy of RT-qPCR assay (environmentally-contaminated oyster and wastewater samples)

Log concentrations of FRNA bacteriophage GA determined from the analysis of 10 oyster samples using the RT-qPCR assay and the plaque assay with oligonucleotide probe hybridisation are shown in Table 3.8. Concentrations determined by the RT-qPCR assay were significantly greater than those determined by the plaque assay ($P<0.001$).

Table 3.8 Concentrations of FRNA bacteriophage in oysters

	Sample number										Mean
	1	2	3	4	5	6	7	8	9	10	
Plaque assay Log ₁₀ PFU 100 g ⁻¹	3.08	3.48	3.50	3.41	3.38	3.13	2.65	2.65	3.08	2.48	3.08
RT-qPCR Log ₁₀ genome copies 100 g ⁻¹	3.52	4.12	4.63	5.06	4.49	4.44	3.62	3.97	4.21	3.53	4.16

Concentrations of FRNA bacteriophage GA in influent and effluent wastewater are shown in Table 3.9. Concentrations determined by the RT-qPCR assay were significantly different in influent ($P=0.04$) and effluent ($P=0.01$) samples from the plaque assay and yielded greater concentrations in all samples analysed.

Table 3.9 Concentrations of FRNA bacteriophage in wastewater

	Log ₁₀ FRNA bacteriophage GA 100 ml ⁻¹			
	Influent		Effluent	
	Plaque assay	RT-qPCR	Plaque assay	RT-qPCR
	5.16	6.56	2.60	4.79
	3.90	6.02	2.90	5.11
	5.68	6.38	5.36	5.65
	5.36	5.65	3.20	4.59
	5.49	6.05	3.34	4.43
Mean	5.11	6.93	3.48	4.91

3.3.6.3 Accuracy of RT-qPCR in spiked matrices

Results from the assessment of RT-qPCR accuracy in spiked oyster HP and SYNTHES samples are shown in Table 3.10 and Table 3.11, respectively. In oyster HP, the RT-qPCR assay detected FRNA bacteriophage GA at all levels of spike used and all results were within 15% of the intended concentrations. The mean analytical recovery of the RT-qPCR was 101% with a range of 88-115%. In SYNTHES, the RT-qPCR assay detected FRNA bacteriophage GA at all levels of spike. The mean accuracy of the RT-qPCR was 93% with a range of 73-116%.

Table 3.10 Accuracy of RT-qPCR assay in oyster HP

Sample 1			Sample 2			Sample 3		
Log ₁₀ genome copies g ⁻¹			Log ₁₀ genome copies g ⁻¹		% recovery	Log ₁₀ genome copies g ⁻¹		% recovery
Spike*	HP	% recovery	Spike*	HP		Spike*	HP	
7.52	7.34	97.58	7.14	7.20	100.86	7.69	7.55	98.21
6.56	6.73	102.67	6.82	6.72	98.44	6.84	6.04	88.26
5.56	5.78	104.02	5.53	5.30	95.90	5.11	5.20	101.71
4.05	4.62	114.02	4.04	4.20	104.03	3.97	3.90	98.28
3.82	3.93	102.86	3.28	3.21	97.97	3.14	3.34	106.15
2.73	2.87	105.28	2.98	3.44	115.27	2.80	2.62	93.45

Table 3.11 Accuracy of RT-qPCR assay in SYNTHES

Sample 1			Sample 2			Sample 3		
Log ₁₀ genome copies 100 ml ⁻¹			Log ₁₀ genome copies 100 ml ⁻¹			Log ₁₀ genome copies 100 ml ⁻¹		
Spike	SYNTHES	% recovery	Spike	SYNTHES	% recovery	Spike	SYNTHES	% recovery
8.64	7.98	92.45	8.84	8.36	94.58	8.54	8.36	97.91
7.27	6.85	94.27	7.12	6.78	95.25	5.82	6.78	116.55
5.16	4.61	89.39	5.24	4.67	89.23	4.94	4.67	94.67
3.04	2.22	73.02	2.67	2.28	85.40	2.37	2.28	96.26

3.4 Discussion

FRNA bacteriophage are attractive surrogates for NoV in environmental samples as they can be easily enumerated (Doré et al., 2000; Grabow, 2001). The inactivation kinetics for NoV have not been investigated thoroughly as RT-qPCR provides the only suitable detection method (Flannery et al., 2013). For this thesis, it was important to select and validate an RT-qPCR assay for FRNA bacteriophage. When used in conjunction with a plaque assay, this RT-qPCR assay would provide potential information on the inactivation kinetics for enteric RNA viruses such as NoV during WWTPs and in the marine environment.

During this study, a one-step RT-qPCR assay for FRNA bacteriophage GA based on a previously published two-step multiplex RT-PCR assay for the four genogroups of FRNA bacteriophage was developed. Using a plaque assay in conjunction with oligonucleotide probe hybridisation, FRNA bacteriophage GA was the most prevalent genogroup in wastewater. This finding was in accordance with other studies (Beekwilder et al., 1996; Cole et al., 2003; Schaper et al., 2002; Wolf et al., 2008). The previously published (Wolf et al., 2008) primers and probes targeting FRNA bacteriophage GA were found to be highly specific for their target RNA.

A plasmid containing the target sequence was used for quantification of FRNA bacteriophage GA in wastewater and oyster samples. Previous RT-qPCR assays for FRNA bacteriophage have used standards prepared from cultures of FRNA bacteriophage (Ogorzaly and Gantzer, 2006). However, the difference in the ratio of viral RNA (i.e. non infectious viruses) to infectious viruses in these standards may introduce bias to the assay. Multiplex RT-PCR assays are unsuitable for quantification of the target as the various primer sets can compete for reagents in the reaction mix (Dorak, 2007). DNA plasmids containing the target sequence have previously been used to provide an accurate and reliable standard for various enteric viruses (Jothikumar et al., 2006; Victoria et al., 2009; Wolf et al., 2008). When performed on serial dilutions of FRNA bacteriophage GA plasmid, the RT-qPCR assay demonstrated a linear range in excess of concentrations likely to be present in environmental samples. The efficiency of the RT-qPCR assay was similar to the efficiency reported by Wolf et al., and was in the range of those reported of other

RT-PCR assays for FRNA bacteriophage (Kirs and Smith, 2007; Ogorzaly and Gantzer, 2006).

The RT-qPCR assay was found to demonstrate suitable precision in oyster and wastewater samples. In the assessment of inter-assay variability in wastewater, no significant difference was found between results obtained by two analysts. However, in the assessment of intra-assay variability, the %CV achieved in oyster and wastewater samples was greater than that observed in the inter-assay variability. Spiked SYNTHES was used to determine the inter-assay variability, thus, it is possible that viruses were more homogenous than those in the naturally-contaminated samples used in the assessment of intra-assay variability. Despite the differences in %CV for inter and intra assay variability, the overall %CV was less than 10%, a value generally considered as acceptable for PCR assays (Bookout et al., 2006; Lipp et al., 2005; Shanks et al., 2011).

A newly generated culture of FRNA bacteriophage GA was used to assess the accuracy of the RT-qPCR assay and concentrations were not significantly different when compared with a plaque assay. However, in oyster and wastewater samples, a discrepancy between concentrations using the plaque assay and the RT-qPCR assay was found. The RT-qPCR assay consistently yielded greater concentrations than the plaque assay. The difference between concentrations achieved by the RT-qPCR and plaque assays in general can be explained by a number of reasons. During virus replication, many copies of the virus genome are created prior to being packaged within the viral capsids (Dimmock et al., 2009). Thus, within an infected cell, many copies of target RNA can be present and subsequently detected using RT-qPCR. While +sense viral RNA has been shown to be infectious when introduced into cells (Collis et al., 1992; Guix et al., 2007), the naked RNA molecules are incapable of entering cells without their intact capsid and do not constitute an infectious virus. In addition, it has long been documented that during multiple passages of viruses in cell culture, defective interfering virus particles are produced (Huang and Baltimore, 1977). These defective interfering particles may have similar physico chemical properties to infectious viruses (i.e. an intact capsid) yet are non-infectious due to incomplete genomes. Furthermore, naked RNA has been shown to

remain stable in marine and fresh waters (Dancer et al., 2010; Simonet and Gantzer, 2006) and can be detected using RT-qPCR long after infectivity has been lost (Flannery et al., 2013b).

In light of the discrepancy between concentrations determined using plaque assay and RT-qPCR, further validation was carried out using the RT-qPCR in isolation. In the absence of certified reference material for FRNA bacteriophage, accuracy of the RT-qPCR assay was assessed using stocks of FRNA bacteriophage GA. The RT-qPCR assay yielded concentrations of spiked FRNA bacteriophage GA in both wastewater and qPCR corresponded with those achieved in similar assays for enteric viruses such as HAV and NoV (Costafreda et al., 2006; Le Guyader et al., 2009). Therefore the method was considered acceptable for use throughout this research project.

The LOD and LOQ of the RT-qPCR assay were not evaluated fully in this study and only the tLOD and an assumed LOQ were reported. To fully determine the LOD and LOQ of the RT-qPCR assay, samples containing concentrations of FRNA bacteriophage GA below those used for this validation study would need to be analysed. However, by comparison with other RT-qPCR assays for FRNA bacteriophage (O'Connell et al., 2006; Wolf et al., 2008), it is reasonable to assume that the LOQ will lie below the concentration cited in this chapter. The determination of LOD and LOQ is most significant in assays which are used for regulatory decisions (National Association of Testing Authorities, Australia, 2012), thus their calculation did not preclude the use of the RT-qPCR in further studies.

In conclusion, FRNA bacteriophage GA was identified as the most appropriate virus indicator for the development of an RT-qPCR assay for use in wastewater and oyster samples. The assay was shown to detect FRNA bacteriophage GA in naturally and artificially contaminated oyster and wastewater samples. Although FRNA bacteriophage have been proposed to indicate the survival of NoV during wastewater treatment (Flannery et al., 2012), an RT-qPCR similar to that used for NoV would be useful to demonstrate the disparity between both detection methods and may provide additional information on the reduction of enteric viruses to WWTP operators and risk management agencies.

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*Chapter 4. Selection and evaluation of an
adsorption-elution procedure to concentrate
norovirus and FRNA bacteriophage from
wastewater*

Abstract

The absence of widely-used standardised methods for concentrating viruses from wastewater has hampered efforts to study the survival of enteric viruses such as norovirus (NoV) during wastewater treatment. Three previously published viral concentration procedures were investigated; polyethylene glycol precipitation with centrifugation (PEG) and two adsorption-elution (AE) procedures involving the use of either electropositive (AEP) or electronegative (AEN) membrane filters. In natural and seeded wastewater, the PEG method gave poor recoveries of NoV and concentrated RT-qPCR inhibitors. A comparison of the AEN and AEP procedures showed that both methods performed similarly, the AEN procedure was selected for further evaluation as the method was easier to perform and the filters were more-readily available from the supplier. The repeatability of the AEN procedure was assessed using three wastewater samples that were concentrated five times and were analysed for NoV GII. The mean %CV achieved by the AEN procedure was 4.36% (range 3.18-6.11%). Reproducibility was determined by two analysts carrying out the AEN procedure using synthetic wastewater (SYNTHES) spiked with a known concentration of FRNA bacteriophage GA. The mean %CV was less than 5% and no significant difference was found between concentrations detected by RT-qPCR following virus concentration by both analysts ($P=0.851$). An initial assessment of analytical recovery was carried out by comparing FRNA bacteriophage concentrations in wastewater using direct enumeration (DE) and the AEN procedure. The AEN procedure gave mean recoveries of 94% and 86% in untreated and secondary treated wastewaters, respectively. Further assessment of analytical recovery involved seeding SYNTHES with NoV and FRNA bacteriophage GA. The mean analytical recovery of the AEN procedure for FRNA bacteriophage GA and NoV GII was 105% and 110%, respectively. This evaluation demonstrated that the AEN procedure provided an accurate and precise procedure for the concentration of NoV and FRNA bacteriophage in wastewater.

4.1 Introduction

Enteric viruses such as NoV are excreted in the faeces of infected persons at high concentrations (10^8 g^{-1}); however, concentrations present in wastewater may be too low for direct detection using RT-qPCR (Hurst et al., 2007). Interest in the transmission route of PV in the mid 20th century prompted the development of primary concentration procedures for viruses from water (Ikner et al., 2012). Since that time, a number of approaches have been developed to concentrate viruses in water samples and fall into six different categories based on the mechanisms involved (APHA, 2005; Hurst et al., 2007). The advantages and disadvantages of each approach are outlined in Table 4.1.

Initial approaches to concentrate viruses were phase separation techniques that involved the use of dextran or polyethylene glycol (PEG) which were further developed to include semi-permeable membranes (Shuval et al., 1969). However, at the time it was noted that virus concentration methods involving the use of PEG simultaneously concentrate toxic substances that were inhibitory to cell culture (Wyn-Jones and Sellwood, 2001; Ikner et al., 2012). Direct flocculation with beef extract was also investigated as an approach to concentrate viruses however the method also concentrated cell-culture inhibitors (Ikner et al., 2012). Ultra centrifugation was investigated as another approach that has been used successfully for concentrating NoV in wastewater (Carlos et al., 2013), however, the high equipment costs are generally prohibitive for widespread use (Cashdollar and Wymer, 2013). Affinity chromatography has also been investigated as a virus concentration method; however, the generation of specific antibodies was often difficult and the method was not useful for concentrating more than one virus type per samples (Brown et al., 1979).

Adsorption-elution (AE) procedures are generally the most commonly employed virus concentration method for use in recovering viruses from large volumes of water and involve the use of cartridge or membrane type filters (Wyn-Jones and Sellwood, 2001; APHA, 2005). During AE procedures viruses are adsorbed onto the filter matrix during the passage of virus-containing water samples through

the filters. The adsorption of viruses onto filters is based on electrostatic and hydrophobic interactions that predominate at low and high pH levels, respectively (Hurst et al., 2007). The recovery of viruses from these filters is achieved by either passing a solution (termed an elution buffer) through the filter to disrupt these interactions or by dissolution of the filter (APHA, 2005).

Table 4.1 Approaches for primary concentration of viruses from water and wastewater

Approach	Mechanism of concentration	Advantages	Disadvantages
Polymeric phase separation and hydroextraction	A hygroscopic polymer such as PEG) is added to water samples and viruses are concentrated through gravimetric separation. The use of hydro-permeable membranes may be used in conjunction with PEG	Can achieve good virus recovery	Concentrates inhibiting substances
Ultracentrifugation or centrifugal ultrafiltration	Concentration of viruses is achieved through a number of centrifugation procedures. May involve a number of filters with decreasing pore size of the filter and leads to an overall reduction in sample volume.	Good virus recovery	Expensive with long processing times
Phase separation and direct flocculation	The use of chemicals causes the separation of viruses into two phases- one which contains the viruses through the use of flocculating agents.	Good virus recovery	Time consuming, useful for small volumes
Affinity chromatography	Concentration achieved by passing water samples through a sucrose gradient of covalently linked antibodies directed towards the viral capsid. Viruses can be eluted by changing the ionic composition of the gradient.	Good virus recovery	Difficult to obtain antibodies and can inactivate viruses
Passive adsorption	Viruses adhere to cotton or gauze pads via either naturally occurring adsorption/absorption interactions (in passive adsorption) or by attenuating the sample prior to filtration(in passive adsorption)	Inexpensive	Poor recovery of viruses
Directed adsorption	Viruses adsorb to filters on the basis of their charge	Good virus recovery	Can co-concentrate organic solids

Sources (APHA, 2005; Cashdollar and Wymer, 2013; Hurst et al., 2007; Ikner et al., 2012)

For AE procedures, two types of adsorbent filters are used; electropositive or electronegative (sometimes referred to as type HA) filters that have a net negative charge. Electropositive membranes are composed of fibreglass or cellulose acetate and contain a positively-charged polymeric resin. Electronegative membranes are also composed of cellulose or fibreglass. They are termed electronegative when compared to the liquid they are used to concentrate viruses from i.e. liquids of low pH (Hurst et al., 2007). The addition of HCl to the water samples has been shown to improve viral recoveries from electronegative membrane filters (Katayama et al., 2002; Kitajima et al., 2012). In addition, multivalent salts (sodium, magnesium and aluminium chlorides) added to the water sample improves the adsorption of viruses onto electronegative membrane filters (Ikner et al., 2012). Haramoto et al., found that magnesium chloride was the most effective salt for adsorbing PV and FRNA bacteriophage Q β from tap and river water onto electronegative filter membranes (Haramoto et al., 2012). Electropositive membrane filters have been shown to give recoveries comparable with those of electronegative membrane filters (Sobsey et al., 1981) and can adsorb viruses over a wider pH range than electronegative membranes without the addition of polyvalent salts (Ikner et al., 2012). In 2011, the US EPA published an adsorption elution method for the detection of NoV and culturable EV in source and drinking water (Fout et al., 2011).

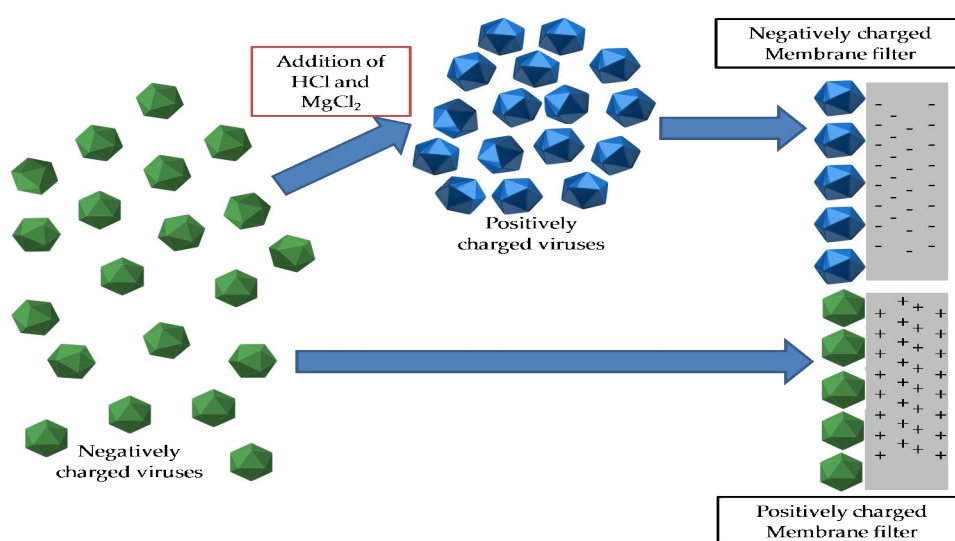


Figure 4.1 Directed adsorption-elution of viruses onto membrane filters

The adsorption of viruses to filters is a reversible process, the reverse of which is termed elution (Hurst et al., 2007). Elution is achieved by exposing the adsorbing matrix (the electropositive or electronegative filter) to an aqueous solution which contain either proteinaceous or chemical substances that causes viruses to desorb from the filter matrix (APHA, 2005). Chemical substances that have been used include chelating agents such as EDTA or detergents (APHA, 2005). Glycine has been widely used to recover viruses following primary concentration procedures (Ikner et al., 2012) as it demonstrates a high buffering capacity and a strong negative charge under alkaline conditions (McMurry and Begley, 2005). Beef extract is the predominant proteinaceous material used for viral elution and works through competition with viruses for binding sites on the adsorbent filter matrix (Ikner et al., 2012). Beef extract at alkaline pH is stipulated as the elution buffer in the US EPA method for NoV and EV in water (Fout et al., 2011). It has been demonstrated that using beef extract at alkaline pH with contact times of 20 minutes were sufficient to yield maximal virus recoveries (Rao et al., 1972).

