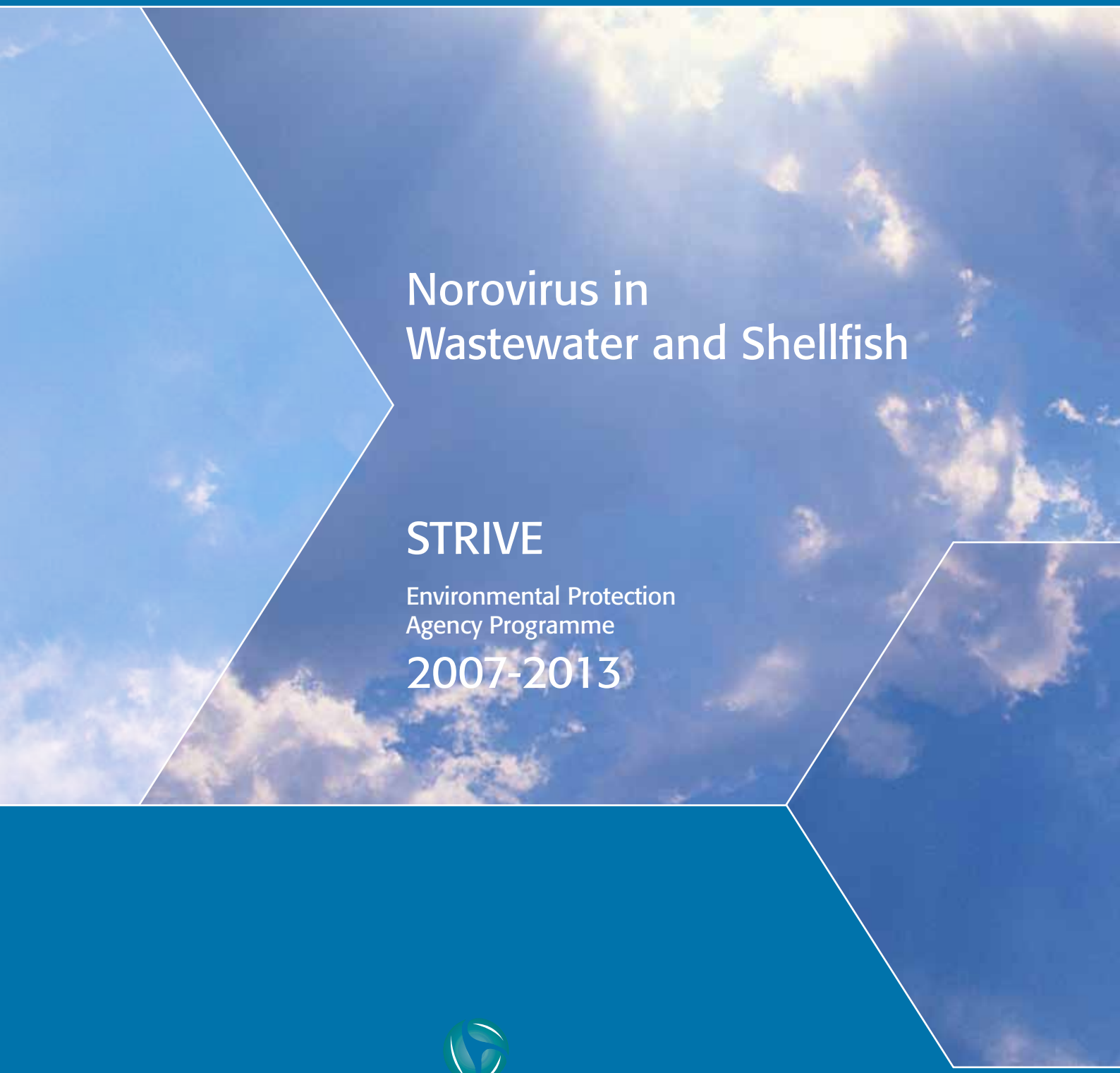
Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

# STRIVE
## Report Series No.109



# Norovirus in Wastewater and Shellfish

## STRIVE

Environmental Protection Agency Programme

2007-2013

SSTI Strategy for Science, Technology and Innovation

**Comhshaol, Pobal agus Rialtas Áitiúil**
Environment, Community and Local Government

# Environmental Protection Agency

The Environmental Protection Agency (EPA) is a statutory body responsible for protecting the environment in Ireland. We regulate and police activities that might otherwise cause pollution. We ensure there is solid information on environmental trends so that necessary actions are taken. Our priorities are protecting the Irish environment and ensuring that development is sustainable.

The EPA is an independent public body established in July 1993 under the Environmental Protection Agency Act, 1992. Its sponsor in Government is the Department of the Environment, Community and Local Government.

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- Office of Environmental Enforcement
- Office of Environmental Assessment
- Office of Communications and Corporate Services

The EPA is assisted by an Advisory Committee of twelve members who meet several times a year to discuss issues of concern and offer advice to the Board.

**EPA STRIVE Programme 2007–2013**

# Norovirus in Wastewater and Shellfish

## Assessing the Impact of Wastewater Treatment Plant Effluent on Norovirus Contamination in Shellfisheries

## (2008-EH-MS-7-S3)

## STRIVE Report

Prepared for the Environmental Protection Agency

by

Marine Institute

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## ACKNOWLEDGEMENTS

This report is published as part of the Science, Technology, Research and Innovation for the Environment (STRIVE) Programme 2007–2013. The programme is financed by the Irish Government under the National Development Plan 2007–2013. It is administered on behalf of the Department of the Environment, Community and Local Government by the Environmental Protection Agency which has the statutory function of co-ordinating and promoting environmental research.

The authors are grateful to the Environmental Protection Agency (EPA) for generous financial support. Numerous people helped out in many ways: in particular, the Steering Committee, the EPA Board, EPA staff, interns, and Economic and Social Research Institute staff.

## DISCLAIMER



The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

**EPA STRIVE PROGRAMME 2007–2013**

Published by the Environmental Protection Agency, Ireland

ISBN: 978-1-84095-503-3

Price: Free **Online version**

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# Executive Summary

Norovirus (NoV) is the most common cause of viral gastroenteritis in the developed world. It is spread through the faecal–oral route and generally causes self-limiting diarrhoea in healthy individuals, although more serious illness may occur in immunocompromised individuals. Municipal wastewater contains significant concentrations of NoV. Therefore, the discharge of municipal wastewater into the marine environment, as practised throughout the world, has implications for the virus quality of shellfish production areas. Bivalve shellfish, such as oysters and mussels, can accumulate NoV from contaminated water. Oysters are frequently consumed raw and have been associated with outbreaks of gastroenteritis when they are grown in areas impacted by municipal wastewater discharges. Wastewater treatment plants (WWTPs) can be effective in reducing the bacterial concentration in wastewater and are considered an important control point in protecting the microbiological quality of shellfish production areas. However, the extent to which wastewater treatment (WWT) reduces NoV concentrations is unclear. This study used a real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) to investigate the reduction of NoV during the treatment provided by two WWTPs and the subsequent accumulation in oysters. The use of UV disinfection processes was also investigated to determine whether an additional level of NoV reduction could be achieved. In addition, the contribution of combined sewer overflow (CSO) discharges to NoV contamination in oysters was investigated. Finally, the survival of NoV in sea water under winter and summer conditions was also investigated. In addition to directly detecting NoV, F-specific RNA (FRNA) bacteriophage was used as an indicator of virus behaviour throughout the study. Concentrations of FRNA bacteriophage were determined using both a RT-qPCR and a plaque assay to provide a direct comparison between an infectivity assay and the RT-qPCR assay.