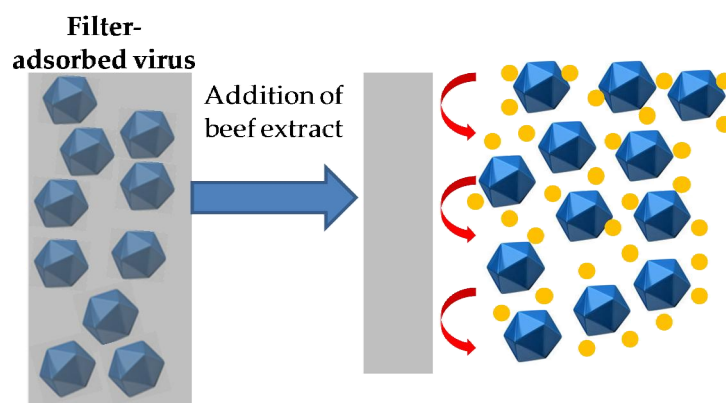


Figure 4.2 Elution of viruses from membrane filters

Virus eluates are generally reduced in volume by a secondary concentration procedure such as organic flocculation or ultracentrifugation. Organic flocculation involves reducing the pH of the beef extract eluate to create a virus-beef extract floc that can be concentrated through centrifugation (Hurst et al., 2007). However, it was demonstrated that organic flocculation does not concentrate all viruses to the same extent (Sobsey and Glass, 1984). In addition, organic substances that are present in the

virus-protein flocs are inhibitory to PCR (Schwab et al., 1993). Centrifugal ultrafiltration involves the centrifugation of eluate using a cellulose membrane to retain viruses based according to the nominal molecular weight limit (NMWL). An ultrafiltration membrane with a stated NMWL should retain most dissolved macromolecules with molecular weights greater than the NMWL. Centrifugal ultrafiltration devices with a 50 kDa NMWL were previously used as a secondary concentration procedure for NoV, FRNA bacteriophage Q β and PV following adsorption elution procedures (Haramoto et al., 2012, 2009; Katayama et al., 2002). Following concentration, the viral retentate can be resuspended into a suitable buffer for downstream processing.

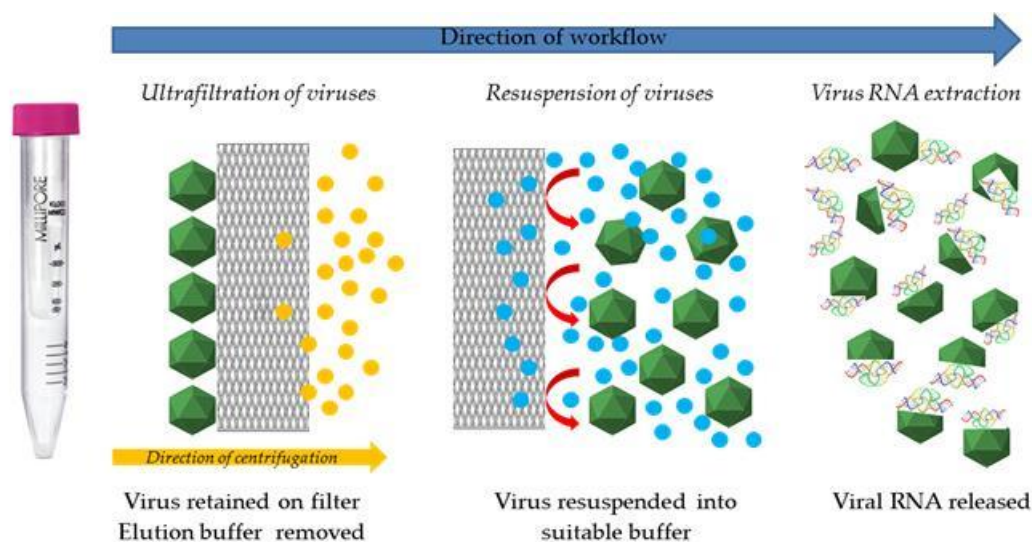


Figure 4.3 Elution of viruses from ultrafiltration membranes

Aside from the potential low concentrations of enteric viruses in wastewater, direct detection using current techniques is oftentimes not possible due to the presence of inhibiting substances (Ikner et al., 2012). Substances that have been shown to be inhibitory to PCR that arise from water or wastewater to be concentrated include haemoglobin, bile salts, urea, polysaccharides, proteins and proteinases (Bessetti, 2007). An advantage of centrifugal ultrafiltration methods is that viruses are simultaneously concentrated whilst eliminating smaller organic molecules and salts (Ikner et al., 2012).

Three methods were evaluated for the concentration of NoV in wastewater for use throughout this research project. Since passive adsorption procedures have been

reported to give poor recoveries of viruses (Ikner et al., 2012), these methods were deemed unreliable for use. Ultracentrifugation facilities were not available in the laboratory, thus, this method of virus concentration was not considered further. As antibodies to target all strains of NoV are not available (Atmar et al., 2011), the development of a concentration procedure based on affinity chromatography was not considered.

The three methods that were considered for evaluation were; precipitation with polyethylene glycol (PEG) and AE procedures using either electropositive (AEP) or electronegative (AEN) membrane filters. Da Silva et al., investigated the removal of NoV during wastewater treatment using PEG precipitation and found that the method successfully allowed for quantification of NoV in all wastewater types (da Silva et al., 2007). The use of adsorption elution procedures to concentrate viruses from water is more frequently reported in the literature. Iwai et al., (2009) used both PEG precipitation and an AEN procedure to concentrate NoV in wastewater and found that while both methods detected similar concentrations of NoV, the AEN procedure was more sensitive. Haramoto et al., (2009) compared an AEN procedure with an AEP procedure to concentrate NoV and PV from different types of water. The authors found that the AEN procedure yielded greater concentrations of viruses than the AEP procedure and that the use of $MgCl_2$ provided better recoveries than $AlCl_3$.

In this chapter, three viral concentration methods were evaluated for their suitability to concentrate NoV and FRNA bacteriophage in wastewater. As there is no culture method for NoV available, FRNA bacteriophage of the family *Leviviridae* (discussed in Chapter 1 of this thesis) are considered to be good indicators of NoV as they have similar characteristic to NoV and can easily be enumerated using a standardised procedure. Methods were initially selected on the basis of the detection of NoV in wastewater and the lack of carryover of PCR inhibiting substances. Following the selection of the most suitable viral concentration procedure, an evaluation of virus recovery and reproducibility/repeatability associated with the procedure was carried out. The repeatability of the concentration procedure was assessed through repeated individual measures of the NoV in wastewater samples. Reproducibility was assessed by two analysts carrying out the viral concentration

procedure on the same spiked synthetic wastewater sample. In the absence of wastewater that was negative for NoV or FRNA bacteriophage, a synthetic wastewater was sourced. SYNTHES is a synthetic wastewater that has been reported to have physicochemical properties similar to domestic wastewater and has been used as a suitable wastewater matrix in anaerobic digestion (Aiyuk and Verstraete, 2004). A measure of the variability of the assay (through repeatability and reproducibility) is expressed as the percentage coefficient of variation (%CV). The %CV is the percentage ratio of the standard deviation to the mean and allows the standard deviation to be placed in context. The assessment of analytical recovery was carried out using samples seeded with known concentrations of virus (NoV and FRNA bacteriophage) and would be reported as a percentage of the nominal value.

4.2 Materials and methods

4.2.1 Approach to assessment

An initial evaluation was carried out of the concentration procedures ability to recover virus from wastewater without co-concentrating substances inhibitory to the RT-PCR. The most appropriate method was then evaluated through an assessment of repeatability, reproducibility and analytical recovery as described below.

4.2.1.1 *Co-concentration of substances inhibitory to RT-PCR*

To control for the presence of RT-PCR inhibitors, 5 μl of sample RNA was added to a further two wells of a reaction plate to which 1 μl of NoV external control (EC) RNA (10^7 genome copies. μl^{-1}) was added. A log dilution series of EC RNA ranging from 10^7 to 10^4 RNA transcripts μl^{-1} was included on each RT-qPCR run. The mean C_q value obtained for samples that included the EC RNA was used to calculate the quantity of EC RNA detected in the sample, which was then used to estimate PCR amplification efficiency, which was expressed as a percentage. No EC RNA was available for FRNA bacteriophage; therefore, the assessment of RT-PCR inhibition was made based on NoV RT-PCR inhibition.

4.2.1.2 Detection of NoV GII in wastewater using three different viral concentration procedures

An assessment of whether the three virus concentration procedures were appropriate to concentrate viruses from wastewater was made. Each concentration procedure was carried out in duplicate on one sample of influent and one sample of secondary treated wastewater. Following RNA extraction, each sample was analysed in duplicate for NoV GII and an assessment of PCR inhibition was included using EC RNA.

4.2.1.3 Detection of NoV from spiked water and wastewater using PEG, AEP and AEN procedures

Further assessment of the virus concentration procedures was carried out using NoV GII positive faecal material that was spiked at different concentrations into 40 ml molecular biology grade water, influent wastewater and secondary treated effluent. Each sample was analysed in duplicate for NoV GII and an assessment of PCR inhibition was included using EC RNA.

4.2.1.4 Comparison of AEP and AEN procedures in wastewater

The PEG precipitation procedure was found to co-concentrate PCR inhibitors, thus it was decided that the adsorption elution procedures would be more suitable for use in concentrating viruses from wastewater. An influent wastewater sample from WWTP1 was used to compare the two adsorption elution procedures. Each sample was analysed in duplicate for NoV GI and NoV GII.

4.2.1.5 *Assessment of repeatability of AEN procedure*

An assessment of repeatability of the AEN procedure was carried out using three samples of untreated wastewater from WWTP2. Each sample was analysed five times for NoV GII and the %CV was determined.

4.2.1.6 *Reproducibility of AEN procedure*

Two different analysts were involved in determining the reproducibility of the AEN procedure. Five-hundred millilitres of SYNTHES was spiked with a stock of FRNA bacteriophage GA ($8.36 \log_{10}$ genome copies ml^{-1}) that was divided into two aliquots of 250 ml. The AEN procedure was carried out on the 250 ml spiked SYNTHES 5 times by two analysts and the %CV for each analyst was calculated.

4.2.1.7 *Analytical recovery of infectious FRNA bacteriophage using AEN procedure*

An initial assessment of the analytical recover of the AEN procedure was carried out using infectious FRNA bacteriophage. Concentrations of FRNA bacteriophage in wastewater determined using direct enumeration were compared with concentrations determined using the AEN procedure. An assumption was made that direct enumeration (DE) is the most accurate method for FRNA bacteriophage in wastewater. The % recovery achieved by the AEN procedure for each wastewater sample was calculated as follows:

$$\% \text{ recovery} = \left(\frac{\log_{10} \text{ concentration AEN}}{\log_{10} \text{ concentration DE}} \right) \times 100$$

A total of 24 wastewater samples (12 influent and 12 effluent) from WWTP1 were processed using DE and the AEN procedure. For DE of FRNA bacteriophage, 1 ml of neat or appropriately diluted (using 0.1% Neutralised Bacteriological peptone) wastewater was assayed using the standard ISO procedure (Anonymous, 1995). For the enumeration of FRNA bacteriophage following the adsorption-elution procedure, viral concentrates (550 μl) were brought to a volume of 1 ml using 0.1% Neutralised

Bacteriological peptone and appropriate dilutions were assayed as per the standard ISO procedure (Anonymous, 1995).

Further determination of the analytical recovery of the AEN procedure for FRNA bacteriophage GA and NoV GII was carried out in SYNTHES as described below;

$$\% \text{ recovery} = \left(\frac{\log_{10} \text{ concentration AEN}}{\log_{10} \text{ concentration of spike per 100 ml SYNTHES}} \right) \times 100$$

Concentrations of FRNA bacteriophage GA used in the assessment of reproducibility were used to determine the analytical recovery. For NoV, 500 ml SYNTHES was spiked with three concentrations of NoV GII (5.29, 3.06 and 2.21 log₁₀ genome copies. ml⁻¹) and was analysed using the AEN procedure in duplicate.

4.3 Results

4.3.1 Detection of NoV GII in wastewater using PEG, AEP and AEN procedures

NoV GII was not detected in influent and secondary treated wastewater following application of the PEG procedure (Table 4.2). In influent wastewater, NoV GII was detected at 3.83 and 4.29 log₁₀ genome copies. 100 ml⁻¹ for the AEP and AEN procedures, respectively. In secondary treated wastewater, NoV GII was detected using the AEP and AEN procedures at 2.75 and 2.43 log₁₀ genome copies. 100 ml⁻¹, respectively. The PEG procedure gave the lowest amplification efficiency while the AEP and AEN procedures gave amplification efficiencies >90% in both sample types.

Table 4.2 Comparison of three virus concentration procedures on wastewater samples

	Log ₁₀ genome copies 100 ml ⁻¹ NoV GII (amplification efficiency %)		
	PEG precipitation	AEP	AEN
Influent wastewater	Undetected (1.41)	3.83 (91.25)	4.29 (112.67)
Secondary treated wastewater	Undetected (3.12)	2.75 (94.63)	2.43 (100.00)

4.3.2 Detection of NoV GII in spiked water and wastewater using PEG, AEP and AEN procedures

Using the PEG procedure, NoV GII was detected in the spiked water and effluent samples however NoV GII was not detected in the influent wastewater samples (Table 4.3). The AEP procedure recovered NoV in all samples types; however, did not recover NoV at the lower spike level. The AEN procedure detected NoV GII in all sample types, at both levels of spike. With the exception of the lower spike in water, the AEN detected NoV GII at greater concentrations than the PEG or AEP procedures. The PEG procedure yielded acceptable amplification efficiency in the water samples only, the AEP and AEN procedures gave acceptable amplification efficiencies in all sample types (>93.58%).

Table 4.3 Detection of NoV GII using the PEG, AEN and AEP procedures

Sample type	NoV spike (μ l)	PEG precipitation		AEP procedure		AEN procedure	
		Log ₁₀ genome copies 100 ml ⁻¹	Amplification efficiency (%)	Log ₁₀ genome copies 100 ml ⁻¹	Amplification efficiency (%)	Log ₁₀ genome copies 100 ml ⁻¹	Amplification efficiency (%)
Water	40	6.53	138	5.42	99.89	6.51	97.59
	4	5.79	88.76	Undetected	97.58	4.82	93.58
Influent	40	Undetected	0	5.97	101.55	5.71	100.15
	4	Undetected	0	Undetected	100.05	3.29	100.55
Effluent	40	5.79	0	5.94	100.05	6.50	99.90
	4	3.58	0	Undetected	99.04	3.74	99.54

4.3.3 Comparison of AEP and AEN procedures in wastewater

The results from the direct comparison of the AEP and AEN procedures in wastewater are shown in Table 4.4. Comparable concentrations of NoV GI and NoV GII were detected in duplicate wastewater samples following the application of the AEP and AEN procedures. Both the AEP and AEN procedures performed similarly to one another and yielded amplification efficiencies >90%.

Table 4.4 Comparison of AEP and AEN procedures in wastewater

Concentration procedure	Sample replicate	Log ₁₀ genome copies 100 ml ⁻¹ (amplification efficiency %)	
		NoV GI	NoV GII
AEP	1	4.20 (97.75)	4.71 (95.20)
	2	3.99 (99.38)	4.22 (96.83)
AEN	1	3.93 (100.37)	4.22 (99.12)
	2	4.04 (99.44)	4.40 (93.49)

The AEP procedure was found to be more time consuming to perform as the time required to filter each sample was greater than that for the AEN procedure. In addition, the electropositive filter membranes were difficult to obtain commercially. Therefore given the comparable performance of the two methods, the AEN procedure was selected over the AEP procedure as the method of choice for further evaluation because of these logistical reasons

4.3.4 Repeatability of AEN procedure

The results from the assessment of repeatability of the AEN procedure on three influent wastewater samples are shown in Table 4.5. NoV GII concentrations in the three wastewater samples ranged from 3.61 to 4.99 log₁₀ genome copies. 100 ml⁻¹. The mean %CV achieved by the AEN procedure was 4.36% (range 3.18-6.11%).

Table 4.5 Assessment of repeatability of AEN procedure

	Replicate NoV GII log ₁₀ genome copies 100 ml ⁻¹					Mean	SD	%CV
	1	2	3	4	5			
Sample 1	3.81	3.76	3.61	3.92	3.68	3.76	0.12	3.18
Sample 2	4.43	4.38	4.36	4.92	4.88	4.59	0.28	6.11
Sample 3	4.66	4.79	4.50	4.78	4.99	4.74	0.18	3.81

4.3.5 Reproducibility of AEN procedure

Table 4.6 shows the results from the assessment of reproducibility of the AEN procedure. The mean concentration of FRNA bacteriophage GA was 8.79 and 8.82 for analysts A and B, respectively. No significant difference was found between concentrations achieved by both analysts using the AEN procedure (P=0.851).

Table 4.6 Assessment of reproducibility of AEN procedure for FRNA bacteriophage GA in SYNTHES

Analyst	Replicate log ₁₀ genome copies 100 ml ⁻¹					Mean	SD	%CV
	1	2	3	4	5			
Analyst A	8.41	9.05	8.89	8.54	9.08	8.79	0.30	3.45
Analyst B	8.53	8.80	9.02	8.44	9.33	8.82	0.36	4.12

4.3.6 Analytical recovery of the AEN procedure

An initial assessment of analytical recovery was carried out using infectious FRNA bacteriophage. Table 4.7 shows the concentrations of FRNA bacteriophage determined using the AEN procedure and DE in influent and effluent wastewater. In influent wastewater, mean FRNA bacteriophage concentrations of $4.57 \log_{10}$ PFU 100 ml^{-1} and $4.32 \log_{10}$ PFU 100 ml^{-1} was obtained using DE and the AEN procedure, respectively. In effluent wastewater, mean FRNA bacteriophage concentrations of $4.33 \log_{10}$ PFU 100 ml^{-1} and $3.79 \log_{10}$ PFU 100 ml^{-1} was obtained using DE and the AEN procedure, respectively. Using a paired t-test, no significant difference was observed between the AEP procedure and DE in influent ($P=0.28$) and effluent ($P=0.062$) wastewater. Assuming that DE provides the most accurate concentration of FRNA bacteriophage in the wastewater samples, the mean recovery using the AEN procedure was 94.78% and 86.34% in influent and effluent wastewater, respectively.

Table 4.7 Analytical recovery of infectious FRNA bacteriophage using the AEN procedure

	Wastewater sample												Mean
	1	2	3	4	5	6	7	8	9	10	11	12	
Influent													
DE (Log ₁₀ PFU 100 ml ⁻¹)	4.93	3.60	6.11	3.80	4.73	2.70	6.54	4.23	5.77	3.11	5.74	3.63	4.57
AEN (Log ₁₀ PFU 100 ml ⁻¹)	5.40	3.58	4.57	3.26	5.83	2.11	5.40	3.66	5.87	3.80	5.65	2.72	4.32
% recovery	109.53	99.44	74.80	85.79	123.26	78.15	82.57	86.52	101.73	122.19	98.43	74.93	94.78
Effluent													
DE (Log ₁₀ PFU 100 ml ⁻¹)	6.11	4.23	5.60	3.44	3.62	3.48	5.36	2.00	5.76	3.42	5.29	3.62	4.33
AEN (Log ₁₀ PFU 100 ml ⁻¹)	6.40	3.98	5.18	2.60	4.27	3.16	4.07	1.21	3.97	1.37	4.78	4.51	3.79
% recovery	104.75	94.09	92.50	75.58	117.96	90.80	75.93	60.50	68.92	40.06	90.36	124.59	86.34

The results from the assessment of reproducibility were also used to determine the analytical recovery of the AEN procedure and are shown in Table 4.8. The concentration of FRNA bacteriophage GA that was spiked into SYNTHES equated to an intended final concentration of 8.36 log₁₀ genome copies. 100 ml⁻¹. For both analysts, the AEN procedure demonstrated a mean recovery of 105% (range 100.62 to 111.63%).

Table 4.8 Analytical recovery of FRNA bacteriophage GA in SYNTHES

Spike log ₁₀ genome copies 100 ml ⁻¹	SYNTHES log ₁₀ genome copies 100 ml ⁻¹		% recovery	
	Analyst		Analyst	
	A	B	A	B
8.36	8.41	8.53	100.62	102.01
8.36	9.05	8.80	108.20	105.24
8.36	8.89	9.02	106.37	107.91
8.36	8.54	8.44	102.11	100.91
8.36	9.08	9.33	108.60	111.63

Table 4.9 shows duplicate concentrations of NoV GII detected in spiked SYNTHES using the AEN procedure. NoV GII was recovered at all levels of spike used with a range of recoveries between 83% and 142 % and a mean recovery of NoV GII was 110%.

Table 4.9 Analytical recovery of NoV GII in SYNTHES

NoV GII log ₁₀ genome copies 100 ml ⁻¹		
Spike	SYNTHES	% recovery
5.29	4.43	84
	4.41	83
3.06	3.73	122
	3.01	98
2.21	2.99	135
	3.14	142

4.4 Discussion

Despite many enteric viruses being excreted in the faeces of infected persons at high titres, they may be present in wastewater at low concentrations. This presents a challenge for the detection of viruses in wastewater. For this thesis, it was necessary to select and evaluate a viral concentration procedure. In this chapter, three different virus concentration procedures were assessed for their suitability for analysis of wastewater for NoV to be used in further studies.