During the study, NoV was detected in wastewater year-round but concentrations peaked during the winter months in line with peaks of infection in the general population at this time of year. There was a clear correlation with NoV concentrations in oysters, which also peaked during the winter. The reductions of NoV and FRNA bacteriophage concentrations during WWT processes were similar with approximately 1 $\log_{10}$ (90%) reduction with approximately a further 0.5 $\log_{10}$ (67%) reduction achieved through ultraviolet (UV) disinfection as judged by the RT-qPCR assays. However, reductions in FRNA bacteriophage concentrations were significantly greater when judged by the infectivity assay. During secondary WWT, infectious FRNA bacteriophage was reduced by >2 $\log_{10}$ (>99%) with the potential to achieve a further reduction of almost 2 $\log_{10}$ (>99%) following UV disinfection. This study concludes that RT-qPCR underestimates the reduction of infectious virus during WWT and cannot be considered a suitable tool to measure the extent of virus removal achieved. Using FRNA bacteriophage, it was demonstrated that a 4 $\log_{10}$ (99.99%) reduction of infectious virus can be achieved through secondary treatment and UV disinfection combined.

This study investigated the impact of CSO discharge events at a secondary WWTP with UV disinfection. Analysis of the CSO discharge effluent using the RT-qPCR and infectivity assays for FRNA bacteriophage indicated a significantly higher ratio of infectious virus to non-infectious virus than in treated wastewater effluent. Therefore, the discharge of effluent following CSO discharge events may lead to the introduction of significant concentrations of infectious virus into shellfisheries where such discharges impact them. Procedures relating to CSO discharges into receiving waterbodies, including the marine environment, are set out in the Department of the Environment and Local Government (DELG) document *Procedures and Criteria in relation to Storm Water Overflows* (1995). This document is used by the Environmental Protection Agency (EPA) as procedural compliance criteria in its Wastewater Discharge Authorisation when licensing/ permitting CSO discharges from agglomerations. However, the DELG document does

not take specific account of virus contamination in shellfisheries and its effectiveness at controlling NoV contamination in shellfisheries is unknown. It should be noted that it was beyond the scope of this project to assess the impact of complying with the arrangement set out in the DELG document on NoV contamination in shellfisheries. Further work is required to fully establish the impact of CSO discharge events on infectious virus concentrations in shellfish production areas.

During the project, the survival of viruses during typical summer and winter conditions in sea water was studied in a laboratory setting. Using the RT-qPCR method, NoV and FRNA bacteriophage appeared to persist for extended periods under both winter and summer conditions. However, using the FRNA bacteriophage infectivity assay, shorter die-off periods were observed. FRNA bacteriophage displayed significantly greater persistence under winter conditions, with a die-off rate almost nine times greater than under summer conditions. If a similar effect is true for NoV, the extended survival periods during the winter may, in part, explain the higher incidence of oyster-related NoV outbreaks that occur annually during this period.

Overall, this project demonstrates that RT-qPCR is an unsuitable method to determine the extent of infectious NoV reduction during WWT or environmental survival because it detects both infectious and non-infectious virus particles. Significant reductions of infectious FRNA bacteriophage were achieved by secondary treatment in association with UV disinfection. Therefore, it is concluded that optimally performing WWT represents a significant barrier to the introduction of infectious viruses into shellfish production areas. In the continuing absence of an infectivity assay, it is proposed that FRNA bacteriophage is an appropriate indicator of infectious virus reduction for NoV and an ongoing monitoring programme to determine the efficiency of virus removal in WWTPs in Ireland should be introduced.

# 1 Introduction and Background

## 1.1 Public Health Risks Associated with Bivalve Shellfish Contaminated with Municipal Wastewater

Filter-feeding bivalve shellfish, such as oysters and mussels, can accumulate human pathogenic viruses when grown in environments impacted by discharges from urban wastewater agglomerations and other sources of human faecal material. The consumption of shellfish contaminated with human pathogenic viruses has been responsible for numerous outbreaks of illness and represents a significant risk to public health (Lees, 2000). The risks associated with such bivalve shellfish are well documented. In Europe, as elsewhere in the world, extensive regulations exist to control these risks. The sanitary quality of shellfish harvesting areas in Europe is assessed by bacterial (*Escherichia coli*) monitoring. Depending on the result of this monitoring, shellfish growing areas are awarded a classification. The classification determines the level of treatment required before shellfish may be placed on the market (Table 1.1). In addition, shellfish must meet bacterial end-product standards of <230 *E. coli*/100 g and the absence of salmonella in 25 g of shellfish flesh before consumption.

These controls have been effective in virtually eliminating the bacterial illness associated with shellfish consumption. However, outbreaks of viral illness associated with the consumption of live bivalve molluscs have continued to occur throughout Europe (Gill et al., 1983; Rippey, 1994; Le Guyader et al., 1996; Doyle et al., 2004). This includes gastroenteritis outbreaks associated with oysters even when shellfish are compliant with all existing regulatory requirements (Doré et al., 2010; Westrell et al., 2010). Acute gastroenteritis caused by norovirus (NoV) is the major virus risk associated with raw oyster consumption in Northern Europe (Lees, 2000). In a recently reported opinion by the European Food Safety Authority (EFSA), raw shellfish containing NoV was identified as a high-risk food–virus combination (EFSA, 2011). Therefore, NoV-contaminated bivalve molluscs, particularly oysters when consumed raw, continue to be a significant and clearly identified public health risk.

## 1.2 Norovirus

NoV is now recognised as the major cause of acute gastroenteritis in the human population. It has been estimated to be responsible for up to 23 million cases of gastroenteritis each year in the USA (Mead et al., 1999). Following an incubation period of 24–60 h, illness usually involves a mild self-limiting infection that lasts 12–24 h. Symptoms include diarrhoea, nausea, vomiting and abdominal pain. More rarely, fever and headache are reported. Transmission of NoV is via the faecal–oral route either directly or via contaminated food or water. The virus is highly infectious, requiring a low infective dose, maybe <10 infectious virus particles (Green, 1997). Person-to-person transmission is most common, especially in closed communities and outbreaks are often associated with highly publicised closures of hospital wards and other care settings. Infections in Ireland and other countries demonstrate a strong seasonal distribution with illness peaking in the

**Table 1.1. Criteria for the classification of bivalve mollusc harvesting areas under Regulation (EC) No. 854/2004 and, by cross-reference, in the Council Regulation on microbiological criteria for foodstuffs.**

| Category | Microbiological standard | Treatment required |
|---|---|---|
| **Class A** | <230 *E. coli*/100 g | May go direct for human consumption |
| **Class B** | <4,600 *E. coli/100 g* (on 90% of sampling occasions) | Must be depurated, heat treated or relayed to meet Class A requirements |
| **Class C** | <46,000 *E. coli*/100 g | Relay for 2 months to meet Class A or B requirements, may also be heat treated |
| **Prohibited** | >46,000 *E. coli*/100 g | Harvesting prohibited |

winter months (December through to March). Because of this seasonal distribution, acute gastroenteritis caused by NoV is often described as winter vomiting disease and NoV as the winter vomiting bug. Occasionally, infection may be more severe and hospitalisation of elderly and debilitated individuals may be required. NoV gastroenteritis has, on rare occasions, been associated with death in such situations (Harris et al., 2008). Further studies in the UK have identified excess deaths in the community associated with epidemic years of gastroenteritis (Harris et al., 2007). Because symptoms are generally mild, illness is significantly under-reported. An extensive study in the UK estimates that for every reported case a further 1,500 cases may be unreported (FSA, 2000). The extent of community illness varies annually and epidemic years have been identified in 2002, 2004 and 2006 (Kroneman et al., 2006). Epidemic years have been associated with the emergence of new NoV variant genotypes. Genetic analysis has indicated that new variants result from recombination of different genotypes of NoV (Lopman et al., 2009).