An initial assessment of the three concentration methods was carried out to determine whether the methods were capable of concentrating NoV from naturally contaminated wastewater. The PEG procedure did not allow for the detection of NoV GII in naturally contaminated wastewater; however, was successful in spiked water and wastewater matrices. Despite this, the PEG method gave unacceptable RT-PCR efficiencies when used on wastewater samples. This finding is in accordance with previous studies demonstrating that virus concentration procedures based on PEG precipitation concentrate substances that are inhibitory to cell culture and PCR (Ikner et al., 2012). Despite this, Da Silva et al., (2007) successfully used PEG precipitation to measure NoV concentrations in a number of WWTPs and found that the method gave acceptable RT-PCR efficiency (>10%) in 95% of samples. The RT-PCR efficiency considered acceptable in this study was >25% and was consistently achieved by both the AEP and AEN procedures.

The two adsorption-elution procedures evaluated in this study did not co-concentrate RT-PCR inhibitors and this has been reported previously by Haramoto et al., (Haramoto et al., 2009). In general, both the AEP and AEN procedures were easier to perform than the PEG procedure and could be performed in less than 2 hours compared with >24 hours for PEG. This and the fact that both the AEP and AEN procedures successfully detected NoV in spiked and unspiked wastewater supports the frequency of their use in the literature (Cashdollar and Wymer, 2013; Ikner et al., 2012). The AEP procedure yielded marginally greater concentrations of NoV GII in spiked wastewater samples than the AEN procedure. Conversely, in unspiked wastewater samples, the AEN procedure detected NoV GI and NoV GII at greater concentrations than the AEP procedure. The limited number of samples used in the comparison of the two

concentrations procedures precluded statistical analysis. In unspiked wastewater, both methods yielded concentrations of NoV within 5% of each other. However, as the AEP procedure took longer to perform than the AEN procedure and as the electropositive filters were difficult to obtain commercially, the AEN procedure was selected for further evaluation.

To evaluate the AEN procedure, repeatability and reproducibility was assessed using a synthetic wastewater (SYNTHESE) spiked with NoV GII-positive faecal material or FRNA bacteriophage. Although other surrogate viruses have been used when developing and evaluating virus concentration methods (such as PV and AdV) (Haramoto et al., 2012; Calgua et al., 2008), FRNA are ubiquitous in wastewater and are easily enumerated (Grabow, 2001). Given the frequent use of FRNA bacteriophage as surrogates for NoV in the available literature, they were considered suitable for use in the evaluation of the AEN procedure and throughout this thesis. The mean %CV of the AEN procedure for both repeatability and reproducibility was less than 5%, however, the performance of adsorption-elution procedures (in terms of repeatability and reproducibility) are rarely reported in the available literature therefore, it is difficult to place the results in this study in context. A %CV of less than 15% is considered suitable when assessed on samples containing a range of target concentrations (U.S. Department of Health and Human Services, 2001) as was used in this evaluation.

An assessment of analytical recovery of the AEN procedure was carried out using a plaque assay for FRNA bacteriophage. The mean analytical recovery of FRNA bacteriophage achieved using the AEN procedure was 86% however in some samples, the analytical recovery was greater than 100%. This is not entirely surprising as aggregates of viruses have been observed to occur in water and wastewater (Gerba et al., 1978; Mattle et al., 2011) and may lead to underestimations as determined using a plaque assay (Teunis et al., 2005). However, the range of recoveries demonstrated here using infectious FRNA bacteriophage was similar to the range previously reported for PV (23-80%) in wastewater and river samples (Haramoto et al., 2012, 2008; Katayama et al., 2008) and for FRNA bacteriophage Q β (27-68%) in river water (Haramoto et al., 2012).

The mean analytical recoveries of the AEN procedure for FRNA bacteriophage GA and NoV GII determined using RT-qPCR was 105% and 110%, respectively.

Haramoto et al., found that the recovery of PV and NoV using RT-qPCR, were not significantly different when using an AEN procedure and reported recoveries of NoV and PV greater than 160% in filtered water (Haramoto et al., 2009). In another study, the mean recovery of NoV and PV in wastewater were 110% and 91%, respectively (Haramoto et al., 2008). For similar reasons to the analytical recovery in excess of 100% observed in this study for infectious bacteriophage, it is also possible that aggregates of virus existed in the spike material (Teunis et al., 2008) that did not undergo a thorough RNA extraction. Alternatively, the concentration of virus in the spike material was simply too great to be fully extracted using the magnetic extraction reagents. In addition, the overestimation of analytical recovery may be accounted for by the inherent variability of RT-PCR (Bustin and Nolan, 2004).

A synthetic wastewater SYNTHES was used in the absence of virus negative wastewater, that was previously developed for use in anaerobic digestion whereby COD removal and methane production was investigated (Aiyuk and Verstraete, 2004). It is possible that in the absence of the other materials (fats, haemoglobin, proteins and faecal solids) present in municipal wastewater, SYNTHES may not provide the most accurate synthetic wastewater matrix to assess viral concentration procedures. Notwithstanding, in a meta-analysis of virus concentration techniques, it was found that the properties of the target virus have more of an influence on the performance of the virus concentration procedure than the sample matrix, volume or filter type (Cashdollar and Wymer, 2013). It is possible that the AEN procedure evaluated in this study is suitable for the concentration of ssRNA enteric viruses however, may not be suitable for other enteric viruses such as adenoviruses. The analysis of wastewater for NoV yielded concentrations of $>10^3$ per litre which is in agreement with concentrations reported in the literature (Haramoto et al., 2012; Kitajima et al., 2012; Prado et al., 2011) and supports the suitability of the AEN procedure to be used in this context. Furthermore, the AEN procedure produced repeatable results for FRNA bacteriophage and NoV GII in wastewater and could be reproducibly applied within the laboratory. Thus, the AEN procedure was deemed appropriate for use throughout this research project.

4.5 References

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*Chapter 5. Concentration of norovirus during
wastewater treatment and the impact on oyster
contamination*

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Abstract

Concentrations of *E. coli*, FRNA bacteriophage, norovirus genogroup I (NoV GI) and II (NoV GII) in wastewater were monitored weekly over a one-year period at a wastewater treatment plant (WWTP) providing secondary treatment. A total of 49 samples of influent, primary and secondary-treated wastewater were analyzed. Using a real-time RT-qPCR, mean NoV GI and NoV GII concentrations detected in effluent wastewater were 2.53 and 2.63 log₁₀ virus genome copies 100 ml⁻¹ respectively. Mean NoV concentrations in wastewater during the winter period (January to March inclusive) (n=12) were 0.82 (NoV GI) and 1.41 (NoV GII) log units greater than mean concentrations for the rest of the year (n=37). The mean reduction of NoV GI and GII during treatment was 0.80 and 0.92 log units respectively with no significant difference detected in the extent of NoV reductions due to season. No seasonal trend was detected in the concentrations of *E. coli* or FRNA bacteriophage in wastewater influent and showed mean reductions of 1.49 and 2.13 log units respectively. Mean concentrations of 3.56 and 3.72 log₁₀ virus genome copies 100 ml⁻¹ for NoV GI and GII respectively were detected in oysters sampled adjacent to the WWTP discharge. A strong seasonal trend was observed and concentrations of NoV GI and GII detected in oyster were correlated with concentrations detected in the wastewater effluent. No seasonal difference was detected in concentrations of *E. coli* or FRNA bacteriophage detected in oysters.

5.1 Introduction

Norovirus (NoV) is the most common cause of outbreaks of acute gastroenteritis in Ireland (Cloak et al., 2010) and is the major cause of acute non-bacterial gastroenteritis in adults worldwide (Mead et al., 1999). In general NoV causes mild illness involving diarrhoea and vomiting although symptoms can be more severe in vulnerable groups such as the elderly (Mead et al., 1999). NoV is spread by the faecal oral route and has been demonstrated to be highly infectious particularly in enclosed settings such as schools, hospitals, care homes, cruise ships and domestic residence (Mead et al., 1999; Estes et al., 2006; Harris et al., 2007). The NoV genus comprises non-enveloped, positive-sense RNA viruses of the family *Caliciviridae*. The genus norovirus is genetically diverse and is divided into 5 different genogroups based on the sequence similarity of the capsid protein (Karst, 2010). Each genogroup has a varying number of genotypes; NoV genogroup I (NoV GI) and NoV genogroup II (NoV GII) contain the majority of NoV genotypes that have been implicated as causing illness in humans (Siebenga et al., 2010). NoV GII, and in particular variants of the NoV GII genotype 4, are most commonly associated with human illness in clinical and community outbreaks (Atmar and Estes, 2006; Lopman et al., 2009; Siebenga et al., 2010).

NoV can be shed in large numbers (up to 10^8 viruses g^{-1}) in the faeces of infected individuals (Lees, 2000) and can continue to be excreted for up to two weeks post-resolution of symptoms (Okhuysen et al., 1995), NoV is, therefore, commonly present in municipal wastewater (da Silva et al., 2007; Katayama et al., 2008; Nordgren et al., 2009). The discharge of municipal wastewater into aquatic environments is practised throughout the world and the link between wastewater discharge and the contamination of bivalve molluscan shellfish is well established (Lees, 2000). Such contamination occurs because bivalve molluscan shellfish such as oysters are filter feeders and can accumulate microorganisms particularly when grown in sewage contaminated water (Lees, 2000). Oysters can become contaminated with NoV in this manner and have been linked to numerous outbreaks of gastroenteritis in consumers (Perret and Kudesia, 1995; Ang, 1998; Doré et al., 2010; Westrell et al., 2010). This public health problem is recognised worldwide and sanitary regulations based on bacterial standards exist to control the risk. However, despite compliance with the existing bacterial standards, NoV

contaminated oysters continue to cause illness on an ongoing basis (Doré et al., 2010; Westrell et al., 2010). The environmental discharge of wastewater is also closely regulated to limit this impact. In Europe, designated sensitive marine sites such as shellfish harvesting areas are protected under the appropriate environmental regulations such as the Shellfish Waters Directive (2006/113/EC) (Anonymous, 2006). Authorities must endeavour to ensure that shellfish harvested from designated shellfisheries comply with the relevant bacterial standards. Wastewater treatment can be considered to be a significant control point to limit the extent of microbial contamination of the marine environment and achieve compliance with both food safety and environmental bacterial standards.

The impact of wastewater treatment on faecal indicator organisms such as *Escherichia coli* (*E. coli*) has been extensively studied and comprehensive data exists on the removal of such organisms through wastewater treatment (Lucena et al., 2004; Wery et al., 2009). Similarly, the survival of faecal indicator organisms in the marine environment is well described (Bitton, 2005). Therefore it is possible to accurately predict the likely microbiological impact of a wastewater treatment plant (WWTP) discharge on a shellfishery in terms of faecal bacteria allowing the likelihood of compliance with the regulatory limits to be determined. Data from previous studies generally indicate that concentrations of enteric viruses may be reduced to a lesser extent than bacteria during the wastewater treatment processes (Havelaar et al., 1993; Haramoto et al., 2006); limited data, however, exists concerning the extent of NoV removal during WWTP treatment. The lack of such data is primarily due to the absence of a reliable culture system for NoV and has led to the use of viral indicator organisms.

FRNA bacteriophage of the family *Leviviridae* have been used as surrogates for enteric viruses in wastewater (Havelaar et al., 1993) and in shellfish (Doré et al., 2000). The detection of NoV in environmental samples using molecular techniques has traditionally been difficult because of the relatively low target concentrations involved and the inhibitory substances present in such samples (Le Guyader et al., 2009). Recently, robust real-time RT-qPCR procedures have been used for the quantitation of NoV in shellfish (Le Guyader et al., 2009) and wastewater (da Silva et al., 2007; Nordgren et al., 2009). da Silva et al., (2007) monitored wastewater effluents to assess the

removal of NoV during different wastewater treatment processes using real-time RT-qPCR and found that all processes studied reduced the NoV concentrations discharged into receiving waters. Nordgren et al., (2009) monitored the concentrations of NoV in wastewater effluents over a one year period and found that NoV GII demonstrated a seasonal trend with greater concentrations detected in the winter months. In addition, both NoV GI and GII reductions during wastewater treatment were similar. However, no quantitative studies assessing the reduction of NoV through a WWTP and subsequent transmission to shellfish are present in the available literature. The aim of this study was to evaluate the reduction of NoV GI and NoV GII through a WWTP providing secondary treatment and to evaluate the impact of the discharge on the concentrations of NoV in oysters adjacent to the outfall.

5.2 Materials and methods

5.2.1 Wastewater Treatment Plant and Wastewater Sampling

The WWTP studied treated wastewater from a population equivalent (P.E.) of 91600 and received an average daily volume of incoming wastewater of 45000 m³. Preliminary treatment at the plant provided screening and grit removal. This was followed by treatment with a conventional activated sludge system including primary settlement, aeration and final settlement. The final effluent was discharged into the sea through a 400 m long outfall pipe at a depth of 10 m.

One litre, 24-hour composite samples of influent and final effluent were taken on a weekly basis. In addition, a one litre grab sample of wastewater was collected following primary treatment. All wastewater samples were collected in polyethylene bottles and transported under ambient temperatures to the laboratory within one hour of collection. Wastewater sampling commenced in June 2009 and ended in May 2010 (n=49).

5.2.2 Concentration procedure for wastewater sample NoV analysis

A conventional filter adsorption-elution method was used for the concentration of wastewater samples and was based on previously described methods (APHA, 2005; Katayama et al., 2008). Four hundred microlitres of 2.5M MgCl₂ (Sigma-Aldrich, United Kingdom) was added to a single, 40 ml sample volume of wastewater to obtain a final concentration of 25 mM MgCl₂. The sample was then adjusted to between pH 3.5 and pH 6.0 with 1M HCl (Sigma-Aldrich) and mixed on a rocking platform for 45 minutes. The sample was then passed through a glass fibre pre-filter (Millipore, Billerica, MA) placed directly on a bacteriological membrane filter (0.45 µm pore size and 90mm diameter; Millipore) attached to a plastic magnetic filter holder (Pall, Port Washington, NY). The filters were then washed once using 25 ml 0.14M NaCl and dried of excess wash solution prior to placing the bacteriological membrane filter in 4 ml 50 mM glycine-NaOH buffer pH 9.5 and shaking at 500 rpm for 20 min. The virus eluate was transferred to a tube containing 100 µl of 1 M HCl (pH 1.0) followed by centrifugation using an Amicon® Ultra-4 centrifugal filter unit (Millipore) at 4000 x g for 10 min. The

filter unit was washed in 550 μl of molecular biology grade water and the virus concentrate ($>500 \mu\text{l}$) stored at -20°C prior to RNA extraction.

5.2.3 Oyster sampling

Oysters from a batch previously demonstrated to be free from microbial contamination (*E. coli*, FRNA bacteriophage and NoV) were suspended in mesh bags 1m below the water surface directly above the WWTP outfall. Oysters were deployed at the outfall for one month before sampling commenced. Samples of 24 oysters were collected each week and transported to the laboratory within 2 hours under chilled conditions ($<15^{\circ}\text{C}$). Each week oyster samples were collected five days before the wastewater samples were collected. Oyster sampling commenced in July 2009 and ended in May 2010 (n=38).

5.2.4 Preparation of oyster samples for *E. coli*, FRNA bacteriophage and NoV analysis

Upon receipt into the laboratory any dead or open oysters not responding to percussion were discarded. Oyster samples were analyzed for *E. coli* and FRNA bacteriophage within 24 hours of receipt using previously published methods (Anonymous, 1995, 2005). For *E. coli* and FRNA bacteriophage analysis, 10 oysters were thoroughly cleaned under running potable water, the meat and intravalvular fluid was homogenized using a blender and diluted 1:3 with 0.1% (w/v) neutralised bacteriological peptone (Oxoid, Cambridge, U.K.) (Anonymous, 2003). For FRNA bacteriophage analysis, 50 ml of the diluted homogenate was centrifuged at $2000 \times g$ for 10 min and the supernatant retained for testing.

For NoV analysis a further 10 oysters were opened and the hepatopancreas from each oyster was dissected and was finely chopped. Two grams of oyster hepatopancreas was weighed and 2 ml of $100 \mu\text{g ml}^{-1}$ Proteinase K solution (30 U mg^{-1} ; Sigma-Aldrich) was added. Fifty microlitres of Mengo virus strain MC₀ was added at this stage as an internal positive control (IPC) virus controlling for the virus extraction efficiency similar to that described by Costafreda et al., (Costafreda et al., 2006). The sample was then incubated at 37°C with shaking at 150 rpm for 1 hour followed by incubation at 60°C for 15 min. The sample was then centrifuged at $3000 \times g$ for 5 min and the supernatant was

retained for RNA extraction. The homogenates were either stored at 4°C prior to RNA extraction within 24 hours, or stored at -80°C where RNA extraction was undertaken within 1 month.

5.2.5 *E. coli* enumeration in wastewater and bivalve molluscan shellfish

Appropriate log dilutions of influent and effluent wastewater samples respectively and diluted shellfish homogenates were assayed for *E. coli* using a standardized five-tube three-dilution most probable number (MPN) method (Anonymous, 2005). This procedure is the mandatory method used in Europe to classify shellfish harvesting areas. The diluted wastewater and homogenates were inoculated into 10 ml volumes of minerals modified glutamate broth MMGB (CM0607, Oxoid) and were incubated at 37°C for 24 ± 2 hours. The presence of *E. coli* was subsequently confirmed by subculturing tubes indicating acid production onto TBX agar (CM0945, Oxoid) at 44°C for 24 ± 2 hours. The limit of detection (LOD) of the assay was an MPN of 20 *E. coli* 100 g⁻¹ and 20 *E. coli* 100 ml⁻¹ for shellfish and wastewater samples respectively.

5.2.6 FRNA bacteriophage enumeration in wastewater and bivalve molluscan shellfish

The diluted wastewater samples and shellfish homogenate were analyzed for FRNA bacteriophage using a standardized procedure (Anonymous, 1995) that uses the *Salmonella enterica* serovar Typhimurium WG49 host (Havelaar and Hogeboom, 1984). *S. Typhimurium* has been genetically engineered by the inclusion of an F-pili producing plasmid and has been shown to reliably select for FRNA bacteriophage and demonstrate negligible interference from somatic bacteriophage (Havelaar et al., 1993). Briefly, to 2.5 ml of molten 1% tryptone yeast-extract glucose agar held at 45°C, was added 1 ml volumes of appropriately diluted sample and 1 ml of host culture (>10⁶ CFU ml⁻¹). This mixture was poured onto 2 % tryptone yeast-extract glucose agar plates and incubated overnight at 37°C. Characteristic plaques were counted and each plaque was assumed to originate from one FRNA bacteriophage. The results were expressed as the number of plaque-forming units (PFU) 100 g⁻¹. The LOD of the assay for shellfish and wastewater samples was 30 PFU 100 g⁻¹ and 10 PFU 100 ml⁻¹, respectively.

5.2.7 NoV RNA extraction procedure for shellfish and wastewater extracts

RNA was extracted from 500 μl of wastewater extract or shellfish Proteinase K extract using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. Viral RNA was eluted into 100 μl of elution buffer (bioMérieux). A single negative RNA extraction control (using water only) was processed alongside shellfish and wastewater samples to be extracted. The eluted RNA was stored at -80°C until analysis using real-time RT-qPCR was undertaken.

5.2.8 RT-qPCR controls and standards

Plasmids carrying the NoV GI and GII target sequences (supplied by Dr. Françoise S. LeGuyader, Ifremer, Nantes, France) were used to prepare standards for quantitation and controls for determining RT-PCR inhibition. Plasmids were transformed in competent cells to create dsDNA and purified as described by Le Guyader et al., 2009 (Le Guyader et al., 2009). From the purified dsDNA, single-use aliquots containing 10^5 genome copies μl^{-1} .

NoV GI and NoV GII were prepared for quantitation in the RT-qPCR. From the dsDNA plasmids, external control (EC) RNA was prepared using the same procedure as Le Guyader et al., 2009 (Le Guyader et al., 2009) and were divided into single-use aliquots of 10^7 genome copies μl^{-1} for NoV GI and GII for use in determining RT-PCR inhibition. The dsDNA and EC RNA standards were stored at -20°C for a period of less than 6 months at which time a new batch was prepared containing the same concentration.