## 1.3 Methods for NoV Detection in Environmental Samples and Approaches for Studying NoV Behaviour

Historically, it has not been possible to culture NoV, making it difficult to undertake quantitative analysis. However, recently successful cultivation of NoV has been reported (Straub et al., 2007, 2011). This involved the use of a highly specialised three-dimensional model of the small intestinal epithelium to demonstrate in vitro replication of NoV. This work has not yet been reproduced by other laboratories. It does not, therefore, provide a reproducible quantitative assay that could be usefully employed to detect and quantify NoV.

Detection methods therefore continue to rely on the use of molecular procedures. Because NoV has a low infectious dose, procedures must be sensitive to enable detection of low-level NoV contamination that may be present in environmental samples. NoV is a genetically diverse genera of viruses with two major human genogroups – Genogroup I (GI) and Genogroup II (GII) – each comprising a significant number of genotypes making the development of consensus primers difficult. Because of the requirement to detect low levels of NoV in environmental samples, detection methods have been based on amplification procedures principally using polymerase chain reaction (PCR) (Lees et al., 1995; Henshilwood et al., 2003; Le Guyader et al., 2003). More recently, a real-time reverse transcription quantitative PCR (RT-qPCR) procedure for NoV was developed that allows virus quantification (Jothikumar et al., 2005) and has been further refined to make use of virus extraction controls to ensure reproducible results. Currently, a working group under the direction of the European Committee for Standardisation (CEN) is finalising full validation of these procedures (Lees and TAG4, 2010). The virus extraction procedure has been developed specifically for food matrices. A variety of virus extraction procedures for wastewater have been developed for both NoV and other human viruses. These are suitable for use with the real-time RT-qPCR procedure developed by the CEN working group. Therefore a reliable and robust real-time RT-qPCR procedure suitable for quantitative detection of NoV is available and this provides a tool to investigate levels of NoV in wastewater and the marine environment.

As a result of the difficulty in detecting NoV in environmental settings, a number of surrogate organisms have been proposed to investigate NoV occurrence and survival characteristics. F-specific RNA (FRNA) bacteriophage are single-stranded RNA viruses infecting bacteria that have been used in numerous studies to investigate human RNA virus survival and persistence in wastewater (Havelaar et al., 1985), the marine environment and shellfish (Doré and Lees, 1995; Doré et al., 2000). They share a similar structure to NoV, are naturally present in wastewater, are simple to enumerate (Havelaar and Hogeboom, 1984; Havelaar et al., 1985) and an ISO method for their detection exists. Recently, real-time RT-qPCR procedures have been developed for FRNA bacteriophage (O'Connell et al., 2006; Orgorzaly and Gantzer, 2006; Wolf et al., 2008). At this point in time, the use of FRNA bacteriophage probably represents as good an infectious surrogate as any for human NoV during wastewater treatment (WWT) and subsequent

uptake in oysters. In addition, as there are concerns over the fact that PCR methods detect both infectious and non-infectious virus particles, FRNA bacteriophage provide a useful model for comparing RT-qPCR detection with an infectivity assay.

## 1.4 NoV and Shellfish

NoV contamination of oysters has been widely reported from a number of countries. A recent study in the UK reported that NoV was detected in 76.2% of Pacific oyster (*Crassostrea gigas*) samples tested (Lowther et al., 2012b). A study in Ireland indicated that 31% of oyster samples analysed contained NoV (Flannery et al., 2009). Similar rates of detection occur in other countries. Therefore, it is clear that NoV may be detected in oysters regularly. Recently introduced real-time RT-qPCR procedures have allowed concentrations of NoV present in oysters to be determined. The European Food Safety Authority recently reported concentrations of >1,000 genome copies/g of NoV present in 37.2%, 44.4% and 7.7% of oyster samples analysed in the UK, Ireland and France, respectively (EFSA, 2012).

It has also been reported that shellfish contaminated by NoV may contain a number of different NoV genotypes and that this can result in multiple NoV infections in individual shellfish consumers (Sugieda et al., 1996; Le Guyader et al., 2006). Multiple infections following shellfish consumption may provide an opportunity for genomic recombination between NoV genotypes. This can result in the development of new NoV variants which initiate community epidemics (Costantini et al., 2006). Because shellfish are traded widely internationally, they can also act as vectors for transmitting NoV strains around the world. Therefore, apart from the immediate risk of causing illness in consumers, NoV-contaminated shellfish are considered important for the potential emergence of new variants of NoV and the international spread of existing NoV strains.

## 1.5 Control of NoV Contamination of Shellfisheries

Control of microbiological contamination of the marine environment to acceptable levels is generally reliant on three issues. These are, firstly, reduction of microbial loading through effective WWT, secondly, dilution through dispersion and, thirdly, natural die-off in the marine environment. Understanding the survival and removal characteristics of a microorganism during each process is critical to determine measures to limit contamination of shellfish production areas. These characteristics are well documented for a number of the principal regulatory bacterial indicator organisms (notably *E. coli* and faecal coliforms). This allows the impact of wastewater disposal to be modelled to ensure compliance with regulatory requirements. Survival characteristics of a number of human viruses and proposed human virus indicators (most notably FRNA bacteriophage) have also been investigated and documented. Their survival characteristics during WWT not only differ markedly from bacteria but also between the virus types. For example, in one study investigating virus persistence in the liquid phase of a pilot-scale activated sludge plant, initial levels of hepatitis A virus (HAV) (an RNA virus) were reduced by 85% after 24 h compared with a reduction of just 68% for the FRNA bacteriophage MS2 (Arraj et al., 2005). In this case, FRNA bacteriophage was considered to be a conservative indicator of HAV removal during WWT.

Traditionally the microbiological impact of wastewater on the aquatic environment has been assessed using bacterial indicator organisms and standards based on bacterial limits. However, a number of studies have documented that the behaviour of bacterial indicators does not reflect that of human viruses. Viruses have been shown to have different survival characteristics than bacteria through the WWT process and in sea water (Mocé-Llivina et al., 2002; Sinton et al., 2002; Allwood et al., 2003).

## 1.6 NoV Removal during WWT

Despite its importance as a human pathogen, only limited data are available on the survival of NoV during WWT. Prior to this project, no data on NoV concentrations in wastewater either before or after WWT were available within Ireland. Workers in France (da Silva et al., 2007) quantified NoV levels in wastewater influent and effluent using real-time RT-qPCR procedures. They investigated a number of treatment types at four sites and detected NoV in 88%

of influent samples compared with 24% of effluent samples. In the Netherlands, untreated and treated wastewater was investigated at two wastewater treatment plants (WWTPs) over a year-long period (van den Berg et al., 2005). Average reductions in each plant were between 2 and 3 $\log_{10}$ of detectable PCR units, with final effluents containing $10^2$–$10^3$ PCR-detectable units per litre of effluent. A limited number of studies have also been undertaken in Japan. In one study using non-quantitative procedures, NoV was detected in 100% and 89% of untreated and treated wastewater samples, respectively (Ueki et al., 2005). In a further study, Haramoto et al. (2006) used real-time RT-qPCR to investigate NoV concentrations in influent and effluent wastewater at a WWTP. Seasonal differences were detected in concentrations of NoV in both influent and effluent, with peaks detected in the winter months. Differences were observed between GI and GII survival during WWT, with NoV GI possibly showing greater resistance.

UV disinfection is increasingly being used to treat final wastewater effluents in order to reduce the microbiological load in treated wastewater discharged to sensitive aquatic environments. UV disinfection is an effective procedure for reducing bacterial indicator organisms to achieve environmental bacterial standards. However, several studies have demonstrated that viruses are more resistant to UV irradiation than vegetative bacterial cells (Thurston-Enriquez et al., 2003; Koivunen and Heinonen-Tanski, 2005). The majority of studies into virus inactivation by UV irradiation have investigated laboratory-grown suspensions of cells seeded into water or buffers. This may be unrepresentative of inactivation in wastewater effluent where virus shielding and other intrinsic properties of the wastewater effluent may reduce inactivation. Limited studies of virus inactivation in wastewater effluents have been undertaken (Tree et al., 1997, 2005). One study in wastewater effluent demonstrated that *E. coli* was considerably more susceptible to UV irradiation than viruses (feline calicivirus, FRNA bacteriophage MS2 and poliovirus), with a four times greater dose of UV irradiation required to cause a 4 $\log_{10}$ reduction of feline calicivirus than *E. coli* (Tree et al., 2005). There were also differences in UV irradiation resistance between the viruses with the FRNA bacteriophage MS2 demonstrated as the most resistant organism. Therefore, there are clear differences in resistance to inactivation by UV irradiation between bacteria and viruses and also between different virus types. Prior to the start of this study, the authors were not aware of any data on the inactivation of NoV by UV irradiation in wastewater effluents.

Outbreaks of shellfish-associated gastroenteritis have been attributed to the overflow of dilute untreated wastewater due to high rainfall events (Murphy and Grohmann, 1980). Specific studies have also indicated that NoV contamination of shellfisheries can occur as a result of combined sewer overflows (CSOs) (Keaveney et al., 2006). Although fairly extensive data exist on the bacterial quality of storm-water overflow effluents more limited data exist on the viral loading. In particular, no information on the NoV content of storm overflow effluent was available. Therefore, it has not been possible to directly establish the relative contribution of CSO discharge events compared with continuous treated effluent discharges on NoV contamination in shellfisheries.

## 1.7 NoV Survival in Sea Water

The authors are aware of only one data set relating directly to NoV survival in sea water (Anonymous, 2004). Using a recently developed quantitative real-time RT-qPCR procedure for a specific GI NoV strain (Valetta) and a specific GII NoV genotype (Grimsby), laboratory studies investigated the survival of the two strains in sea water. Results for these strains (which may or may not be representative of other NoV strains) demonstrated extended survival times during winter conditions compared with summer conditions. Sunlight was found to play a significant part in the inactivation of both strains of NoV. A further limited number of studies have been conducted in fresh waters and drinking water. In one study, using a real-time RT-qPCR method, levels of NoV persisted in tap water and mineral water for over 80 days (Ngazoa et al., 2007). Another study indicated no significant reduction in NoV over a 262-day period at 10°C using real-time RT-qPCR (Gassilloud et al., 2003). A number of studies using various general virus surrogates have been undertaken to investigate the survival of viruses in sea water. In general, these studies highlight considerably

increased survival of human enteric viruses over that of bacteria in the marine environment. Factors that have been identified to influence both virus and bacterial survival include temperature, salinity and turbidity.

## 1.8 Background Summary

In summary, prior to the commencement of this project it was the case that:

- NoV contamination of shellfish production areas producing bivalve shellfish that are consumed raw or lightly cooked represents a significant public health risk and may facilitate the international spread of a new recombinant NoV in the community
- Limited international data existed on the survival of NoV during WWT and in the marine environment
- There were no data in the Irish setting in relation to NoV occurrence in wastewater, survival during WWT or survival in the marine environment
- No data existed on the effect of UV treatment on NoV inactivation
- The relative impact of CSO discharge events and continuous treated wastewater discharges on NoV contamination in shellfish was unclear
- Investigation into aspects of NoV survival in environmental settings has been limited because of a lack of reliable quantitative assays to detect NoV in environmental samples
- A reliable quantitative real-time RT-qPCR method to detect NoV in shellfish had been developed and is in use though it is undergoing international validation
- The extent to which real-time RT-qPCR methods detect non-infectious virus particles was unclear.

## 1.9 Aims and Objectives

The overall aim of the project was to provide a robust data set on the survival of NoV during WWT and in the marine environment in the Irish setting. This will allow regulatory authorities and planners to make informed decisions on suitable locations for new bivalve shellfish growing areas, the level of WWT required and on the location of wastewater outfalls to prevent or reduce NoV contamination in shellfisheries and other sensitive marine environments. Protection of shellfisheries will increase public health protection. Specific objectives were to:

- Quantify the level of NoV found in wastewater influent, intermediate stages and effluent during WWT and identify the extent of NoV removal during WWT
- Determine the relative contribution of combined sewer overflow discharges and continuous treated wastewater inputs to NoV contamination in shellfisheries
- Establish the time required to reduce 90% of NoV ($T_{90}$ values) in sea water under typical winter and summer conditions
- Determine the extent of the reduction of NoV levels using UV treatment.

# 2 Materials and Methods

## 2.1 Laboratory Methods

### *2.1.1 Concentration of wastewater samples for NoV and FRNA bacteriophage (GA) real-time RT-qPCR 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 2.5 M $MgCl_2$ (Sigma–Aldrich, UK) were added to a single, 40-ml sample volume of wastewater to obtain a final concentration of 25 mM $MgCl_2$. The sample was then adjusted to between pH 3.5 and pH 6.0 with 1 M HCl (Sigma–Aldrich) and mixed on a rocking platform for 45 min. 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 90 mm diameter; Millipore) attached to a plastic magnetic filter holder (Pall, Port Washington, NY). The filters were then washed once using 25 ml 0.14 M 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 rev./min 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 4,000 × *g* for 10 min. The filter unit was washed in 550 µl of molecular biology grade water and the virus concentrate (>500 µl) was stored at –20°C prior to RNA extraction.

### *2.1.2 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 analysed for *E. coli* and FRNA bacteriophage within 24 h 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 were homogenised using a blender and diluted 1:3 with 0.1% (w/v) neutralised bacteriological peptone (Oxoid, Cambridge, UK) (Anonymous, 2003). For FRNA bacteriophage analysis, 50 ml of the diluted homogenate were centrifuged at 2,000 × *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 finely chopped. Two grams of oyster hepatopancreas were weighed to which 2 ml of 100 µg/ml Proteinase K solution (30 U/mg; Sigma-Aldrich) were added. Fifty microlitres of Mengo virus strain $MC_0$ were 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. (2006). The sample was then incubated at 37°C, with shaking at 150 rev./min 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 supernatants were either stored at 4°C prior to RNA extraction within 24 h, or stored at –80°C where RNA extraction was undertaken within 1 month.

### *2.1.3* **E. coli** *enumeration in wastewater and oysters*

Appropriate $log_{10}$ dilutions of untreated influent and treated effluent wastewater samples, respectively, and diluted oyster homogenates were assayed for *E. coli* using a standardised 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 h. The presence of *E. coli* was subsequently confirmed by subculturing tubes indicating acid production onto Tryptone Bile X-glucuronide (TBX) agar (CM0945, Oxoid) at 44°C for 22 ± 2 h. The limit of detection (LOD) of the assay was an MPN of 20 *E. coli*/100 g and 20 *E. coli*/100 ml for shellfish and wastewater samples, respectively.