5.2.9 Determination of NoV GI and GII using one-step RT-qPCR

For NoV GI and NoV GII analysis of wastewater and shellfish samples, duplicate $5\mu\text{l}$ aliquots of sample RNA was added to adjacent wells of a 96-well optical reaction plate. This was followed by 20 μl of the appropriate one-step reaction mix prepared using RNA Ultrasense one-step RT-qPCR system (Invitrogen, Carlsbad, CA) containing 1 \times reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 \times μl Rox and 1.25 μl of enzyme mix. For NoV GI analysis, previously described primers

QNIF4 (da Silva et al., 2007), NV1LCR and probe NVGG1p (Svraka et al., 2007) and for NoV GII analysis, primers QNIF2 (Loisy et al., 2005), COG2R (Kageyama et al., 2003) and probe QNIFS (Loisy et al., 2005) were used. In addition, no template controls were included for NoV GI, GII and IPC virus on the same 96-well plate. The plate was incubated at 55°C for 60 min, 95°C for 5 min and then 45 cycles of 95°C for 15s, 60°C for 1 min and 65°C for 1 min on an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA).

To control for the presence of RT-PCR inhibitors 5 µl of sample RNA was added to a further two wells to which 1 µl of EC RNA (10^7 genome copies μl^{-1}) was added. A log dilution series of the NoV GI and GII EC RNA ranging from 10^7 to 10^4 copies μl^{-1} was included on each RT-qPCR run. The mean Ct value obtained for samples that included the EC RNA was used to calculate the quantity of EC RNA detected in the sample which was then used to estimate PCR amplification efficiency which was expressed as a percentage. Wastewater and oyster samples with an amplification efficiency greater than 25% were accepted for inclusion in this study.

For extraction efficiency, samples seeded with the IPC, Mengo virus, were subjected to RT-qPCR for Mengo virus. Twenty microlitres of a one-step reaction mix prepared with the same one-step RT-qPCR system containing the same concentrations of reaction mix, primers, probe, Rox and enzyme mix as was used for NoV analysis. Duplicate 5µl aliquots of sample or extraction control RNA were added to the adjacent wells of the 96-well plate. Forward (Mengo209) and reverse (Mengo110) primers and probe (Mengo147) used were the same as those described by Pintó et al., 2009 (Pintó et al., 2009). The Ct value of the sample was compared to a standard curve obtained by preparing log dilutions from the same batch of Mengo virus as was used to seed samples for analysis, and was subsequently expressed as percentage extraction efficiency. Samples with an extraction efficiency of greater than 1% were accepted for inclusion in this study.

To enable quantification of NoV RNA in copies per µl, a log dilution series of the GI and GII DNA plasmids (range 1×10^0 to 1×10^5 copies per µl) was included in duplicate on each RT-qPCR run. The number of RNA copies in NoV positive samples was determined by comparing the Ct value to the standard curves. The final

concentration was then adjusted to reflect the volume of RNA analyzed and was expressed as detectable virus genome copies g⁻¹ hepatopancreas or detectable virus genome copies 100 ml⁻¹ wastewater. The LOD for NoV GI and GII was 20 detectable virus genome copies g⁻¹ and 25 detectable virus genome copies 100 ml⁻¹ for shellfish and wastewater samples respectively.

5.2.10 Calculation of log reductions of *E. coli*, FRNA bacteriophage and NoV through the wastewater treatment process

The reductions by the wastewater treatment process were calculated using the following equation: $\text{Log reductions} = \log_{10} (N_{\text{inf}}/N_{\text{eff}})$, where: N_{inf} = concentration of microbial parameter (MPN *E. coli* 100 ml⁻¹, FRNA bacteriophage PFU 100 ml⁻¹ and NoV genome copies 100 ml⁻¹) detected in influent wastewater; N_{eff} = concentration of microbial parameter (MPN *E. coli* 100 ml⁻¹, FRNA bacteriophage PFU 100 ml⁻¹ and NoV genome copies 100 ml⁻¹) detected in primary or secondary treated effluent wastewater. For the samples with negative results (n=2), the log reductions could not be determined; however, the minimum log reductions were estimated by applying a value of the detection limit of the assay. Minitab statistical software version 15 (Minitab Inc., PA, USA) was used for the data analysis whereby all data was initially assessed for normality (Anderson Darling) and then log transformed to achieve a normal distribution.

5.3 Results

5.3.1 Concentrations of microbes detected in wastewater

E. coli, FRNA bacteriophage, NoV GI and GII concentrations detected in all influent, post-primary and post-secondary treated effluents are shown in Table 5.1. *E. coli* concentrations ranged from 3.73 to 7.54 log₁₀ MPN 100 ml⁻¹ in influent wastewater and underwent a mean log reduction of 1.49 log₁₀ MPN 100 ml⁻¹ during the entire treatment process. The mean reduction of FRNA bacteriophage was 2.13 log₁₀ PFU 100 ml⁻¹ with mean concentrations of 5.54, 5.46 and 3.41 log₁₀ PFU 100 ml⁻¹ detected in influent, primary treated and secondary treated effluent respectively. No correlation was found between concentrations of *E. coli* and FRNA bacteriophage with either NoV GI or NoV GII levels in influent and effluent wastewater ($r < 0.07$ in all instances).

NoV GI and GII was detected in influent and effluent wastewater on all sampling occasions throughout the sampling period. Mean concentrations of NoV GI and NoV GII detected in influent wastewater were 3.32 and 3.55 log₁₀ genome copies 100 ml⁻¹ respectively. Mean concentrations of NoV GI and NoV GII detected in effluent wastewater were 2.53 and 2.63 log₁₀ genome copies 100 ml⁻¹, respectively. NoV GII concentrations in influent wastewater were significantly greater ($p < 0.05$) than concentrations of NoV GI and the mean concentrations of NoV GII were 0.23 log₁₀ genome copies 100 ml⁻¹ higher than NoV GI concentrations.

The mean NoV GI and GII reduction during the entire treatment process was 0.80 and 0.92 log₁₀ virus genome copies respectively. Although the mean log₁₀ reduction achieved throughout the study period was 0.12 greater for NoV GII compared with NoV GI, this difference was not statistically different ($p = 0.25$). Mean log₁₀ reductions for all microorganisms ranged from 0.13 (NoV GI) to 0.32 (FRNA bacteriophage) log₁₀ units following primary treatment (Table 5.1) Table 5.1.

Table 5.1. Mean log₁₀ concentrations of *E. coli*, FRNA bacteriophage and NoV GI and GII wastewater treatment stages and associated mean log reductions

	Wastewater Treatment Stage				
	Influent	Post primary settlement		Post final settlement	
n = 49	Concentration ± SD	Concentration ± SD	Reduction ± SD	Concentration ± SD	Reduction ^a ± SD
<i>E. coli</i> (MPN 100 ml ⁻¹)	6.54 ± 0.59 (3.73-7.54)	6.38 ± 0.51 (4.54-7.38)	0.16 ± 0.64	5.06 ± 0.58 (3.54-6.20)	1.49 ± 0.63
FRNA bacteriophage (PFU 100 ml ⁻¹)	5.54 ± 0.51 (3.87-6.82)	5.23 ± 0.55 (3.41-5.96)	0.32 ± 0.55	3.41 ± 0.77 (2.00-5.84)	2.13 ± 0.76
NoV GI (genome copies 100 ml ⁻¹)	3.32 ± 0.64 (2.05-4.76)	3.17 ± 0.71 (1.62-4.57)	0.13 ± 0.64	2.53 ± 0.57 (1.26-4.06)	0.80 ± 0.49
NoV GII (genome copies 100 ml ⁻¹)	3.55 ± 0.89 (1.81-5.34)	3.40 ± 0.84 (1.46-5.51)	0.14 ± 0.65	2.63 ± 0.71 (1.51-4.08)	0.92 ± 0.76

^aThe reduction shown is the total reduction provided by the entire treatment process

5.3.2 Seasonal variation in NoV concentrations

NoV GI and GII concentrations detected in the influent wastewater during the winter period were significantly higher ($p < 0.05$) than during the rest of the year (Table 5.2). Mean concentrations of NoV GI and GII in the influent wastewater for the period January to March inclusive ($n=12$) were 0.82 and 1.41 \log_{10} virus genome copies 100 ml^{-1} greater than mean concentrations for the rest of the year ($n=37$) respectively. No significant difference was detected in the extent of NoV reductions during treatment due to season and consequently NoV concentrations in the final effluent were also significantly higher ($p < 0.05$) during the January to March period (Table 5.2) than during the rest of the year. The ratio of NoV GI to GII detected in wastewater also varied by season. Throughout the period January-March 2010, NoV GII concentrations were on average 0.49 \log_{10} higher than NoV GI concentrations in effluent wastewater and 0.63 \log_{10} higher in influent wastewater. The mean difference between NoV GI and GII concentrations at this time of year was highly significant ($p < 0.05$). However, no significant difference was detected between NoV GI and GII concentrations during the rest of the year (April-December 2009). Unlike NoV concentrations, no seasonal trend was detected in the concentration of FRNA bacteriophage or *E. coli* in wastewater influent or effluent.

Table 5.2 Mean \log_{10} concentrations of NoV GI and GII in influent and effluent wastewater by season

NoV genogroup	Season	Mean concentration (\log_{10} virus genome copies 100 ml^{-1}) \pm SD	
		Influent	Effluent
GI	April-Dec. ^a	3.12 \pm 0.55	2.32 \pm 0.68
	Jan.-Mar. ^b	3.94 \pm 0.49	3.06 \pm 0.55
GII	April-Dec. ^a	3.20 \pm 0.71	2.27 \pm 0.39
	Jan.-Mar. ^b	4.61 \pm 0.41	3.53 \pm 0.65

^a 37 samples were analyzed during this period

^b 12 samples were analyzed during this period

5.3.3 Oysters

Mean FRNA bacteriophage and *E. coli* concentrations detected in oysters throughout the study period were $4.14 \log_{10}$ PFU 100 g^{-1} (SD ± 0.64) and $3.22 \log_{10}$ MPN 100 g^{-1} (SD ± 0.55) respectively. On a sample by sample basis, *E. coli* concentrations in oysters did not correlate with concentrations of NoV GI or GII ($r = -0.097$; $p = 0.57$) ($r = 0.184$; $p = 0.26$). Similarly, FRNA bacteriophage concentrations did not correlate with NoV GI or GII ($r = 0.015$; $p = 0.93$ or $r = 0.252$; $p = 0.127$ respectively). Unlike for NoV, no seasonal difference was observed in the concentrations of FRNA bacteriophage and *E. coli* in oysters. Weekly concentrations of NoV detected in oysters and wastewater effluent are shown in Figure 5.1

Mean NoV GI and GII concentrations detected in oysters over the year long monitoring were 3.53 and $3.73 \log_{10}$ virus genome copies g^{-1} respectively (Table 5.3). NoV detected in oyster samples displayed a strong seasonal trend with significantly higher concentrations ($p < 0.05$) in the winter compared with the rest of the year. Mean concentrations of NoV GI and GII detected during the January to March period were 1.31 and $1.65 \log_{10}$ virus genome copies g^{-1} greater than concentrations detected during the rest of the year respectively. Log concentrations of NoV in oysters were significantly correlated with concentrations detected in effluent wastewater on a weekly basis (NoV GI $r = 0.48$; $p < 0.05$ and NoV GII $r = 0.68$; $p < 0.05$).

Table 5.3 Mean \log_{10} NoV concentrations in oysters grouped by season

Season (n)	Mean concentration (\log_{10} virus genome copies g^{-1}) \pm SD	
	NoV GI	NoV GII
All data (38)	3.53 ± 0.87	3.73 ± 0.55
April-Dec. (26)	3.12 ± 0.68	3.21 ± 0.56
Jan.-Mar. (12)	4.43 ± 0.50	4.86 ± 0.54

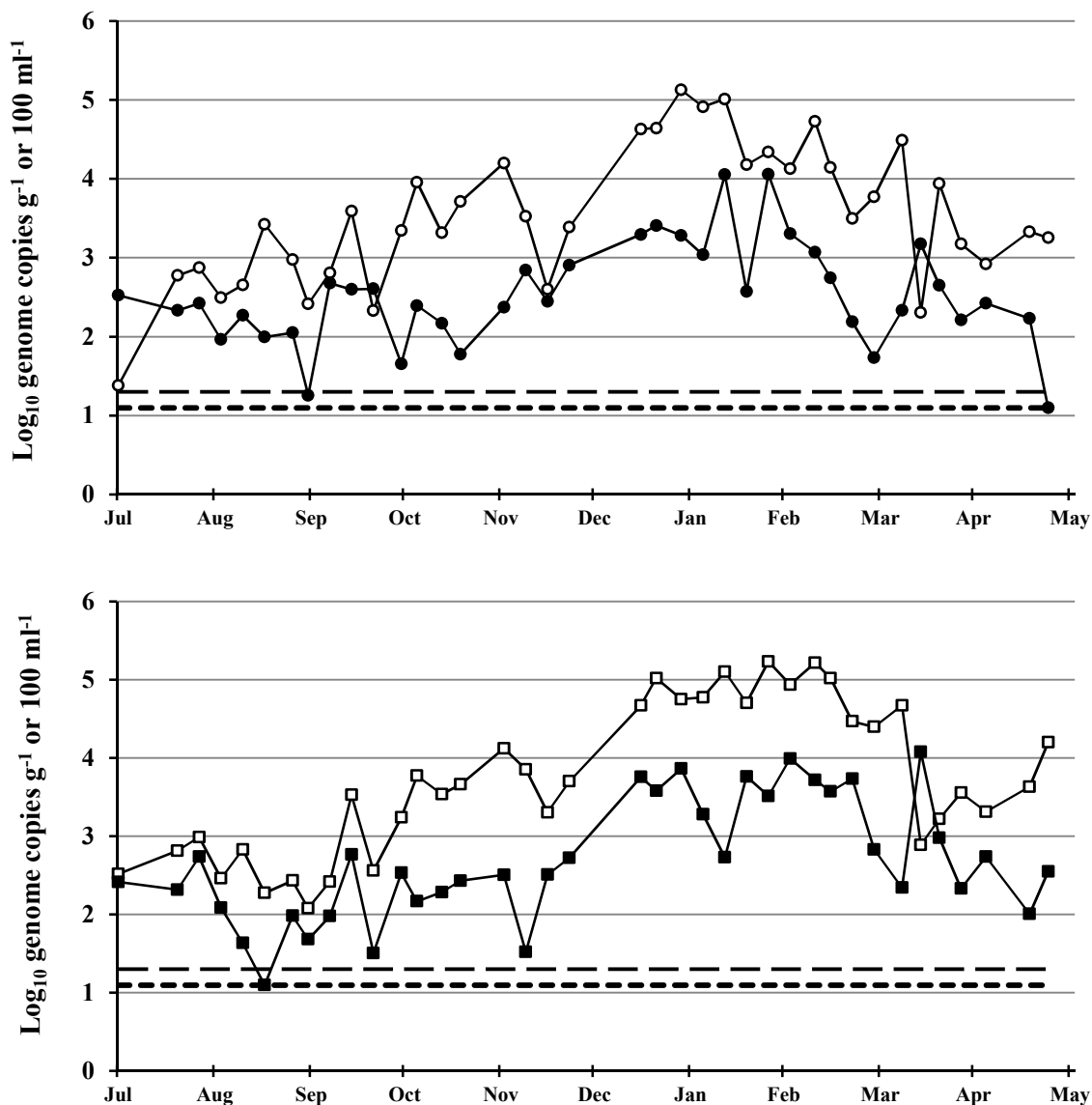


Figure 5.1. Concentrations of NoV GI (A) and NoV GII (B) detected in oysters and effluent wastewater

Concentrations of NoV GI (○) and NoV GII (□) detected in oysters are expressed as log_{10} genome copies g^{-1} oyster hepatopancreas and concentrations of NoV GI (●) and NoV GII (■) in effluent are expressed as log_{10} genome copies 100 ml^{-1} . Dashed lines indicate the limit of detection for shellfish (—) and wastewater analysis (--).

5.4 Discussion

In this study we detected NoV in wastewater from a WWTP on a weekly basis throughout a year-long monitoring period. The use of real-time RT-qPCR in this study demonstrated that NoV was continuously discharged into the marine environment from the WWTP throughout the year. NoV GI and NoV GII was continuously detected in influent wastewater, demonstrating that both NoV genotypes circulate in the human population throughout the year. Whilst NoV was detected in wastewater year-round, the concentrations of NoV GI and GII increased significantly during the period January to March. This increase was most pronounced with NoV GII and is consistent with epidemiological reports that generally record a predominance of NoV GII infections occurring at this time of year (Mounts et al., 2000). NoV related gastroenteritis infections in the community are recognised as being strongly seasonal with peak infections observed during the colder winter months (Mounts et al., 2000; Karst, 2010). During the period January to March 2010, the Health Protection Surveillance Centre in Ireland recorded 1309 cases of NoV infections. Furthermore, 202 cases were recorded in the region where the WWTP investigated in this study is located (Cloak et al., 2010) compared with a total of 60 during the remainder of the year recorded in this area. It is notable that although the majority of NoV infections are generally associated with NoV GII (Siebenga et al., 2010), high concentrations of NoV GI were simultaneously detected in wastewater. The fact that there was a seasonal increase in the concentration of NoV GI detected in the wastewater concurrent with increased NoV GII concentrations during this study would appear to be evidence of a simultaneous increase of NoV GI infections in the community during this period. Given this, it is possible that the significance of symptomatic NoV GI infections in the community is under-estimated or alternatively that there is a significant concentration of shedding of NoV GI in the community associated with increased asymptomatic infections occurring at this time of year.

The concentrations of NoV detected in the present study differ to those found in a number of previous studies investigating the removal of NoV during wastewater treatment. These have indicated that NoV is often absent in wastewater effluent particularly during the summer months (Haramoto et al., 2006; Katayama et al., 2008).

However in a recent year-long study by Nordgren et al., NoV was detected from a WWTP serving a P.E. in excess of 800,000 (Nordgren et al., 2009). It may be that the detection of NoV throughout the year during the previous study and our investigation may be related to the size of the population served by the WWTP. There is likely to be a greater chance of NoV being present in wastewater from WWTPs serving large populations considering that only a relatively small percentage of the population may be shedding NoV during non-epidemic periods.

The reduction of NoV GI and GII during wastewater treatment was consistent between genogroups irrespective of the initial concentrations of virus present in the influent. This suggests that both genogroups are impacted in a similar manner to one another during the activated sludge treatment process investigated here. Moreover, NoV GI and NoV GII underwent similar reductions, irrespective of the season and NoV was released to the environment with the same seasonal profile as observed for infections in the community. The application of real-time RT-qPCR procedures in this study indicates that mean reductions for NoV GI and NoV GII concentrations of less than one \log_{10} virus genome copy are achieved through a conventional activated sludge WWTP and falls within the range previously reported (Ottoson et al., 2006; Nordgren et al., 2009). This limited reduction means that during the winter period, NoV GI and GII were discharged in wastewater effluent at concentrations greater than $3 \log_{10}$ virus genome copies 100 ml^{-1} . Concentrations recorded post primary treatment for all microbiological parameters in this study indicate that minimal reduction is achieved by this process. In this study, the majority of the reduction achieved for each of the parameters investigated was observed during the activated sludge, secondary treatment process.

Recently a specialised tissue culture system for the detection of NoV has been reported (Straub et al., 2011). However, this has not been used to investigate NoV concentrations in environmental samples and currently it is not possible to directly investigate the viability of NoV in wastewater effluent. The absence of a reliable tissue culture system has led to the adoption of virus surrogates for use in inactivation studies (Tree et al., 1997; Nuanualsuwan et al., 2002; de Roda Husman et al., 2004). FRNA bacteriophage have been proposed as surrogates for enteric viruses in a range

of settings including shellfish harvesting areas and wastewater treatment processes (Havelaar et al., 1993; Doré et al., 2000; Flannery et al., 2009; Skraber et al., 2009). The mean \log_{10} FRNA bacteriophage reduction observed during this study was $2.11 \log_{10}$ which is significantly greater than that observed for NoV and is consistent with other reports (Arraj et al., 2005; Zhang and Farahbakhsh, 2007; Carducci et al., 2008). We employed a direct agar overlay plaque assay to detect only viable FRNA bacteriophage and this may account for the greater reduction observed over NoV rather than a true difference between the level of reduction for the two viruses. It has been demonstrated that real-time RT-qPCR procedures may detect both infectious and non-infectious virus particles (Nuanualsuwan and Cliver, 2003; Pecson et al., 2009, 2011). It is possible; therefore, that inactivated NoV may be detected by the real-time RT-qPCR method used here. Therefore, the results from our study and others (da Silva et al., 2007; Katayama et al., 2008; Nordgren et al., 2009) may overestimate the number of infectious virus present in the final effluent and thus underestimate the reduction of viable viruses and the infectious risk. Pecson et al., (Pecson et al., 2011) found that a 4-log reduction in infectious bacteriophage MS2 when exposed to UV irradiation produced a real-time PCR signal loss of just $0.11 \log_{10}$. Therefore in this study, it was not possible to determine whether the reductions of NoV are representative of the actual level of NoV reduction that would be observed if a viability assay was used to detect infectious NoV. It is clear that relying solely on real-time PCR to determine the viral reduction during wastewater treatment may be misleading and in the absence of a culture system for NoV, a surrogate culturable virus may provide a better indication of the reduction of infectious viruses throughout wastewater treatment processes. FRNA bacteriophage may prove useful for this purpose until such time that a reliable culture system for NoV or procedures to estimate concentrations of viable NoV become available.

In Ireland, as in the rest of the European Union, *E. coli* is used as the bacterial indicator organism to assess the sanitary quality of bivalve molluscan shellfish. Monthly sampling of the oysters in this study would have showed compliance with a category B harvesting area (<4600 MPN *E. coli* 100 g^{-1} in 90% of samples) meaning that the oysters could be sold for consumption following minimal treatment such as

depuration (Lees, 2000). Given the minimal reduction of NoV provided by the WWTP, elevated concentrations of NoV were detected in oysters harvested adjacent to the outfall throughout the year. These concentrations would be consistent with those that have caused illness in consumers (Doré et al., 2010) and demonstrates the inadequacy of *E. coli* to assess the NoV risk associated with oysters. As alternatives to *E. coli*, FRNA bacteriophage have been proposed as a viral surrogate to indicate the presence of NoV in oysters previously (Doré et al., 2000; Flannery et al., 2009) and thus were included in this study. However, no seasonal trend was observed during our study as has been observed by others (Miossec et al., 2001) and oysters were contaminated to consistent concentrations year round and did not demonstrate an increased risk of higher concentrations of NoV being present during the winter months. This questions their suitability of use as an indicator of NoV in oysters. However, it has been proposed that FRNA bacteriophage may provide useful information on the viral contamination of shellfish in areas that are infrequently impacted by sewage rather than in areas undergoing continuous wastewater inputs as studied here (Flannery et al., 2009).

This study provides a comprehensive dataset concerning the concentrations of NoV GI and GII in a WWTP providing secondary treatment and the effect of effluent on NoV concentrations in shellfish. As wastewater treatment is considered an important control in reducing the microbial contamination of aquatic environments to acceptable concentrations, the actual reduction provided by treatment processes has implications for plant operators and water management agencies. The data from this and other studies (Katayama et al., 2008; Nordgren et al., 2009) demonstrates that conventional wastewater treatment processes cannot be relied upon in isolation to prevent the contamination of the marine environment and thus oysters with NoV as determined using real-time PCR. As yet, methods are not available to differentiate infectious from non-infectious NoV and the detection of NoV in oysters using current procedures may overestimate the infectious risk. It is probable that low concentrations of NoV, as determined using real-time PCR, may not have an impact on consumer health. Therefore, results from widespread general monitoring of oysters need to be placed in context and should be considered to be one element of a more comprehensive risk-based approach to managing NoV contamination in shellfisheries.

A more useful approach may be to target at risk harvest areas identified through the use of sanitary surveys and areas known to be at risk of contamination by municipal wastewater to mitigate the risk of NoV contamination from oysters.

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*Chapter 6. Norovirus and FRNA bacteriophage
determined by RT-qPCR and infectious FRNA
bacteriophage in wastewater and oysters*

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Abstract

Norovirus (NoV), the leading cause of adult non-bacterial gastroenteritis can be commonly detected in wastewater but the extent of NoV removal provided by wastewater treatment plants (WWTPs) is unclear. We monitored a newly commissioned WWTP with UV disinfection on a weekly basis over a six month period for NoV using RT-qPCR and for FRNA bacteriophage GA using both RT-qPCR (total concentration) and a plaque assay (infectious concentration). Mean concentrations of NoV GI and GII in influent wastewater were reduced by 0.25 and 0.41 \log_{10} genome copies 100 ml^{-1} , respectively by the WWTP. The mean concentration of total FRNA bacteriophage GA was reduced by 0.35 \log genome copies 100 ml^{-1} compared to a reduction of infectious FRNA bacteriophage GA of 2.13 \log PFU 100 ml^{-1} . A significant difference between concentrations of infectious and total FRNA bacteriophage GA was observed in treated, but not in untreated wastewaters. We conclude that RT-qPCR in isolation underestimates the reduction of infectious virus during wastewater treatment. We further compared the concentrations of infectious virus in combined sewer overflow (CSO) and UV treated effluents using FRNA bacteriophage GA. A greater percentage (98%) of infectious virus is released in CSO discharges than UV treated effluent (44%). Following a CSO discharge, concentrations of NoV GII and infectious FRNA bacteriophage GA in oysters increased from less than the limit of detection to 3150 genome copies 100 g^{-1} and 1050 PFU 100 g^{-1} respectively.

6.1 Introduction

Norovirus (NoV) is the most significant cause of non-bacterial gastroenteritis in the developed world (Mead et al., 1999). NoV infections cause a number of symptoms including diarrhoea, nausea, vomiting and abdominal pain (Turcios et al., 2006), which can have complications for immunocompromised individuals (Schwartz et al., 2011). Faeces from infected persons can contain NoV concentrations of up to 10^8 copies g^{-1} (Lee et al., 2007) and thus NoV is commonly present in municipal wastewater (Da Silva et al., 2007; Flannery et al., 2012; Katayama et al., 2002). The discharge of municipal wastewater into the marine environment is a common practice worldwide and is a major source of faecal contamination in bivalve shellfish (Lees, 2000). Bivalve shellfish, such as oysters, can accumulate NoV when grown in wastewater-contaminated waters and can transmit the virus when consumed (Lees, 2000).

Wastewater treatment is a critical control point employed to reduce the extent of microbiological and other contaminants prior to the release of wastewater to the environment. However, enteric viruses can persist in treated wastewater (Flannery et al., 2012; Haramoto et al., 2006; Katayama et al., 2008; Nordgren et al., 2009). The introduction of additional treatment processes such as ultraviolet (UV) disinfection can be employed to further reduce microbial concentrations and this process is widely used in Europe (Bixio et al., 2006). In addition to the impact of treated wastewater, significant microbiological contamination can occur as result of untreated wastewater originating from combined sewer overflows (CSOs) (Maalouf et al., 2010). Wastewater treatment plants (WWTPs) are designed to treat a finite volume of wastewater and generally have a capacity to store excessive volumes of wastewater in holding tanks during periods of increased rainfall. Under normal circumstances stored wastewater is then returned to the treatment process when flows return to normal levels. However, under prolonged or extreme rainfall events this storage capacity can be exceeded and may lead to the discharge of untreated effluent. Such occurrences have been associated with increased enteric viral concentrations in shellfish and subsequent outbreaks of NoV gastroenteritis (Grodzki et al., 2012; Le Guyader et al., 2008, 2006).

The assessment of faecal contamination in shellfish and water is often carried out using bacterial indicator organisms such as *Escherichia coli*. However, bacterial

indicator organisms have generally been shown to be poor indicators of viral persistence in environmentally contaminated shellfish (Doré and Lees, 1995; Flannery et al., 2009). It is therefore recognised that *E. coli* monitoring does not fully determine the virus risk associated with faecally contaminated bivalve shellfish. Currently it is not possible to routinely culture NoV and molecular methods, such as real-time reverse-transcription quantitative PCR (RT-qPCR), provide the most reliable approach for the detection and quantification of NoV in environmental samples. However, RT-qPCR detection does not differentiate between infectious and non-infectious viruses (Nuanualsuwan et al., 2002; Pecson et al., 2011). This makes it difficult to interpret the infectious risk associated with concentrations of NoV detected in environmental samples and it is recognised that on occasion RT-qPCR procedures may overestimate the infectious risk in shellfish (EFSA 2012).

F-specific RNA (FRNA) bacteriophage of the family *Leviviridae* are also commonly present in wastewater and have been proposed as suitable surrogates of human enteric viruses. They have been used previously as an indicator of viral reduction during disinfection processes (Allwood et al., 2005; Nappier et al., 2006; Nuanualsuwan et al., 2002; Park et al., 2011) and proposed as an index of enteric virus contamination in shellfish (Doré et al., 2000; Flannery et al., 2009). FRNA bacteriophage contain two genera, *Levivirus* and *Allolevivirus*, that can be subdivided into four genogroups that are present in either animal (genogroups I and IV) or human waste (genogroups II and III) (Beekwilder et al., 1996; Sundram et al., 2006). Infectious FRNA bacteriophage can be readily detected using a standard plaque assay (Anonymous, 1995) and when coupled with in situ hybridisation procedures, it is possible to differentiate between the genogroups of FRNA bacteriophage (Beekwilder et al., 1996; Sundram et al., 2006). FRNA bacteriophage GA is a human-specific strain within *Levivirus* genogroup II and both in situ hybridisation (Beekwilder et al., 1996; Sundram et al., 2006) and RT-qPCR procedures for *Levivirus* genogroup II (including GA) exist (Wolf et al., 2008). Therefore the opportunity exists for a direct comparison between concentrations of FRNA bacteriophage GA determined by RT-qPCR and plaque assay. Such a comparison could provide a useful indicator towards extent of

the possible overestimation of infectious NoV afforded by the use of RT-qPCR procedures with environmental samples.

In this study we used RT-qPCR to determine concentrations of NoV and FRNA bacteriophage GA in influent, secondary treated and UV treated effluent at a WWTP over a six month period. We also monitored the subsequent impact of the wastewater effluent on an oyster harvest area. In wastewater and oysters, we directly compared the concentration of FRNA bacteriophage GA present as determined by RT-qPCR (hereafter termed total concentration) and a plaque assay (hereafter termed infectious concentration) to characterise the potential overestimation of infectious virus in environmental samples. In addition, we applied the same methodologies to investigate the impact of CSO discharges from the same WWTP on the oyster harvest area.

6.2 Materials and methods

6.2.1 Wastewater treatment plant

A WWTP that was newly commissioned in December 2011 was monitored in this study. The WWTP treated wastewater from a population equivalent (P.E.) of 10,000 and received an average daily incoming wastewater volume of 13,500 m³. Preliminary treatment at the plant provided screening and grit removal, followed by phosphate removal through ferric sulphate addition. Secondary treatment was carried out in one of four sequencing batch reactors. The secondary treated effluent was then passed through a Trojan UV 3000 disinfection unit and discharged to estuarine waters. The plant was capable of holding 1600 m³ of combined storm water and influent wastewater before treatment was by-passed and untreated effluent was discharged. UV treated effluent was discharged to estuarine waters 380 m downstream from a commercial oyster harvesting area. CSO discharges were released through a separate pipe 570 m downstream from the shellfish harvesting area.

6.2.1.1 Wastewater and oyster sampling January-June 2011

Initial monitoring of the WWTP commenced in January 2011 and ended in June 2011. One litre 24-hour composite samples of influent, secondary treated and UV treated effluent were taken on a weekly basis (n=22) during this period. Oyster samples (n=25) consisting of 24 oysters were collected alongside wastewater samples. Wastewater and oyster samples were transported to the laboratory under chilled conditions (<15°C). All samples were received in the laboratory within 24 hours of collection.

6.2.1.2 CSO monitoring September-December 2011

Monitoring for CSO events commenced on 29th September 2011 and ended on 20th December 2011. One litre, 24-hour composite samples of UV treated effluent were taken on a fortnightly basis over the study period (n=8) to generate background data. Four CSO discharges occurred during the monitoring period and 1 litre composite samples of CSO effluent discharged during these events were collected. All wastewater samples were collected in high density polyethylene bottles and transported to the

laboratory under chilled temperatures (<15°C) within twenty four hours of collection. Oyster samples (n=5) were taken following an alert of an imminent CSO discharge event in September 2011 and all samples were transported to the laboratory within 24 hours of collection under chilled temperatures (<15°C).

6.2.2 Oyster sample processing

Upon arrival to the laboratory, 10 oysters were shucked and the whole flesh and liquor diluted 1:3 with neutralised bacteriological peptone (Oxoid) for *E. coli* and FRNA bacteriophage analysis (Anonymous, 2005). For NoV analysis, a further 10 oysters were shucked and the hepatopancreas (HP) dissected out and chopped finely. Two gram of oyster HP was treated with 2 ml Proteinase K (100 µg ml⁻¹) and 50 µl Mengo virus strain MC₀ was added as an internal process control. The sample was incubated at 37°C under continuous shaking at 150 rpm for 1 hour followed by incubation at 60°C for 15 minutes. The sample was centrifuged at 3000 × g for 5 minutes and the supernatant retained for RNA extraction.

6.2.3 Concentration of wastewater for viral analysis

A conventional filter adsorption-elution method was used for the concentration of wastewater samples and carried out using a previously described method (Flannery et al., 2012). Briefly, following the addition of 2.5 M MgCl₂ (Sigma-Aldrich, UK), 40 ml wastewater samples were passed through a type HA negatively charged membrane filter (0.45 µm pore size and 90mm diameter; Millipore). Sodium chloride (0.14 M) was passed through the filter and the filter placed in 4 ml of glycine-NaOH buffer pH 9.5, under constant shaking at 500 rpm for 20 minutes. The eluate was concentrated using an Amicon® Ultra-4 centrifugal filter unit (Millipore) at 4000 × g for 10 minutes. Viruses were resuspended from the filter walls using 550 µl of molecular biology grade water and were stored at -20°C for less than 1 month prior to RNA extraction.

6.2.4 Viral genomic extraction

Viral RNA was extracted from oyster HP supernatants and wastewater concentrates using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, France) following the manufacturer's

instructions. Five hundred microlitres of shellfish or wastewater extract was used per extraction and viral RNA was eluted into 100 μ l of elution buffer (bioMérieux). The eluted RNA was stored at -80°C until RT-qPCR analysis was undertaken (<1 month). A single water aliquot (free from NoV or FRNA bacteriophage GA RNA) was processed alongside shellfish and wastewater samples as a negative RNA extraction control.

6.2.5 Preparation of double stranded DNA plasmids for quantification of NoV and FRNA bacteriophage GA in wastewater and oysters

Double stranded (ds) DNA plasmids for quantification of NoV GI and GII were prepared as previously described (Flannery et al., 2012). For quantification of bacteriophage GA, PCR products from the RT-qPCR were purified using the Wizard® SV PCR Purification kit (Promega) and cloned using the pGEM-T Easy vector system (Promega). Transformant clones were screened and purified using the PureYield™ plasmid miniprep kit (Promega). The purified plasmid was quantified using spectrophotometry at 260 nm and subsequently diluted to 10^5 copies/ μ l for use as quantitative standard in the RT-qPCR. From the NoV GI and NoV GII dsDNA plasmids, external control (EC) RNA transcripts were prepared as described previously (Le Guyader et al., 2009) for use in determining RT-qPCR inhibition.

6.2.6 One-step RT-qPCR assay for NoV in wastewater and oysters

RT-qPCR analysis was carried out using an Applied Biosystems AB7500 instrument. Twenty microlitres of the NoV GI or NoV GII one-step reaction mix was prepared using RNA Ultrasense one-step qRT-PCR system (Invitrogen) containing 1 \times reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 \times μ l Rox and 1.25 μ l of enzyme. Previously described primers QNIF4 (Da Silva et al., 2007), NV1LCR and probe NVGG1p (Svraka et al., 2007) were used for NoV GI analysis, and primers QNIF2 (Loisy et al., 2005), COG2R (Kageyama et al., 2003) and probe QNIFS (Loisy et al., 2005) used for NoV GII analysis. Duplicate 5 μ l aliquots of sample RNA was added to adjacent wells of a 96-well optical reaction plate in addition to no template controls. Reaction conditions for NoV GI, NoV GII and IPC were the same;

initial incubation at 55°C for 60 min followed by 95°C for 5 min and then 45 cycles of 95°C for 15s, 60°C for 1 min and finally 65°C for 1 min.

To control for the presence of RT-qPCR inhibitors 5 µl of sample RNA was added to a further two wells to which 1 µl of EC RNA (10^7 genome copies μl^{-1}) was added. A log dilution series of the NoV GI and GII EC RNA ranging from 10^7 to 10^4 copies μl^{-1} was included on each RT-qPCR run. The mean Cq value obtained for samples that included the EC RNA was used to calculate the quantity of EC RNA detected in the sample which was then used to estimate the percentage PCR amplification efficiency. Samples with an amplification efficiency of less than 25% were not accepted and in such cases the RNA was reanalysed at a 1:10 dilution.

For extraction efficiency (determined using the IPC; Mengo virus) previously described forward (Mengo209) and reverse (Mengo110) primers and probe (Mengo147) were used (Pintó et al., 2009). The Cq value of the sample was compared to a standard curve obtained by preparing log dilutions from the same batch of Mengo virus as was used to spike samples for analysis. This was expressed as percentage extraction efficiency and samples with an extraction efficiency of less than 1% were not accepted. A log dilution series of the NoV GI and NoV GII dsDNA plasmids (range 1×10^1 to 1×10^5 copies μl^{-1}) was included in duplicate on each RT-qPCR plate. NoV was quantified by comparing the Cq value to the standard curves in copies per µl, and then adjusted to reflect the volume of RNA analysed (expressed as genome copies g^{-1} HP or genome copies 100 ml^{-1} wastewater). The LOD for the assay was determined as 20 detectable genome copies g^{-1} HP and 25 detectable genome copies 100 ml^{-1} for shellfish and wastewater samples respectively.

6.2.7 One-step RT-qPCR assay for FRNA bacteriophage GA in wastewater and oysters

Twenty microlitres of the FRNA bacteriophage GA one-step reaction mix was prepared using RNA Ultrasense one-step RT-qPCR system (Invitrogen) containing 1 × reaction mix, 2.5 µl EXO IPC reaction mix, 0.5 µl EXO IPC DNA, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 × µl Rox and 1.25 µl of enzyme. Previously described Levivirus genogroup II forward and reverse primers and probe were used (Wolf et al., 2008). Reaction conditions for FRNA bacteriophage GA

involved an initial incubation at 55°C followed by 95°C for 5 min and then 45 cycles of 95°C for 15 s and 58°C for 1 min (Wolf et al., 2008).

The TaqMan Exogenous Internal Positive Control kit (Applied Biosystems) was used to control for PCR inhibition. For quantification of FRNA bacteriophage GA, a log dilution series of the dsDNA plasmids (range 1×10^1 to 1×10^5 copies μl^{-1}) was included in duplicate on each RT-qPCR plate and Cq values were compared with sample Cq values. The LOD the assay was determined as 20 detectable genome copies g^{-1} HP and 25 detectable genome copies 100 ml^{-1} for oyster and wastewater samples respectively.

6.2.8 FRNA bacteriophage plaque assay

FRNA bacteriophage were enumerated using a standardised double agar layer method (Anonymous, 1995) that used the host strain *Salmonella enterica* serovar Typhimurium WG49 as described by (Havelaar and Hogeboom, 1984). Host cells were first cultivated for 4–6 h at 37°C in tryptone yeast-extract glucose broth (to yield $>10^6$ CFU ml^{-1}) and then mixed with 2.5 ml of tryptone yeast-extract glucose 1% agar (soft agar) and 1 ml shellfish homogenate or appropriately diluted wastewater. The mixture was then poured onto the surface of the hard agar (tryptone yeast-extract glucose 2% agar). After overnight incubation at 37°C, FRNA bacteriophage were identified as semi-transparent plaques that were then counted and multiplied by the dilution factor to obtain a titre in plaque forming units (PFU) 100 ml^{-1} . The LOD of the assay was determined as 30 PFU 100 g^{-1} and 10 PFU 100 ml^{-1} for oyster and wastewater samples, respectively.