### 2.1.4 Enumeration of FRNA bacteriophage in wastewater and oysters using the plaque assay

The diluted wastewater samples and oyster homogenate were analysed for FRNA bacteriophage using a standardised 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 (>$10^6$ cfu/ml). 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. The LOD of the assay for shellfish and wastewater samples was 30 pfu/100 g and 10 pfu/100 ml, respectively.

### 2.1.5 Genotyping of FRNA bacteriophage using oligonucleotide probes

All FRNA bacteriophage plaques detected in each wastewater and shellfish sample were transferred using an applicator stick onto Hybond N+ nylon transfer membranes (Amersham). Genotyping was carried out as described by Sundram et al. (2006), with some modifications. Briefly, membranes containing FRNA bacteriophage were submerged in denaturing solution (0.05 M NaOH, 0.15 M NaCl) for 1 min followed by neutralisation in 0.1 M sodium acetate for 5 min prior to fixation of coliphage RNA by baking at 80°C for 30 min. RNA hybridisation was carried out using DIG Easy Hyb (Roche) and the membrane was probed with a 5-pmol/ml digoxigenin (DIG)-labelled oligonucleotide probe (5'-AATCGTTCAGGAAGTGA GATTCAAAACC-3') designed by Beekwilder et al. (1996) (Eurogentec). Washing and blocking of the membranes were carried out using the DIG wash and block buffer set (Roche), followed by detection using the DIG Nucleic Acid Detection Kit (Roche) according to the manufacturer's instructions. GA bacteriophage plaques were confirmed as dark purple areas on each membrane and were expressed as GA pfu/100 ml.

### 2.1.6 NoV and GA 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.

### 2.1.7 RT-qPCR controls and standards for NoV and GA real-time assays

Plasmids carrying the NoV GI and GII target sequences (supplied by Dr Francoise 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 double-stranded (ds) DNA 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 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) and were divided into single-use aliquots of $10^7$ genome copies/µl for NoV GI and GII for use in determining RT-PCR inhibition. For quantitation of FRNA bacteriophage GA, PCR products from the real-time RT-qPCR were purified using the Wizard® SV PCR Purification kit (Promega) and, following a tailing procedure, were inserted into pGEM-T Easy vector prior to transformation in *E. coli*. Transformant clones were screened and purified using the PureYield™ plasmid miniprep kit (Promega). Quantitation of plasmid DNA was carried out on a nanodrop spectrophotometer at 260 nm and single-use aliquots containing $10^5$ genome copies/µl were prepared for quantitation in the RT-qPCR. For FRNA coliphage GA,

the TaqMan Exogenous (EXO) IPC kit (Applied Biosystems) was used to control for RT-qPCR inhibition. The dsDNA and EC RNA standards were stored at –20°C.

### *2.1.8 Determination of FRNA bacteriophage GA, NoV GI and GII using one-step RT-qPCR*

For FRNA bacteriophage and NoV GI and GII analysis of wastewater and shellfish samples, duplicate 5-µl aliquots of sample RNA were 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 an RNA Ultrasense one-step RT-qPCR system (Invitrogen, Carlsbad, CA) containing 1 × reaction mix, 500 nM forward primer, 900 nM reverse primer, 250 nM probe, 1 × µl Rox and 1.25 µl of enzyme mix. The reaction mix for FRNA bacteriophage was the same as for NoV, with the inclusion of 2.5 µl EXO IPC mix followed by 0.5 µl of IPC DNA (Applied Biosystems, Foster City, CA, USA). 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. For FRNA bacteriophage GA, primer and probes were the same as those used to detect genogroup II FRNA bacteriophage by Wolf et al. (2008). For NoV analysis, the plate was incubated at 55°C for 60 min, 95°C for 5 min and then 45 cycles of 95°C for 15 s, 60°C for 1 min and 65°C for 1 min on an AB7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). For FRNA bacteriophage GA analysis, reaction conditions were the same as used by Wolf et al. and involved an initial incubation at 55°C for 60 min, followed by 95°C for 5 min, and then 45 cycles of 58°C for 1 min (Wolf et al., 2008). In addition, no template controls were included for each target virus on the 96-well plate.

To control for the presence of RT-PCR inhibitors, 5 µl of sample RNA were added to a further two wells to which 1 µl of EC RNA ($10^7$ genome copies/µl) was added. A log dilution series of the NoV GI and GII EC RNA ranging from $10^7$ to $10^4$ copies/µl was included on each RT-qPCR run. The mean $C_T$ 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. To control for PCR inhibitors in the FRNA bacteriophage GA assay, sample $C_T$ values for the EXO IPC target were compared with two wells containing only IPC DNA and reaction mix. 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 were 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 5-µl aliquots of sample or extraction control RNA were added to the adjacent wells of the 96-well plate. The forward (Mengo209) and reverse (Mengo110) primers and probe (Mengo147) used were the same as those described by Pintó et al. (2009). The $C_T$ 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 viral RNA in genome copies/µl, a log dilution series of the GI and GII DNA plasmids (range $1 \times 10^0$ to $1 \times 10^5$ copies/µl) was included in duplicate on each RT-qPCR run. The number of RNA copies in NoV-positive samples was determined by comparing the $C_T$ value with the standard curves. The final concentration was then adjusted to reflect the volume of RNA analysed and was expressed as detectable virus genome copies per gram hepatopancreas or detectable virus genome copies/100 ml wastewater. The theoretical LOD for FRNA bacteriophage and NoV GI and GII was 20 detectable virus genome copies/g and 25 detectable virus genome copies/100 ml for shellfish and wastewater samples, respectively.

### *2.1.9 NoV sequencing*

#### *2.1.9.1 Nested RT-PCR and cloning*

Viral RNA was extracted from 500 µl of wastewater concentrate using the NucliSENS® miniMAG® extraction platform and NucliSENS® magnetic extraction reagents (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. A negative RNA extraction control (molecular biology grade water) was included alongside each batch of samples to be extracted. Prior to nested RT-PCR, each shellfish sample was extracted at three different dilutions of shellfish proteinase K extract (neat, 1:2, and 4:1) using phosphate-buffered saline (Oxoid, Basingstoke, UK). Viral RNA was eluted into 30 µl of elution buffer and stored at –80$^{o}$C until further analysis.

To reduce the occurrence of non-specific amplification, 8 µl of the RNA were treated with 1 U of RNase-Free DNase (Promega) according to the manufacturer's protocol. Reverse transcription (RT) was performed by adding 15 µl of DNase-treated RNA to 15 µl of the RT master mix as described by Nishida et al. (2007). Five microlitres of cDNA were added to 45 µl of a reaction mixture consisting of 10 mM Tris–HCl (pH = 8.3), 50 mM KCl, 20 µM of dNTPs, 2 µM of each primer, 2.5 U of AmpliTaq® DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and 2.5 mM of $MgCl_2$. The primers used for the NoV GI and GII reactions were COG1F, G1-SKR and COG2F, G2-SKR, respectively (Kojima et al., 2002; Kageyama et al., 2003). One microlitre of the first round PCR product was added to 49 µl of a reaction mixture containing 10 mM Tris–HCl (pH = 8.3), 50 mM KCl, 20 µM of dNTPs, 0.4 µM of each primer, 2.5 U of AmpliTaq® DNA Polymerase and 2.5 mM of $MgCl_2$. In the nested RT-PCR, the pair of primers used were GISKF, GISKR for NoV GI and GIISKF, GIISKR for NoV GII (Kojima et al., 2002). The PCR was performed under the following conditions: an initial denaturation at 95°C for 5 min, 40 amplification cycles with denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, and a final extension of 72°C for 15 min. The size of the amplified DNA fragments was confirmed by gel electrophoresis (2% agarose) and then purified using CHROMA SPIN™ columns (Unitech, Dublin, Ireland). The PCR products were cloned into the pCR®4-TOPO® vector according to the protocol for the TOPO TA Cloning® Kit (Invitrogen), and from four to six clones each from the NoV GI and NoV GII specific PCR products (for each sample) were randomly selected for DNA sequencing.