6.2.9 Genotyping of FRNA bacteriophage GA using in-situ probe hybridisation

For each wastewater and shellfish sample, all plaques from the plates used to enumerate FRNA bacteriophage were transferred using an applicator stick onto Hybond N+ nylon transfer membranes (Amersham, Freiburg, Germany). Genotyping was carried out as described by (Sundram et al., 2006) with some modifications. Briefly, membranes containing bacteriophage RNA were submerged in denaturing solution (0.05 M NaOH, 0.15 M NaCl) for 1 minute followed by neutralisation in 0.1 M sodium acetate for 5 minutes prior to fixation of bacteriophage RNA by baking at 80°C for 30 minutes. RNA

hybridisation was carried out using DIG Easy Hyb (Roche, Dublin, Ireland) and the membrane was probed with a 5 pmol ml⁻¹ digoxigenin (DIG) labelled oligonucleotide probe (Eurogentec). The probe used for the detection of FRNA bacteriophage GA was designed by (Beekwilder et al., 1996) to detect bacteriophages within Levivirus genogroup II. Washing and blocking of the membranes was carried out according to manufacturer instructions using the DIG wash and block buffer set (Roche) followed by detection using the DIG Nucleic Acid Detection Kit (Roche). GA bacteriophage plaques were confirmed as dark purple areas on each membrane and were expressed as FRNA bacteriophage GA PFU 100 ml⁻¹ or 100 g⁻¹.

6.2.10 *E. coli* analysis of wastewater and shellfish

Suitable log dilutions of wastewater samples and oyster homogenates using 0.1% neutralized bacteriological peptone (Oxoid, Cambridge, UK) were analysed for *E. coli* using a standardised five-tube three-dilution most probable number (MPN) method (Anonymous, 2005). Briefly, diluted wastewater and oyster homogenates were inoculated into 10 ml volumes of minerals modified glutamate broth (Oxoid) and incubated at 37°C for 24 hours. *E. coli* was subsequently confirmed by subculturing tubes positive for acid production onto plates containing Tryptone bile X-glucuronide agar (Oxoid) at 44°C for 24 hours. The LOD of the assay was a MPN of 20 *E. coli* 100 g⁻¹ and 20 *E. coli* 100 ml⁻¹ for oyster and wastewater samples respectively.

6.2.11 Calculation of NoV and FRNA bacteriophage GA concentrations in oyster whole flesh

To directly compare concentrations of total and infectious virus in oysters, concentrations of NoV or FRNA bacteriophage GA (genome copies g⁻¹ HP) were adjusted to genome copies 100 g⁻¹ whole flesh. This was achieved by multiplying the NoV or GA concentration (genome copies g⁻¹ HP) by the total weight of HP from 10 oysters and then dividing by the whole flesh weight of 10 oysters per sample. The quotient was then multiplied by 100 to achieve genome copies 100 g⁻¹ whole flesh as described below.

$$\frac{\text{Concn. (genome copies g}^{-1}\text{)HP} \times \text{weight of HP (g) in 10 oysters}}{\text{Weight of wholeflesh (g) 10 oysters}} \times 100$$

6.2.12 Calculation of log reductions

For NoV and GA negative wastewater samples, a hypothetical value of 1.25×10^1 genome copies 100 ml^{-1} (corresponding to half the LOD) was ascribed to determine the log reduction values. For wastewater samples, the log reductions for all microbial parameters were determined as the \log_{10} quotient of the concentration before and after treatment (*E. coli* MPN 100 ml^{-1} , FRNA bacteriophage GA PFU 100 ml^{-1} and NoV genome copies 100 ml^{-1}).

6.2.13 Statistical analysis

Minitab statistical software version 15 (*Minitab* Inc., PA, USA) and Sigma Plot software version 11 (Systat Software, Chicago, IL) was used for the data analysis whereby all data was initially assessed for normality (Anderson-Darling) and then log transformed to achieve a more-normal distribution.

6.3 Results

6.3.1 Performance of WWTP and Microbial concentrations in wastewater and oysters (January to June 2011)

Following the completion of the initial monitoring period (January to June 2011) it was confirmed that a total of 41 CSO discharges (Figure 6.1) had occurred during the period. These occurred most frequently in January (n=17) and February (n=11). These CSO discharges were not associated with intense rainfall events but were, rather, attributed to performance testing and operational problems at the newly commissioned WWTP. In addition, in January and February 2011 the recorded mean reduction of biological oxygen demand (BOD) and suspended solids during treatment were outside the specified target values for the WWTP. During the CSO monitoring period (September – December 2011), four CSO discharges occurred at the WWTP all of which were attributed to heavy rainfall. The total volumes discharged during the CSO events in September, October, November and December were 413, 534, 1160 and 1182 m³, respectively.

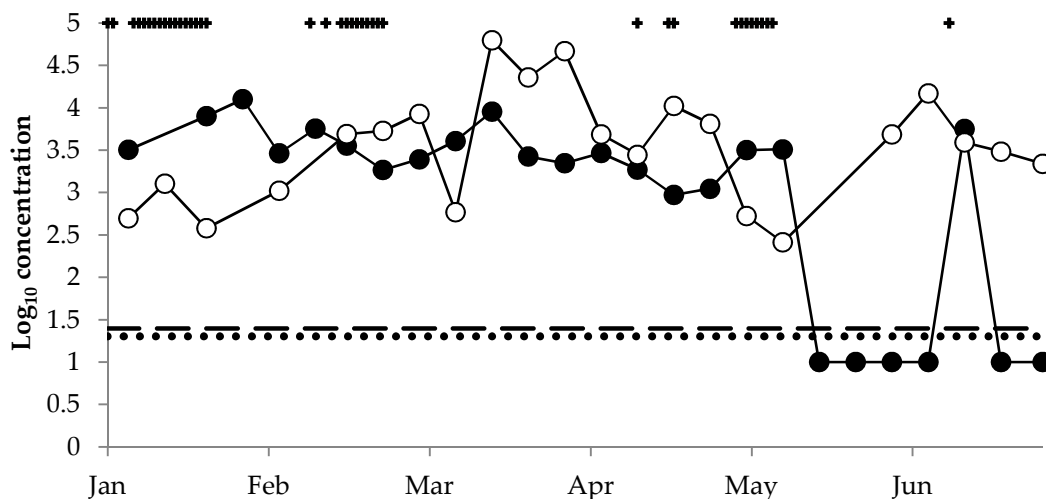


Figure 6.1 Concentrations of NoV GII in UV treated effluent and oysters January-June CSO discharges (+) and concentrations of NoV GII in oysters (●; log₁₀ genome copies g⁻¹ HP) and UV treated effluent (○; log₁₀ genome copies 100 ml⁻¹) that occurred during the sampling period are indicated. The LOD for NoV GII in oysters (dotted line) and wastewater (broken line) are also included.

6.3.2 Microbial concentrations in wastewater (January to June 2011)

Mean concentrations of *E. coli* were 6.18, 4.73 and 2.81 log₁₀ MPN 100 ml⁻¹ in influent, secondary treated and UV treated effluent respectively. Mean concentrations of FRNA bacteriophage were 5.32, 3.81 and 2.69 log₁₀ PFU 100 ml⁻¹ in influent, secondary treated and UV treated effluent, respectively. NoV GI and GII were detected in 61% and 95% of influent wastewater samples, respectively. Mean NoV GII concentrations were significantly greater (paired t-test; P<0.05) than NoV GI in all wastewater types and were 1.12, 1.33 and 1.37 log₁₀ genome copies 100 ml⁻¹ higher in influent, secondary treated and UV treated effluent, respectively. A total mean log reduction of 3.37 log₁₀ MPN 100 ml⁻¹ for *E. coli* and 2.63 log₁₀ PFU 100 ml⁻¹ for FRNA bacteriophage was observed during the treatment process. Of that total reduction, the UV disinfection process was responsible for a mean log reduction of 1.92 log₁₀ MPN 100 ml⁻¹ and 1.12 log₁₀ PFU 100 ml⁻¹ for *E. coli* and FRNA bacteriophage, respectively. Total mean log reductions (0.25 and 0.41 log₁₀ genome copies 100 ml⁻¹ for NoV GI and GII, respectively) were attributed to the secondary treatment process only and no further reduction in NoV concentrations were attributed to the UV disinfection process.

6.3.3 Microbial concentrations in oysters (January to June 2011)

Mean FRNA bacteriophage and *E. coli* concentrations detected in oyster samples (n=25) throughout the study period were 2810 PFU 100 g⁻¹ and 494 MPN 100 g⁻¹, respectively. Mean NoV GI and GII concentrations detected in oysters were 405 and 3090 genome copies g⁻¹ HP, respectively. Peak NoV GII concentrations in UV treated effluent occurred on 16th March (4.79 log₁₀ genome copies 100 ml⁻¹) whereas, the peak concentration of NoV GII in oysters (12600 genome copies g⁻¹) occurred on January 27th (Figure 5.1). No correlation was found between concentrations of NoV GI or NoV GII in UV treated effluent and concentrations detected in oysters. From 18th May, NoV GII concentrations in oysters were below the LOD of the assay until monitoring ended on 29th June with the exception of one sample taken on 15th June. A CSO discharge that was caused by a period of intense rainfall occurred three days prior to the collection of this sample (Figure 6.1).

6.3.4 Comparison of infectious and total FRNA bacteriophage GA concentrations with NoV GII in wastewater and oysters

Mean concentrations of total FRNA bacteriophage GA were 5.11, 4.72 and 4.57 \log_{10} genome copies 100 ml^{-1} in influent, secondary treated and UV treated effluents, respectively. Mean concentrations of infectious FRNA bacteriophage GA were 5.26, 3.57 and 2.96 \log_{10} PFU 100 ml^{-1} . In influent wastewater, concentrations of infectious and total FRNA bacteriophage GA were not significantly different. However a significant difference existed between infectious and total FRNA bacteriophage GA in both secondary treated ($P < 0.03$) and UV treated effluent ($P < 0.03$) (Figure 6.2). Mean concentrations of total FRNA bacteriophage GA were reduced by 0.42 and a further 0.06 \log_{10} genome copies 100 ml^{-1} after secondary and UV treatment, respectively. Infectious FRNA bacteriophage GA was reduced by 1.69 and 0.61 \log_{10} PFU 100 ml^{-1} following the secondary and UV treatment, respectively. In the same wastewater samples, the mean concentration of NoV GII was reduced by 0.35 \log_{10} genome copies 100 ml^{-1} after secondary treatment and no further reduction occurred during UV treatment.

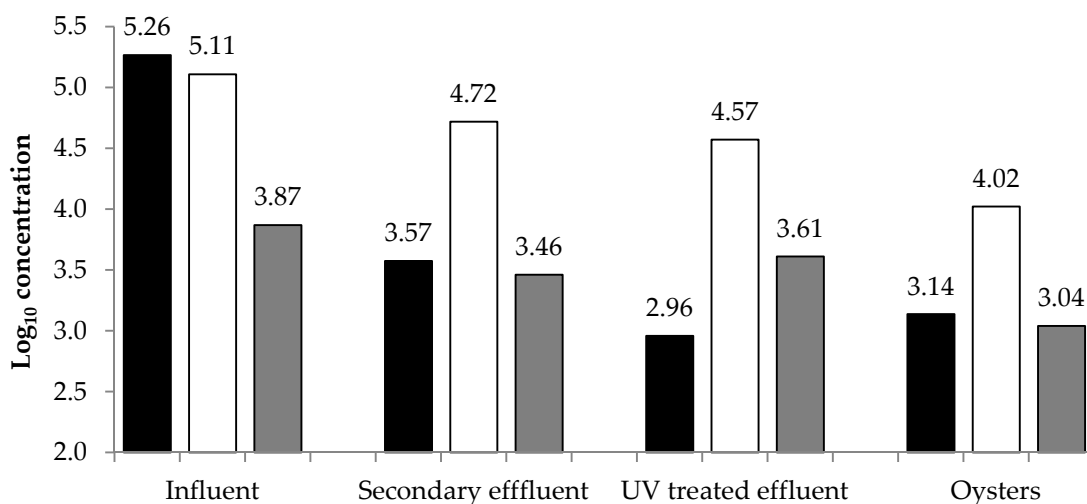


Figure 6.2 Mean concentrations of FRNA bacteriophage GA and NoV in wastewater and oysters from January to June 2011

Concentrations of FRNA bacteriophage GA detected using the plaque assay (black bar), RT-qPCR (white bar) and NoV GII (grey bar) are shown with \log_{10} concentrations in UV treated effluent as PFU 100 ml^{-1} or genome copies 100 ml^{-1} and for oysters, PFU 100 g^{-1} or genome copies 100 g^{-1} whole flesh.

The mean concentration of total FRNA bacteriophage GA detected in oysters was significantly ($P=0.03$) greater than the mean concentration of infectious FRNA bacteriophage GA (Figure 6.2). Mean concentrations of NoV GII in the same oyster samples were $3.04 \log_{10}$ genome copies 100 g^{-1} (range $<\text{LOD}$ - $4.72 \log_{10}$ genome copies 100 g^{-1}) (Figure 6.2). NoV GI was detected at very low concentrations in only 2 of 9 samples and is therefore not considered further here.

6.3.5 Impact of CSO discharges on NoV GII, total and infectious FRNA bacteriophage GA concentrations in oysters

The concentration of NoV GII in an oyster sample taken approximately 3 hours prior to the CSO event in September was below the LOD of the assay (Figure 6.3). In the same sample, the total FRNA bacteriophage GA concentration was 1.13×10^3 genome copies 100 g^{-1} in contrast to infectious concentrations that were $<\text{LOD}$. However, 12 hours after the CSO discharge, an oyster sample contained a NoV GII concentration of 1.57×10^3 genome copies 100 g^{-1} . This sample also contained a total FRNA bacteriophage GA concentration of 3.89×10^3 genome copies 100 g^{-1} and infectious FRNA bacteriophage GA concentrations of 1.05×10^3 PFU 100 g^{-1} . Further oyster samples, taken over a 72 hour period ($n=4$), following the CSO discharge were positive for NoV GII, both total and infectious bacteriophage GA and no temporal trend in concentrations was observed over that period.

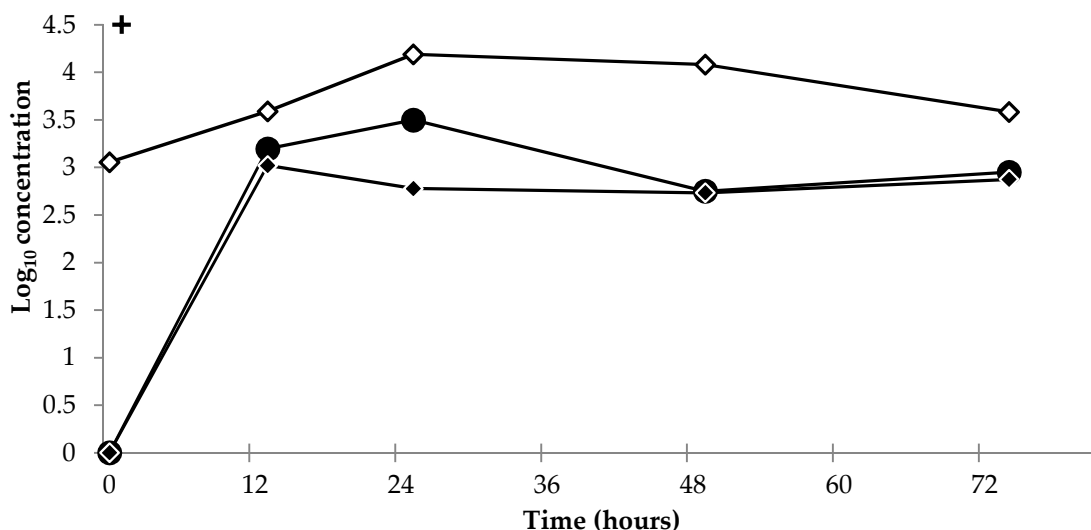


Figure 6.3 CSO discharges and concentrations of NoV GII and FRNA bacteriophage GA in oysters

CSO discharges (+) and concentrations of NoV GII in oysters (●; log₁₀ genome copies 100 g⁻¹ whole flesh) that occurred during the September CSO discharge event are indicated. Concentrations for FRNA bacteriophage GA using the plaque assay (log₁₀ PFU 100 g⁻¹◆) and the RT-qPCR (log₁₀ genome copies 100 g⁻¹;◇) assay are shown.

6.3.6 Comparison of infectious and total concentrations of FRNA bacteriophage GA in CSO and UV treated effluent

The mean concentration of total FRNA bacteriophage GA from the 4 CSO effluent samples (September-December 2011) was 4.36 log₁₀ genome copies 100 ml⁻¹ compared with the mean concentration of infectious FRNA bacteriophage GA of 4.41 log₁₀ pfu 100 ml⁻¹. In UV treated effluent samples (n=8) taken during the same time period, the mean concentration of total FRNA bacteriophage GA was 3.91 log₁₀ genome copies 100 ml⁻¹ compared to concentrations of infectious FRNA bacteriophage GA of 1.75 log₁₀ PFU 100 ml⁻¹. In CSO effluent, no significant difference between concentrations of total and infectious FRNA bacteriophage GA was found however, the difference between concentrations in UV treated effluent was statistically significant (P<0.001).

6.4 Discussion

The discharge of wastewater effluents to the marine environment remains a significant source of NoV contamination in bivalve shellfish. In the absence of a viable cell culture assay, RT-qPCR remains the only practical method to detect NoV in environmental samples. We used a standardised RT-qPCR procedure to investigate concentrations of NoV in wastewater and in oysters from a commercial shellfishery. As it is recognised that RT-qPCR detects both infectious and non-infectious virus particles in environmental samples we monitored for concentrations of total and infectious FRNA bacteriophage in selected samples. This allowed for a direct comparison between total and infectious concentrations of an enteric RNA virus to help place NoV concentrations in context. FRNA bacteriophage have previously been proposed as surrogates for human enteric viruses, both as an indicator during wastewater treatment and an index of contamination in shellfish. Recently, FRNA bacteriophage GA in particular has been found to be a conservative indicator for virus survival during water treatment (Boudaud et al., 2012), therefore, its survival is likely to be similar or greater than NoV.

6.4.1 Microbiological monitoring of wastewater and oysters (January-June 2011)

We commenced monitoring of the WWTP in January and detected NoV in influent wastewater and UV treated effluent throughout the study period. The range of concentrations of NoV in influent wastewater in our study (1×10^2 and 1×10^4 genome copies 100 ml^{-1}) were similar to those previously reported (Flannery et al., 2012; Nordgren et al., 2009; Simmons and Xagorarakis, 2011). The reductions for NoV GI and NoV GII throughout the entire treatment process were lower than reductions reported previously (Flannery et al., 2012; Francy et al., 2012; Lodder and de Roda Husman, 2005; Lowther, 2011). However indicators of plant performance demonstrated sub-optimal operation of the WWTP over the first three months of the study. Given the poor performance of the WWTP, the NoV reductions in our study cannot be considered to be representative of what may be achieved by a WWTP providing UV disinfection when operating correctly.

While peak concentrations of NoV GII in UV treated effluent were detected in March, no concurrent peak in NoV GII concentrations occurred in oysters at this time. This is in

contrast with a previous study whereby NoV concentrations in treated effluent correlated with concentrations in oysters (Flannery et al., 2012). The consistent concentrations of NoV GII in oysters during the initial months of this study may have been as a result of the frequent CSO discharges that were occurring at the WWTP at this time. Towards the end of our study, NoV GII was less than the LOD in a concurrent number of weekly oyster samples yet following a CSO event in June, a marked increase in NoV concentrations was detected in oysters. While it is not possible to definitively state that the CSO discharge was responsible for the increase in NoV concentrations in the oysters, it would appear likely given the proximity of the discharge outfall to the oysters. The detection of NoV following the CSO discharge highlights the potential impact on virus contamination in shellfisheries associated with such discharges.

6.4.2 Comparison of infectious and total FRNA bacteriophage GA in wastewater effluents and oysters

Influent wastewater concentrations of total and infectious FRNA bacteriophage GA were not significantly different prior to treatment, indicating that the majority of viruses detected were infectious. However, an increasing divergence between concentrations of infectious and total FRNA bacteriophage GA existed as wastewater samples were taken through the treatment process. Consequently, the true extent of the reduction of FRNA bacteriophage achieved by both secondary and UV treatment is underestimated when assessed by RT-qPCR. It is not possible to determine whether FRNA bacteriophage GA survival during wastewater treatment accurately indicates NoV survival. However, as FRNA bacteriophage GA is considered to be a conservative indicator of virus survival (Boudaud et al., 2012), it is likely that the reduction of infectious NoV during wastewater treatment is similar or greater than that for FRNA bacteriophage. We found that using RT-qPCR for NoV GII and FRNA bacteriophage GA quantification, similar log reductions were achieved during wastewater treatment. We therefore consider RT-qPCR is likely to be an inappropriate approach for measuring the reduction of NoV or other enteric viruses during treatment. Monitoring for infectious FRNA bacteriophage may provide a more accurate assessment of the efficiency of wastewater treatment processes than RT-qPCR, as well as being less expensive and easier to perform.

In our study, concentrations of total FRNA bacteriophage GA in oysters were significantly greater than concentrations of infectious FRNA bacteriophage GA. Previous studies have demonstrated a high frequency of NoV positive shellfish in commercial harvest areas (Lowther et al., 2012). Although it is not possible to determine the associated health risk of NoV positive oysters, the proposed CEN RT-qPCR method for NoV detection in shellfish (Lees and TAG4, 2010) has been considered suitable for risk assessment by the European Food Safety Authority (EFSA, 2012). Previously, we found that monitoring NoV concentrations in oysters using RT-qPCR could be effective in the management of a harvesting area implicated in an outbreak of gastroenteritis (Doré et al., 2010). In addition, increased concentrations of NoV in oysters have been correlated with an increased risk of NoV illness in consumers (Lowther, 2010). Further work is required to establish the public health risks associated with the consumption of NoV positive shellfish.

6.4.3 Impact of CSO discharges on NoV concentrations in oysters

We found that CSO effluent contains a greater percentage of infectious virus than treated effluent. In an oyster sample taken pre-CSO discharge, we detected a relatively high concentration of FRNA bacteriophage GA using the RT-qPCR (1130 genome copies 100 g^{-1}) in the absence of infectious FRNA bacteriophage GA. This may be as a result of geographically or temporally distant contamination where a high level of natural virus inactivation could have occurred prior to accumulation in oysters. Similarly, this could be representative of contamination with non infectious virus particles originating from successfully treated wastewater. However, twelve hours post-CSO discharge in oysters, the concentrations of infectious FRNA bacteriophage GA increased to levels comparable with total FRNA bacteriophage GA. As concentrations of NoV GII increased from below the LOD to 3150 genome copies 100 g^{-1} in the same samples, it is probable that the concentrations of NoV GII in this sample consisted of a high proportion of infectious NoV. NoV concentrations persisted at similar levels over the following 74 hours and are consistent with concentrations in oysters previously shown to present a risk if consumed raw (Doré et al., 2010; Lowther, 2010). Using FRNA bacteriophage as a model, our results indicate that the proportion of infectious to total virus concentrations in shellfish depends on the conditions under which they were contaminated.

Limited studies have involved the monitoring of wastewater effluents for NoV and FRNA bacteriophage GA using both RT-qPCR and a plaque assay for FRNA bacteriophage. We carried this out in an effort to determine the extent of overestimation of infectious virus by RT-qPCR in environmental samples. Although the WWTP monitored in this study may not be representative of a fully functioning plant, it remains the case that currently available RT-qPCR methods are inappropriate to assess the effectiveness of wastewater treatment processes to reduce infectious NoV in wastewater. There remains a clear requirement for the development of molecular methods to better assess the concentrations of infectious NoV in environmental samples. In the absence of such methods we believe that FRNA bacteriophage monitoring of wastewater using a plaque assay will provide a better assessment of the general efficiency virus removal during wastewater treatment processes. Using FRNA bacteriophage GA as a model we also demonstrated that CSO effluents contain a greater concentration of infectious virus than treated effluents and that CSO discharges therefore can have a significant impact on virus contamination in bivalve shellfish.

6.5 References

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*Chapter 7. Simulated sunlight inactivation of
norovirus and FRNA bacteriophage in seawater*

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Abstract

Aims: To investigate norovirus (NoV) and F-specific (FRNA) bacteriophage inactivation in seawater under simulated sunlight and temperature conditions representative of summer and winter conditions in Ireland.

Methods and Results: Inactivation experiments were carried out using a collimated beam of simulated sunlight and 100 ml of filtered seawater seeded with virus under controlled temperature conditions. NoV concentrations were determined using RT-qPCR and FRNA bacteriophage concentrations were determined using RT-qPCR and by plaque assay. For all virus types, the fluence required to achieve a 90% reduction in detectable viruses (S_{90} value) using RT-qPCR was not significantly different between summer and winter conditions. S_{90} values for FRNA bacteriophage determined by plaque assay were significantly less than those determined by RT-qPCR. Unlike S_{90} values determined by RT-qPCR, a significant difference existed between summer and winter S_{90} values for infectious FRNA bacteriophage.

Conclusions: This study demonstrated that RT-qPCR significantly overestimates the survival of infectious virus and is therefore unsuitable for determining the inactivation rates of viruses in seawater.

Significance and Impact of the Study: Results from this study provide initial data on the inactivation of NoV and FRNA bacteriophage in seawater under representative summer and winter conditions and will be of interest to shellfish and water management agencies alike.

7.1 Introduction

Norovirus (NoV) of the family *Caliciviridae* is the most significant cause of non-bacterial gastroenteritis in the developed world. NoV is shed in the faeces of infected persons at concentrations $>10^8$ g⁻¹ (Lee et al., 2007) and thus can be commonly detected in wastewater (Flannery et al., 2012; Nordgren et al., 2009). Wastewater discharge to marine waters has been associated with the NoV contamination of shellfisheries and the subsequent illness amongst consumers (Lees, 2000). Wastewater treatment is employed worldwide to reduce microbiological contaminants in wastewater prior to discharge to the environment. However, enteric viruses including NoV have been shown to persist in treated wastewater (Flannery et al., 2012; Nordgren et al., 2009). Knowledge of the viral inactivation kinetics for NoV in seawater would prove useful to shellfish and water management agencies to model the likely risk posed by wastewater discharges to shellfish harvest waters.

The sunlight that reaches the earth's surface is comprised of UV, visible and longer wavelengths of light and is the primary virucide in the environment (Lytle and Sagripanti, 2005). Virucidal effects are elicited primarily through direct damage to the nucleic acids of viruses, where pyrimidine dimers or other photo-products form upon exposure of DNA or RNA to sunlight (Sinton et al., 2002). The extent to which NoV is inactivated through sunlight exposure in marine waters is poorly understood. Numerous studies have investigated the inactivation rates for enteric viruses and bacteriophage under UV-C at 254 nm (germicidal wavelengths) and NoV inactivation kinetics have been inferred from data involving FRNA bacteriophage (Simonet and Gantzer, 2006), viruses within the *Caliciviridae* (De Roda Husman et al., 2004; Duizer et al., 2004) and other non-enveloped enteric RNA viruses (Crance et al., 1998; Meng and Gerba, 1996). However, germicidal wavelengths are not found within terrestrial sunlight that is composed of wavelengths greater than 293 nm (Kay et al., 2005). Notwithstanding, it has been shown that these non-germicidal wavelengths can directly damage enteric viruses (Love et al., 2010; Sinton et al., 2002, 1999). One reason for the lack of inactivation kinetic data for NoV specifically is the absence of a reliable cell culture assay. The detection of NoV in environmental samples has relied on molecular methods such as RT-qPCR. However, it has been shown that these

methods do not provide information on viral infectivity (Nuanualsuwan et al., 2002; Pecson et al., 2009).

Using solar simulated light, we determined the T_{90} and S_{90} values for NoV and FRNA bacteriophage GA in seawater under representative summer and winter temperature and sunlight conditions using RT-qPCR. FRNA bacteriophage GA has been found to be a conservative indicator for virus survival during water treatment (Boudaud et al., 2012). With the inclusion of a plaque assay for FRNA bacteriophage GA, we aimed to determine whether RT-qPCR is adequate for viral detection in marine waters and for use in modelling the risk posed by wastewater discharges to shellfish harvest areas.

7.2 Materials and methods

7.2.1 Solar simulator setup

The solar disinfection experiments were performed using an LOT Oriel Low-Cost solar Simulator (LOT Oriel, Leatherhead, UK) equipped with a 150W xenon arc lamp (LSO106, LOT Oriel). The solar simulator was equipped with an Oriel Air Mass 1.5 Global Filter (LSZ189, LOT Oriel) and provided a 35 mm diameter beam of light with a constant intensity of 1000 W m^{-2} at a distance of 80 mm from the light source. A calibrated solar cell (15150-1, LOT Oriel) was used to measure the irradiance of the solar simulator.

Solar radiation data, consisting of the cumulative irradiance received each month in Ireland, were obtained from Met Éireann. From the monthly irradiance the mean daily irradiance (per 24 hour period) was calculated. Mean daily irradiances of 235 W m^{-2} and 56 W m^{-2} were calculated as irradiances typical of summer and winter periods, respectively. To attenuate the irradiance required for summer and winter experiments a collimated beam set-up was used (Qualls and Johnson, 1983) at a distance of 45 cm and 155 cm from the solar simulator, respectively. Mean maximal summer (17°C) and mean minimum winter (10°C) seawater temperatures were obtained from Met Éireann and all experiments were carried out in a darkened, temperature controlled room at $17 \pm 1^{\circ}\text{C}$ for summer experiments and $10 \pm 1^{\circ}\text{C}$ for winter experiments.

7.2.2 Preparation of virus stocks

FRNA bacteriophage GA was propagated in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) WG49 host by broth enrichment (Anonymous, 1995b, p. 10705) and prepared as 10% glycerol/virus stocks. Briefly, following six hours incubation, host cells were lysed with chloroform and stored overnight at 5°C . The aqueous layer was decanted, centrifuged at $2000 \times g$ for 20 minutes and glycerol was added to make a 10% stock. Single use aliquots were prepared and stored at -20°C . The concentrations of the FRNA bacteriophage aliquots were 10^9 PFU ml^{-1} and 10^9 genome copies ml^{-1} determined using the plaque assay and RT-qPCR assay, respectively. For NoV, two separate stool samples, each positive for NoV GI or NoV

GII, was diluted 1:5 with PBS. Following centrifugation at $2000 \times g$ for 20 minutes, the supernatant was divided into single-use aliquots that were stored at -20°C . The concentrations of NoV in each aliquot were determined as 10^3 genome copies ml^{-1} for both NoV GI and NoV GII.

7.2.3 Experimental Setup for virus inactivation

Two millilitres of each virus stock was inoculated into 200 ml of filtered seawater that was then mixed and divided into two 100 ml volumes. For the inactivation studies, 500 ml sterile Pyrex beakers containing 100 ml of inoculated filtered seawater at a depth of 18mm were used for the irradiated samples. For control (dark) conditions, 100 ml of inoculated filtered seawater was added to 500 ml bottles covered in aluminium foil. All inoculated seawater volumes in both the control and irradiated samples were magnetically stirred at 130 rpm throughout the trial. Two millilitre subsamples were removed at timed intervals. For infectious FRNA bacteriophage GA, seawater samples were stored in the dark at 4°C and analysed within 24 hours. For total FRNA bacteriophage GA, NoV GI and NoV GII analysis by RT-qPCR, 1 ml samples were immediately stored at -20°C and were analysed within one week.

7.2.4 FRNA bacteriophage plaque assay

FRNA bacteriophage were enumerated using a double agar layer method (Anonymous, 1995b) using *S. Typhimurium* WG49 as the host strain. Host cells were cultivated for 4–6 h at 37°C in tryptone yeast-extract glucose broth (to yield $>10^6$ CFU ml^{-1}) and then mixed with 2.5 ml of tryptone yeast-extract glucose 1% agar (soft agar) and 1 ml of appropriately diluted seawater. This was then poured onto the hard agar (tryptone yeast-extract glucose 2% agar). Following overnight incubation at 37°C , FRNA bacteriophage GA were identified as semi-transparent plaques that were then counted and multiplied by the dilution factor to obtain the titre in plaque forming units (PFU) ml^{-1} . The limit of detection (LOD) of the assay was determined as 10 PFU ml^{-1} .

7.2.5 Viral genomic extraction

Viral RNA was extracted from 1 ml seawater samples in duplicate using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, France) following the manufacturer's instructions. Five hundred microlitres of seawater was used per extraction and viral RNA was eluted into 100 µl of elution buffer (bioMérieux). The eluted RNA was stored at -80°C until RT-qPCR analysis was undertaken (<1 month). A negative RNA extraction control using molecular biology grade water was processed alongside seawater samples.

7.2.6 Preparation of DNA plasmids

Double stranded (ds) DNA plasmids for quantification of NoV GI and GII were prepared as previously described (Flannery et al., 2012). For quantification of bacteriophage GA, PCR products from the RT-qPCR were purified using the Wizard® SV PCR Purification kit (Promega) and cloned using the pGEM-T Easy vector system (Promega). Transformant clones were screened and purified using the PureYield™ plasmid miniprep kit (Promega). The purified plasmid was quantified using a spectrophotometer at 260 nm and subsequently diluted to 10^5 copies μl^{-1} for use as a quantitative standard in the RT-qPCR.

7.2.7 RT-qPCR assay for NoV and FRNA bacteriophage GA

An Applied Biosystems AB7500 instrument was used for RT-qPCR analysis. Twenty microlitres of the NoV or FRNA bacteriophage GA reaction mix was prepared using RNA Ultrasense one-step qRT-PCR system (Invitrogen) containing 1 × reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 × µl Rox and 1.25 µl of enzyme. Previously described primers QNIF4 (da Silva et al., 2007), NV1LCR and probe NVGG1p (Svraka et al., 2007) were used for NoV GI analysis, and primers QNIF2 (Loisy et al., 2005a), COG2R (Kageyama et al., 2003) and probe QNIFS (Loisy et al., 2005a) used for NoV GII analysis. Previously described Levivirus genogroup II forward and reverse primers and probe were used (Wolf et al., 2008). Duplicate 5µl aliquots of sample RNA was added to adjacent wells of a 96-

well optical reaction plate in addition to no template controls. RT-qPCR inhibitors were controlled for each virus as previously described (Flannery et al., 2012).

NoV GI and GII reaction conditions were; initial incubation at 55°C for 60 min followed by 95°C for 5 min and then 45 cycles of 95°C for 15s, 60°C for 1 min and finally 65°C for 1 min. For FRNA bacteriophage GA, reaction conditions involved an initial incubation at 55°C followed by 95°C for 5 min and then 45 cycles of 95°C for 15 s and 58°C for 1 min. NoV and FRNA bacteriophage GA were quantified by comparing the C_q value to the standard curves in copies per µl, and then adjusted to reflect the volume of RNA analysed (expressed as genome copies ml⁻¹). The LOD of the assay was determined as 20 detectable genome copies ml⁻¹.

7.2.8 Calculation of T_{90} and S_{90} values

To evaluate the log decrease in detectable virus, the virus concentrations in the initial inoculated seawater suspension at time 0 were expressed as N_0 . The concentrations of detectable virus at all subsequent times were expressed as N_t . The linear relationship between log decrease in detectable virus and time is described by the inactivation rate constant (k):

$$\log_{10} \left(\frac{N_t}{N_0} \right) = -k * \text{time}$$

All experiments were performed on three separate occasions. At each sampling occasion, the mean concentration of virus was determined from duplicate analysis. For comparison with other studies, the time taken to achieve a 1 log reduction in virus concentrations (T_{90} value) was calculated. The fluence (MJ m^{-2}) was determined as a product of the irradiance (W m^{-2}) and the exposure time (seconds). The fluence required to for a 90% reduction in detectable virus (S_{90} value) was also determined. The mean data for N_t/N_0 from three independent summer and winter experiments was paired with the corresponding time or fluence and were plotted. Minitab statistical software version 15 (*Minitab Inc., PA, USA*) and Sigma Plot software version 11 (*Systat Software, Chicago, IL*) was used for the statistical analysis. A students t-test was carried out on $\log N_t/N_0$ for each virus to determined whether a difference in S_{90} and T_{90} values existed between summer and winter conditions and between irradiated and control conditions.

7.3 Results

7.3.1 Decrease in total detectable viruses in seawater determined by RT-qPCR

S_{90} and T_{90} values were determined for all virus types under summer and winter conditions (Table 7.1). The S_{90} values for total NoV GI, NoV GII and FRNA bacteriophage GA, as judged by RT-qPCR, were not significantly different ($P > 0.05$) between summer (GI; 18.18 MJ m⁻², GII; 17.30 MJ m⁻², GA; 13.96 MJ m⁻²) and winter (GI; 18.09 MJ m⁻², GII; 16.92 MJ m⁻², GA; 15.73 MJ m⁻²) experiments.

Under summer conditions, the reduction of all virus types as judged by RT-qPCR was significantly greater in the irradiated samples than in the control (dark) samples (Figure 7.1). No significant difference was found between the T_{90} values for NoV GI (21.5 hours) and NoV GII (20.5 hours) under summer conditions. However, the T_{90} value for FRNA bacteriophage GA (T_{90} 16.5 hours) was significantly less than the T_{90} values for NoV GI and GII ($P < 0.01$) under summer conditions.

Under winter conditions, no difference was observed between the T_{90} values for NoV GI (89.3 hours) and NoV GII (83.9 hours) in irradiated samples. However, NoV GI and NoV GII T_{90} values were greater than the T_{90} value for total FRNA bacteriophage GA (78 hours) however this difference was not significant. Similarly in control samples, no significant difference existed between T_{90} values for all virus types detected using RT-qPCR under winter conditions. Under winter conditions using RT-qPCR, the reduction of all viruses in irradiated samples was not significantly different from the reduction in the control samples.

Table 7.1 T_{90} and S_{90} values for viruses in seawater

Virus	Detection method	S_{90} (MJ m ⁻²) ^a		Irradiated T_{90} (h)		Control T_{90} (h)	
		Summer	Winter	Summer	Winter	Summer	Winter
NoV GI	RT-qPCR	18.18	18.09	21.5	89.3	59.8	85.9
NoV GII	RT-qPCR	17.30	16.92	20.5	83.9	41.0	101.5
FRNA phage GA	RT-qPCR	13.96	15.73	16.5	78	50.5	95.0
FRNA phage GA	Plaque assay	0.20	0.86	0.25	4.0	5.2	15.0

^aThe irradiance applied during the experiments were 20 MJ m⁻² day⁻¹ in summer and 5 MJ m⁻² day⁻¹ in winter.