#### *2.1.9.2 DNA sequencing and phylogenetic analysis*

The amplicons were purified using the QIAquick PCR purification kit (Qiagen) and the nucleotide sequence was determined by an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems) using the BigDye Terminator v 3.1 Ready Reaction kit (Applied Biosystems) according to the manufacturer's protocol. Sequences were aligned using the ClustalW algorithm of the MegAlign (DNAstar, Inc., Madison, WI, USA) software and compared with reference strains retrieved from GenBank. The genotype nomenclature of the reference strains has been adopted from the NoV genotyping tool (National Institute of Public Health and the Environment, the Netherlands[1]). A maximum likelihood phylogenetic tree was constructed using PAUP* version 4.0 (Swofford, 2003) and the model of DNA evolution was chosen based on the hierarchical likelihood ratio test using the Modeltest program (Posada and Crandall, 1998). The maximum likelihood phylogenetic tree was based on the TrN+I+G model of substitution for NoV GI and on the TrNef+I+G for NoV GII. The reliability of the generated trees was estimated by bootstrap analysis of 1,000 replicates of the sequence alignment using the neighbour-joining method. Sequences described in the study have GenBank accession numbers JQ362499–JQ362594.

## 2.2 Study Design

### *2.2.1 Monitoring at WWTP1*

#### *2.2.1.1 WWTP and wastewater sampling*

WWTP1 treated wastewater from a population equivalent (PE) of approx. 90,000 and received an average daily volume of incoming wastewater of 45,000 m$^3$. Preliminary treatment at the plant provided screening and grit removal. This was followed by primary WWT and conventional activated sludge treatment (aeration and final settlement). The final treated wastewater effluent was discharged into the

1. http://www.rivm.nl/mpf/norovirus/typingtool

sea through a 400-m-long outfall pipe at a depth of 10 m.

One litre, 24-h composite samples of influent and final effluent were taken on a weekly basis. In addition, a 1-l grab sample of wastewater was collected following primary treatment. All wastewater samples were collected in reusable polyethylene bottles and transported under ambient temperatures to the laboratory within 1 h of collection. Prior to use, polyethylene bottles were sterilised by autoclaving and washed using the laboratory glass washer. Wastewater sampling commenced in June 2009 and ended in May 2010 (n = 49).

#### *2.2.1.2 Oyster sampling*

Pacific oysters (*Crassostrea gigas*) from a batch previously demonstrated to be free from microbial contamination (*E. coli*, FRNA bacteriophage and NoV) were suspended in mesh bags at a depth 1 m below the water surface directly above the WWTP outfall. From previous trials, a depth of 1 m was chosen as it was logistically manageable whilst providing similar levels of microbial contamination to oysters suspended at depths up to 10 m. Oysters were deployed at the outfall for 1 month before sampling commenced. Samples of 24 oysters were collected each week and transported to the laboratory within 2 h, under chilled conditions (<15°C). Each week oyster samples were collected 2 days after the wastewater samples were collected. Oyster sampling commenced in June 2009 and ended in May 2010 (n = 38).

### *2.2.2 Monitoring at WWTP2*

#### *2.2.2.1 WWTP and Wastewater sampling*

WWTP2 treated wastewater from a PE of 10,000 and received an average daily volume of incoming wastewater of 13,500 $m^3$. Preliminary treatment at the plant provided screening and grit removal and this was followed by phosphate removal through the addition of ferric sulphate. Biological treatment was carried out in four sequencing batch reactors. The biologically treated effluent was then passed through a Trojan UV 3000 disinfection unit and discharged to estuarine waters. One-litre, 24-h composite samples of influent, secondary-treated and UV-disinfected final effluent were taken on a weekly basis. All wastewater samples were collected in high-density polyethylene bottles and transported under chilled temperatures (<15°C) to the laboratory within 24 h of collection. The WWTP was newly constructed and began treating wastewater, following commissioning, in December 2010. Following this, the plant underwent full commissioning in January and February 2011. Wastewater sampling commenced in January 2011 and ended in June 2011 (n = 24). A subset of influent, secondary-treated and UV-disinfected final effluent samples were also analysed for GA FRNA bacteriophage by the infectivity assay and RT-qPCR to compare the concentration of infectious and total GA FRNA bacteriophage present (n = 10).

#### *2.2.2.2 Measurement of physicochemical parameters*

In addition to flow rates for influent and effluent (measured using electromagnetic flow meters), physicochemical parameters were measured by the plant operators for the untreated influent and UV-disinfected final effluent. The analyses were performed according to *Standard Methods for the Analysis of Water and Wastewater* by the American Public Health Association (APHA, 2005). Suspended solids analysis was carried out using a filtration method. Chemical oxygen demand (COD) analysis was performed by colorimetric determination using the potassium dichromate method. Biological oxygen demand (BOD) analysis was carried out according to Standard Method 5210:B. Total nitrogen analysis was carried out using the persulphate digestion method. Salinity was recorded for all influent and effluent samples using a conductivity probe with a HACH Lange HQ30D meter in the WWTP laboratory

#### *2.2.2.3 Oyster sampling*

Pacific oysters (*C. gigas*) from a commercial harvesting area located approximately 400 m upstream of the WWTP outfall were monitored for *E. coli*, FRNA bacteriophage and NoV GI and GII on a weekly basis throughout the study. Samples of 24 oysters were collected each week and transported to the laboratory within 24 h, under chilled conditions (<15°C). Each week, oyster samples were collected on the same day as the wastewater samples were collected. Oyster sampling commenced in January 2011 and ended in June 2011 (n = 25). A subset of oysters was also analysed for GA FRNA bacteriophage by the infectivity assay and RT-qPCR to

compare the concentration of infectious and total GA FRNA bacteriophage present.

#### 2.2.2.4 CSO monitoring

During the period from October to December 2011, monitoring of overflow events from the CSO at the WWTP was carried out. The total volume of wastewater discharged for each CSO discharge event was recorded by the WWTP operators. Where a CSO discharge event was detected in real time, a 1-l sample of the CSO discharge effluent was collected. Over the same period, 1-l, 24-h composite samples of UV-disinfected final effluent were taken on a fortnightly basis to determine background concentrations discharged into the marine environment. All wastewater samples were collected in high-density polyethylene bottles and transported under chilled temperatures (<15°C) to the laboratory within 24 h of collection. All wastewater samples were analysed for NoV GI and GII, and GA FRNA bacteriophage by the infectivity assay and RT-qPCR.

During this period, Pacific oysters (*C. gigas*) from a commercial harvesting area located approximately 400 m upstream of the WWTP outfall were monitored for NoV GI and GII fortnightly. Samples of 24 oysters were collected and transported to the laboratory within 24 h, under chilled conditions (<15°C). Additional intensive sampling of oysters was also undertaken in response to CSO discharges.

### 2.2.3 UV disinfection studies at model WWTP

As part of the NoV study, a pilot disinfection system was examined at a municipal WWTP. The UV disinfection unit used at the pilot WWTP was an AquaPro UV 12GPM-HTM (Taiwan, Republic of China). This unit provides a lamp power of 30 mW/s/cm$^2$ (30 mJ) at a wavelength of 253.7 nm and was placed downstream of a sand/anthracite filter. The unit was operated under flow conditions of between 0.3 m$^3$/h and 0.45 m$^3$/h. The UV disinfection unit treated wastewater that had previously undergone biological treatment in a series of horizontal flow, fixed-film reactors. Triplicate samples (1 l in polypropylene bottles) were taken at the influent (post-secondary treatment), post-sand-filter and post-UV-disinfection stages and transported to the laboratory within 2 h.

### 2.2.4 Sea-water survival studies

Monthly solar radiation data for four locations in Ireland were obtained from Met Éireann and were used to determine the mean daily solar radiation that occurs during the summer and winter months. Similarly, mean sea-water temperatures for Irish waters during the summer and winter periods were obtained from Met Éireann. Typical summer conditions were identified as having a daily solar radiation dosage of 1,939 J/cm$^2$ and a mean temperature of 17°C, while typical winter conditions consisted of a daily dosage of 272 J/cm$^2$ and a mean temperature of 10°C.

The solar simulator used in the survival studies was an LOT Oriel Low-cost solar simulator (LOT-Oriel, Surrey, UK) that produced a uniform beam of solar-simulated light. In addition, an air-mass 1.5 global filter was applied to mitigate the lamp output in accordance with typical sunlight intensities received at latitudes similar to that of Ireland. To determine the light intensity, a reference solar cell (LOT-Oriel, Surrey, UK) was calibrated to express irradiance received at the sample surface as a voltage using a digital multimeter (with 1,000 W/m$^2$ equivalent to 89.7 mV). Summer solar radiation of 1,939 J/cm$^2$ equated to an irradiance of 224 W/m$^2$ applied over 24 h and winter solar radiation of 272 J/cm$^2$ equated to an irradiance of 31 W/m$^2$ applied over 24 h.

As it was not possible to reduce the output of the solar simulator lamp, a collimated beam apparatus was set up with the distance from the lamp being used to vary the irradiance applied to the sample surface. A collimated beam of simulated sunlight was achieved using a polypropylene tube that had been previously coated in non-reflective matt-black paint. It was not possible to achieve the desired irradiances; instead, representative dosages of 235 W/m$^2$ and 55 W/m$^2$ were applied for summer and winter experiments, respectively. The sea-water survival experiments were carried out in the absence of light in a refrigerated room at temperatures of 17°C and 10°C for summer and winter experiments, respectively.

A 400-ml sample of sea water (0.10–0.19 NTU) was placed in a beaker at a distance from the solar simulator to achieve the desired irradiance at the surface of the water. The water was magnetically

stirred throughout the experiments and the distance from the light source was maintained constant using a laboratory jack placed underneath the magnetic stirrer to mitigate volume loss upon sampling. Alongside were 400 ml of sea water in dark conditions as a control and were to be sampled at the same intervals as the irradiated sea water. A 1-ml volume of spiking material (wastewater, NoV GII-positive faecal material or FRNA bacteriophage stock culture) was added to 400 ml sea water and, at each sampling time, 10 ml were taken for infectivity analysis while 40 ml were taken for real-time RT-qPCR analysis. For the infectivity assay, samples were taken at time zero and thereafter every 10 min and every 2 h for the summer and winter experiments, respectively. For the real-time RT-qPCR assay, samples were taken at time zero and thereafter every 24 h and every 3 days for the summer and winter experiments, respectively The measured biological response to simulated sunlight was loss of infectivity (ability to form plaques), which was quantified as described in Section 2.1.4. A linear regression line was fitted to concentrations of FRNA bacteriophage at the different sampling occasions with the line equation used to derive the $T_{90}$ values.

## 2.3 Statistical Analysis and Determination of Microbial Log Reductions

To determine the mean concentration of microbial parameters in wastewater and oysters, a value of half the LOD for the assay was applied to negative samples. The average log reduction achieved by a treatment was determined by subtracting the mean concentration post-treatment from the mean concentration prior to treatment.

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

# 3 Results

## 3.1 Monitoring of WWTP1 Providing Secondary Treatment June 2009–May 2010

### *3.1.1 Concentrations of microbial parameters detected in wastewater*

The mean *E. coli*, FRNA bacteriophage, NoV GI and GII concentrations detected in influent, post-primary and post-secondary-treated effluent samples during year-long monitoring at WWTP1 (n = 49) are shown in Table 3.1. The mean concentration of *E. coli* in influent samples (6.54 $\log_{10}$ MPN/100 ml) was reduced by 1.49 $\log_{10}$ (96.6%) during the entire treatment process to give a mean concentration of 5.06 $\log_{10}$ MPN/100 ml in effluent samples. Mean FRNA bacteriophage concentrations were reduced by 2.13 $\log_{10}$ pfu/100 ml (99.2%) during the entire treatment process, with mean concentrations of 5.54, 5.46 and 3.41 $\log_{10}$ pfu/100 ml 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 concentrations in influent and effluent wastewater ($r < 0.07$ in all instances).

NoV GI and GII were detected in influent and effluent wastewater on all sampling occasions throughout the year-long sampling period. Mean concentrations of NoV GI and GII detected in influent wastewater were 3.32 and 3.55 $\log_{10}$ genome copies/100 ml, respectively. Mean concentrations of NoV GI and GII detected in effluent wastewater were 2.53 and 2.63 $\log_{10}$ genome copies/100 ml, respectively. The mean concentration of NoV GII was 0.23 $\log_{10}$ virus genome copies/100 ml greater than the mean NoV GI $\log_{10}$ concentration in influent wastewater. This difference was significant ($P < 0.05$).

$\text{Log}_{10}$ reductions for all microorganisms ranged from 0.15 (NoV GI and GII) to 0.32 (FRNA bacteriophage) $\log_{10}$ units following primary treatment (Table 3.1). The mean NoV GI and GII reduction during the entire treatment process was 0.79 $\log_{10}$ (83.8%) and 0.92 $\log_{10}$ (87.9%) virus genome copies/100 ml,

**Table 3.1. Mean $\log_{10}$ concentrations of *E. coli*, FRNA bacteriophage and NoV GI and GII during wastewater treatment stages at WWTP1.**

| n = 49 | Wastewater treatment stage | | | | | |
|---|---|---|---|---|---|---|
| | Influent | Post-primary settlement | | Final effluent | | |
| | Concentration ± SD (range) | Concentration ± SD (range) | Reduction[1] | Concentration ± SD (range) | Reduction[2] | Percent reduction |
| ***Escherichia coli* (MPN/100 ml)** | 6.54 ± 0.59 (3.73–7.54) | 6.38 ± 0.51 (4.54–7.38) | 0.16 | 5.06 ± 0.58 (3.54–6.20) | 1.49 | 96.8 |
| **FRNA bacteriophage (pfu/100 ml)** | 5.54 ± 0.51 (3.87–6.82) | 5.23 ± 0.55 (3.41–5.96) | 0.32 | 3.41 ± 0.77 (2.00–5.84) | 2.13 | 99.2 |
| **NoV GI (copies/100 ml)** | 3.32 ± 0.64 (2.05–4.76) | 3.17 ± 0.71 (1.62–4.57) | 0.15 | 2.53 ± 0.57 (1.26–4.06) | 0.79 | 83.8 |
| **NoV GII (copies/100 ml)** | 3.55 ± 0.89 (1.81–5.34) | 3.40 ± 0.84 (1.46–5.51) | 0.15 | 2.63 ± 0.71 (1.51–4.08) | 0.92 | 87.9 |

[1]The reduction shown is based on the difference between the mean $\log_{10}$ concentration in influent samples and the mean $\log_{10}$ concentration in post-primary settlement samples.
[2]The reduction shown is based on the difference between the mean $\log_{10}$ concentration in influent samples and the mean $\log_{10}$ concentration in final effluent samples and therefore represents the total average reduction provided by the entire treatment process.

respectively. Although the mean $\log_{10}$ 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).