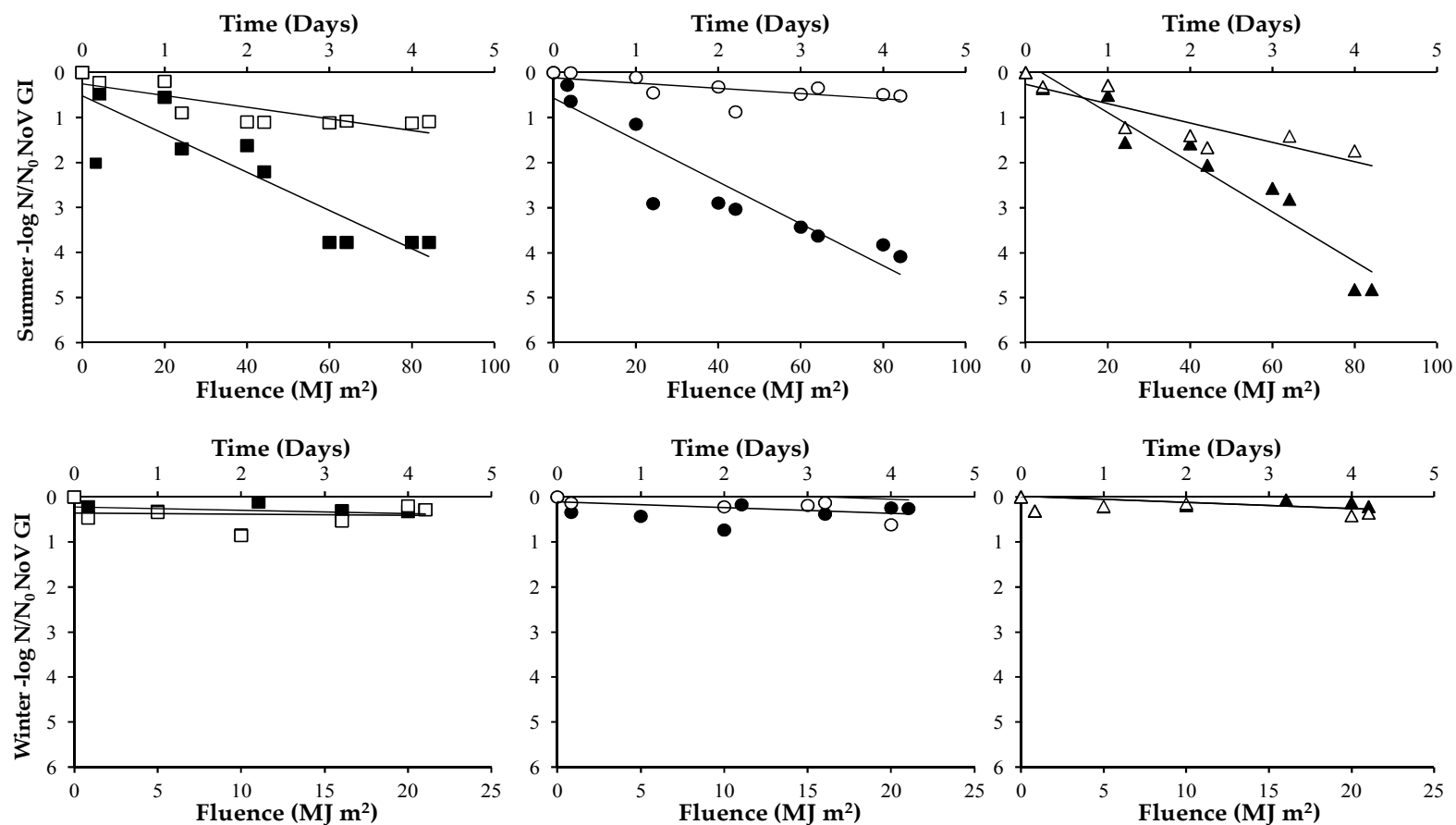


Figure 7.1 Simulated sunlight inactivation of viruses in seawater

Summer and winter $-\log N_t/N_0$ for NoV GI (squares), NoV GII (triangles) and FRNA bacteriophage GA (circles) are plotted against time (days) and fluence (MJ m^{-2}).

7.3.2 Detection of total and infectious FRNA bacteriophage GA in seawater

Figure 7.2 shows the reduction of infectious FRNA bacteriophage GA under summer and winter conditions. The S_{90} values for infectious FRNA bacteriophage GA determined by the plaque assay were significantly less ($P < 0.001$) than those determined using the RT-qPCR at 0.20 and 0.86 MJ m⁻² for summer and winter conditions, respectively. The difference between summer and winter S_{90} values for infectious FRNA bacteriophage GA were significantly different ($P < 0.05$). Under summer and winter conditions, the T_{90} values for infectious FRNA bacteriophage GA in irradiated samples (0.25 and 4.0 hours respectively) were significantly less ($P < 0.05$) than in the control samples (5.2 and 15 hours respectively). The T_{90} values for infectious FRNA bacteriophage GA under winter conditions were significantly greater than summer conditions ($P < 0.05$). Under winter and summer conditions, the T_{90} of total FRNA bacteriophage GA as determined using the RT-qPCR, was significantly different than that determined for infectious FRNA bacteriophage GA ($P < 0.001$).

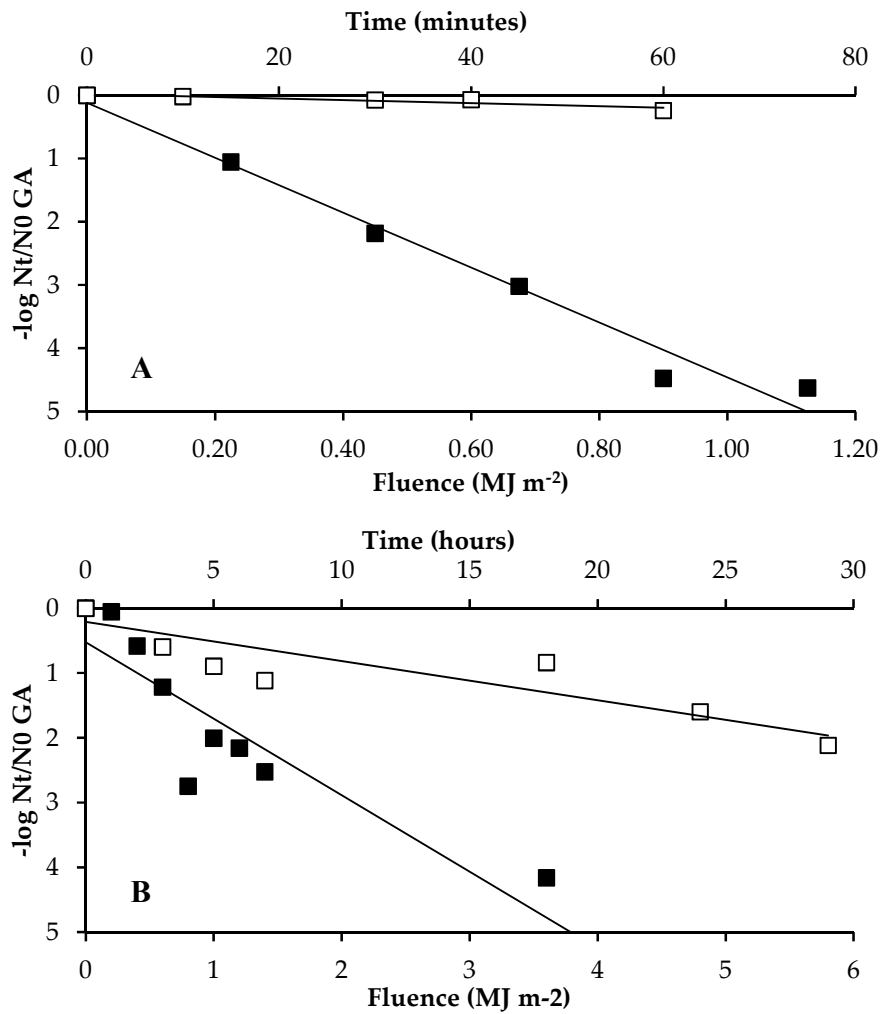


Figure 7.2 Solar simulation inactivation of infectious FRNA bacteriophage

Summer (A) and winter (B) experiments were carried out for 75 minutes and 28 hours respectively. The $\log N_t/N_0$ of viral concentrations in the irradiated samples (shaded squares) and control samples (open squares) are shown.

7.4 Discussion

We determined the S_{90} and T_{90} values for NoV and FRNA bacteriophage GA in seawater using RT-qPCR under simulated sunlight and temperature conditions representative of those found in Ireland during summer and winter periods. No significant difference was observed between S_{90} values under summer and winter conditions for any virus type when determined by the RT-qPCR. This suggests that temperature does not have a significant effect on viral inactivation. Given that the S_{90} values were constant between summer and winter, the T_{90} values for all virus types were greater under winter conditions than under summer conditions. The shorter T_{90} values in summer were a function of the greater irradiance that was applied in the summer experiments and therefore the shorter time required to reach the S_{90} value in these studies.

The S_{90} value for infectious FRNA bacteriophage GA determined by the plaque assay was significantly less than the S_{90} value determined using RT-qPCR in both winter and summer experiments. Clearly the RT-qPCR underestimates the extent of infectious FRNA bacteriophage inactivation under simulated environmental conditions. Primers used in the RT-qPCR target a short region (111-bp) of the ~3.5 kb FRNA bacteriophage GA genome, which is a normal feature of real-time PCR. However, provided this fragment is intact, the virus genome would still be detected and quantified even if there is damage to other regions of the virus genome which could prevent viral replication from occurring. Capsid-directed damage has been shown to occur in water impacted by sunlight due to interactions with reactive oxygen species and natural organic matter (Romero et al., 2011). RT-qPCR also detects virus particles that are not infectious because of damage to the capsid which would prevent virus attachment to host cell receptors (Nuanualsuwan et al., 2002b). It is probable that a similar overestimation of infectious NoV concentrations is provided by RT-qPCR. Therefore using RT-qPCR to determine T_{90} values for virus inactivation should be considered inappropriate. A significant difference existed between summer and winter S_{90} values for infectious FRNA bacteriophage GA suggesting that temperature, possibly associated with capsid damage, as well as sunlight irradiation played a role in virus inactivation. This is in agreement with other studies that have

reported a greater reduction of infectious virus in water under increased temperatures (Duizer et al., 2004b; Sinton et al., 1999b).

Under both summer and winter conditions, S_{90} values for NoV GI and NoV GII were greater than S_{90} values for total FRNA bacteriophage GA determined by RT-qPCR. FRNA bacteriophage and in particular FRNA bacteriophage GA has been proposed to be a conservative indicator of enteric viruses (Boudaud et al., 2012) in water treatment processes. The use of an RT-qPCR assay for FRNA bacteriophage alongside an RT-qPCR assay for NoV in inactivation studies has not been reported previously; therefore FRNA bacteriophage GA may not be as conservative an indicator when used in this context. As the NoV genome is ~4 kb larger than the FRNA bacteriophage GA genome, it may mean that the NoV genome is more-resistant to damage from sunlight.

Few data exist concerning the solar inactivation of viruses in seawater (Love et al., 2010; Sinton et al., 2002b, 1999b). Where such data is available, caution is required in comparing data across studies because of different experimental design and detection methods employed. To apply a standardised experimental regimen, we used a collimated beam set up for the solar simulator, similar to other inactivation studies that used germicidal UV light (de Roda Husman et al., 2004b; Duizer et al., 2004b). We recognise that the data presented here represents an initial assessment of viral inactivation in seawater under simulated sunlight using ideal laboratory conditions. We carried out our experiments using filtered seawater and a shallow depth; therefore, the S_{90} values calculated in this study may represent the highest inactivation rate likely for FRNA bacteriophage under sunlight and temperature conditions representative of summer and winter periods in Ireland.

There is therefore a clear requirement for further studies to determine more representative T_{90} values for NoV and viral surrogates under environmental conditions. Such studies should include consideration of the effect of seawater turbidity and depth. In addition, given the underestimation of infectious virus inactivation provided by RT-qPCR, such studies should consider the application of more appropriate approaches that may distinguish between infectious and non-infectious virus particles. Recently Pecson et

al., proposed a framework to assess UV inactivation of viruses and found that RT-qPCR can be used to provide an accurate estimation of MS2 inactivation under UV light (Pecson et al., 2011). Alternatively, the use of long range RT-qPCR has been shown to reflect the reduction in infectious murine NoV during UV disinfection (Wolf et al., 2009). Both approaches may provide additional relevant data in virus inactivation studies. In this study, FRNA bacteriophage inactivation in seawater determined using a plaque assay occurred at a greater rate than that determined using RT-qPCR. Therefore, RT-qPCR overestimated the survival of FRNA bacteriophage and we consider this method unsuitable for use in determining inactivation rates of viruses in seawater.

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*Chapter 8. Concluding remarks and future
recommendations*

8.1 Concluding remarks

The discharge of municipal wastewater to the marine environment is a cost effective practice that is widely used throughout the world. As it has long been recognised that discharges can reduce the sanitary quality of receiving waters and as consumer demands for shellfish increase, NoV contamination of oysters poses a significant challenge for WWTP operators, the shellfish industry and regulatory authorities. Two major approaches can be investigated to address this problem. Firstly, improved control of contamination inputs that impact on shellfisheries is required. Secondly risk management procedures are required to protect public health when shellfisheries become contaminated with NoV. The research output from this thesis provides information on the survival of NoV during wastewater treatment and in the marine environment. These data will assist policy makers to assess the effectiveness of current WWT processes at preventing NoV contamination in shellfisheries. This may eventually lead to improved pollution management strategies to prevent NoV contamination in marine environments supporting commercial shellfish harvest areas.

The results presented in this thesis are in agreement with the available literature and demonstrate that NoV concentrations in wastewater are reduced but not eliminated following WWT. Therefore, WWTP discharges are a significant cause of NoV contamination in the marine environment. All results from this project indicate that reductions in NoV concentrations during wastewater treatment were less than those for *E. coli* or FRNA bacteriophage during wastewater treatment. Previous studies have shown viruses are reduced less than bacterial indicator organisms during WWTPs. In the studies at two WWTPs in Chapter 4 and Chapter 5, NoV concentrations were reduced less than concentrations of *E. coli* and FRNA bacteriophage with both NoV genogroups undergoing less than a 1-log reduction in concentrations. In both WWTPs, the most significant reduction in NoV concentrations occurred during secondary treatment; with primary treatment and UV disinfection providing little additional reduction. The wastewater effluent discharged from both WWTPs was responsible for the NoV concentrations detected in oysters.

The application of UV disinfection to effluent subjected to an optimally-designed and operated secondary treatment process may improve the reduction of infectious NoV during wastewater treatment. In Chapter 4, it was found that infectious FRNA bacteriophage concentrations can be reduced by greater than 2 log during conventional activated sludge treatment. With the inclusion of UV disinfection as investigated in Chapter 5, an additional reduction of infectious FRNA bacteriophage concentrations of approximately 1 log was demonstrated. Since FRNA bacteriophage may be considered to be appropriate indicators of other enteric viruses such as NoV, it is possible that NoV may behave in a similar manner. Considering that NoV was detected in influent wastewater at approximately 3 log genome copies 100 ml⁻¹, the addition of UV disinfection to a well-functioning secondary treatment process may remove the majority of infectious NoV contamination entering shellfisheries. It is assumed that FRNA bacteriophage accurately indicate the inactivation kinetics of NoV however this has not been determined. Despite this, since FRNA bacteriophage have similar characteristics to NoV and can be easily enumerated, they may provide for the most suitable indicator in this context.

RT-qPCR monitoring of effluent provides little information of the reduction of NoV during wastewater treatment. In Chapter 4, reductions of infectious FRNA bacteriophage concentrations were significantly greater than reduction of NoV concentrations at each stage of treatment. However, as NoV was detected using RT-qPCR, it is likely that the reduction of infectious NoV was underestimated. The study outlined in Chapter 5 aimed to provide further information on the discrepancy between concentrations of enteric viruses determined by RT-qPCR (total virus) and an infectivity assay (infectious virus). Direct comparison was undertaken using FRNA bacteriophage GA as a surrogate for NoV. Assuming that NoV and FRNA behave similarly; the results suggest that infectious NoV concentrations are reduced significantly during wastewater treatment. Thus, RT-qPCR is an inappropriate method for measuring the reduction of infectious NoV in WWTPs. Alternatively monitoring of effluents using a plaque assay for FRNA bacteriophage may provide an appropriate indicator for assessing the reduction of NoV concentrations during wastewater treatment. This approach could be used to

develop process criteria for the reduction of virus concentrations during WWT. WWTPs could be monitored using the FRNA bacteriophage plaque assay to ensure compliance with such a process criteria.

NoV contamination of shellfisheries caused by untreated wastewater via CSOs has been reported previously and has been linked to outbreaks of illness associated with shellfish consumption. In Chapter 5, frequent CSO discharges from the WWTP were linked to increased NoV concentrations in oysters. In this study using FRNA bacteriophage GA, CSO effluent was found to contain a higher percentage of infectious virus than found in treated effluent. Assuming a similar increase in concentrations of infectious NoV are associated CSO effluents, shellfish contaminated by CSO discharges may present a greater public health risk compared to shellfish contaminated with continuous discharges of treated effluent. This has important implications for controlling NoV contamination in shellfisheries. Eliminating the discharging of CSOs is not economically or logistically feasible, however such events should be minimised as far as is practicable. Management strategies are also required post-contamination by such events. Alerting shellfish producers of a CSO event in real-time would allow them the option of introducing additional risk management procedures such as delaying harvest or enhancing post harvest treatment procedures such as depuration.

Despite the fact that RT-qPCR overestimates the concentration of infectious NoV in oysters it remains a useful tool as an index of the risk of NoV infection in oyster consumers. In Chapter 4, NoV concentrations in wastewater effluent and oysters were correlated and a clear peak in concentrations occurred in the winter. This is in agreement with the predominance of oyster-related NoV illness in that occurs during the winter months. It has been demonstrated that the risk of NoV infection from consuming oysters increases with increasing concentrations of NoV determined by RT-qPCR. Therefore RT-qPCR is an appropriate tool in this context. Thus, monitoring of oyster production areas for NoV should prove valuable in identifying areas and periods of increased risk of NoV contamination.

Understanding the survival characteristics of NoV in the marine environment is necessary to further develop mitigation strategies. Dilution and inactivation of bacteria in the marine environment are often factored into the impact assessments for WWTPs and shellfisheries. Currently environmental inactivation characteristics are well-defined for bacteria but less so for enteric viruses such as NoV. The results in Chapter 6 highlight that NoV is stable in seawater, particularly under winter conditions. This also may explain the greater frequency of NoV detection in shellfish that occurs at this time and the elevated concentrations present. However, as shown in this thesis, RT-qPCR overestimates infectious virus concentrations, therefore, further studies involving other culturable NoV (such as MuNoV) may be more appropriate than the FRNA bacteriophage used throughout this thesis. The NoV inactivation values determined probably represent an overestimation of those that may occur in the environment. NoV inactivation occurring at deeper depths in natural seawater is likely to be less than was found in this thesis using shallow depths of filtered seawater. Therefore, the results presented in this thesis should be treated with caution as they are likely to underestimate the survival of infectious NoV in the marine environment. Further studies are required to better characterise NoV inactivation in the marine environment.

The potential implications of NoV contamination in shellfish remain a concern to the shellfish industry and regulatory bodies. Monitoring of WWTP effluent for FRNA bacteriophage is a low-cost way to assess plant performance and routine analysis may allow for optimisation of treatment conditions to reduce infectious NoV. It is not possible to prevent the impact of NoV contamination in shellfisheries entirely (such as that from CSO discharges) therefore, a combination of pollution reduction measures and subsequent risk management procedures in contaminated harvest areas will provide the most suitable approach. The novel data presented in this thesis provides relevant information on the reduction of NoV concentrations during WWT and in the marine environment. This data will prove useful in developing strategies for producing NoV-free shellfish.

8.2 Future recommendations

The results reported in this thesis will feed into the wider understanding of NoV survival during WWTPs and in the marine environment. However significant knowledge gaps remain. Therefore, the following recommendations are proposed based on the findings and conclusions presented in this thesis:

- Monitoring of wastewater using RT-qPCR is inappropriate to assess infectious viral reduction during treatment.
- Infectious FRNA bacteriophage could provide a suitable indicator organism for enteric viruses and could be used to develop guideline operational criteria for virus reduction during wastewater treatment.
- Improvement to existing WWTPs such as the addition of UV disinfection can result in an increased reduction of concentrations of viruses may reduce the risk posed to human health.
- Further development of methods to distinguish between infectious and non-infectious NoV particles in environmental samples is needed. Such methods could provide data that relates NoV concentration in oysters to the risk of infection in consumers.
- NoV monitoring of oyster harvest areas can identify high-risk locations and periods when NoV contamination is elevated. Strategies should be developed and employed by producers during high-risk periods to reduce NoV exposure to consumers.
- CSO effluent contains higher ratios of infectious viruses than treated effluent therefore further studies should be conducted to establish appropriate guidelines to limit the impact of CSO discharges in shellfish production areas.
- Further studies should be undertaken to model the likely fate of NoV in the marine environment to aid in the validation of existing pollution reduction strategies and potential risk management procedures in shellfisheries.

Appendix A: Author publications

J. Flannery, S. Keaveney, P. Rajko-Nenow, V. O'Flaherty, W. Doré (2012). Concentration of norovirus during wastewater treatment and the impact on oyster contamination. Applied and Environmental Microbiology, Volume 78, Issue 9, pages 3400-3406.

J. Flannery, S. Keaveney, P. Rajko-Nenow, V. O'Flaherty, W. Doré (2013). Norovirus and FRNA bacteriophage determined by RT-qPCR and infectious FRNA bacteriophage in wastewater and oysters. Water Research, Volume 47, Issue 14, pages 5222-5231.

J. Flannery, P. Rajko-Nenow, S. Keaveney, V. O'Flaherty, W. Doré (2013). Simulated sunlight inactivation of norovirus and FRNA bacteriophage in seawater. Journal of Applied Microbiology, Volume 115, Issue 3, pages 915-922.