### *3.1.2 Seasonal variation in NoV concentrations*

NoV GI and GII concentrations detected in the influent wastewater during the winter period (January–March) were significantly higher (P < 0.05) than during the rest of the year (Table 3.2). Mean concentrations of NoV GI and GII in the influent wastewater for the period January–March inclusive (n = 12) were 0.82 and 1.41 $\log_{10}$ virus genome copies/100 ml 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–March period (Table 3.2). 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 0.67 $\log_{10}$ higher in influent wastewater and 0.47 $\log_{10}$ higher in effluent wastewater than NoV GI concentrations. Whilst the mean difference between NoV GI and GII concentrations at this time of year was significant (P < 0.05) no significant difference was detected between NoV GI and GII concentrations during the rest of the year (April–December). Unlike NoV concentrations, no seasonal trend was detected in the concentration of FRNA bacteriophage or *E. coli* in wastewater influent or effluent.

### *3.1.3 Oysters*

Mean FRNA bacteriophage and *E. coli* concentrations detected in oysters throughout the study period were 4.14 $\log_{10}$ pfu/100 g (SD ± 0.64) and 3.22 $\log_{10}$ MPN/100 g (SD ± 0.55), respectively. On a sample-by-sample basis, *E. coli* concentrations in oysters did not correlate with concentrations of NoV GI ($r$ = –0.097; P = 0.57) or GII ($r$ = 0.184; P = 0.26). Similarly, FRNA bacteriophage concentrations did not correlate with NoV GI ($r$ = 0.015; P = 0.93) or GII ($r$ = 0.252; P = 0.127). 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 Fig. 3.1. 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, respectively (Table 3.3). NoV detected in oyster samples displayed a strong seasonal trend with significantly higher

**Table 3.2. Mean log10 concentrations of NoV GI and GII in influent and effluent wastewater at WWTP1 by season.**

| NoV genogroup | Season (n) | Mean concentration ($\log_{10}$ genome copies/100 ml) ± SD | |
|---|---|---|---|
| | | Influent | Effluent |
| **GI** | **April–December** (37) | 3.12 ± 0.55 | 2.32 ± 0.68 |
| | **January–March** (12) | 3.94 ± 0.49 | 3.06 ± 0.55 |
| **GII** | **April–December** (37) | 3.20 ± 0.71 | 2.27 ± 0.39 |
| | **January–March** (12) | 4.61 ± 0.41 | 3.53 ± 0.65 |

**Table 3.3. Mean $\log_{10}$ NoV concentrations in oysters by season.**

| | Mean concentration ($\log_{10}$ virus genome copies/g) ± SD | |
|---|---|---|
| **Season (n)** | NoV GI | NoV GII |
| **All data (38)** | 3.53 ± 0.87 | 3.73 ± 0.55 |
| **April–December (26)** | 3.12 ± 0.68 | 3.21 ± 0.56 |
| **January–March (12)** | 4.43 ± 0.50 | 4.86 ± 0.54 |

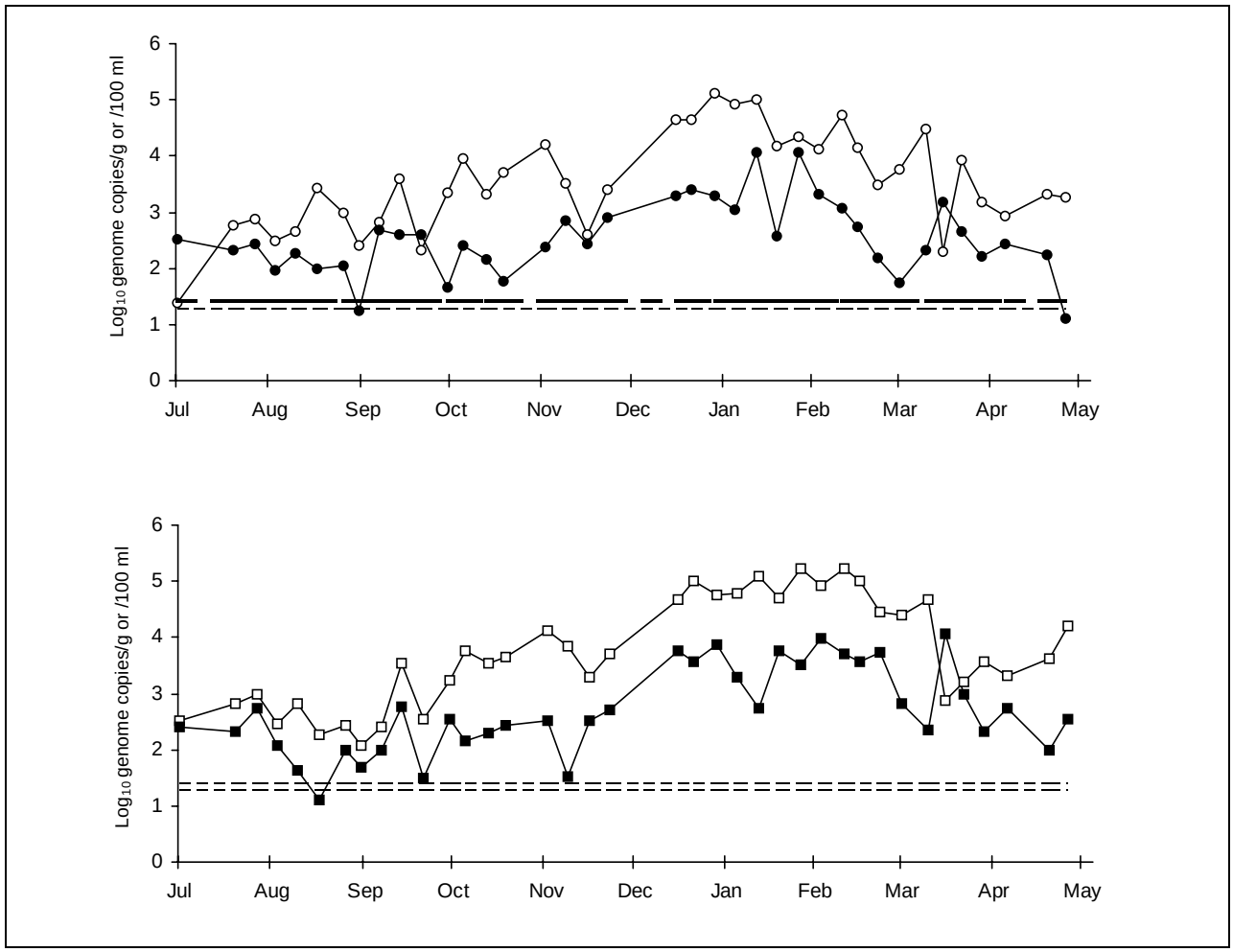


**Figure 3.1. Concentrations of NoV GI (upper graph) and NoV GII (lower graph) detected in oysters and effluent wastewater. Concentrations of NoV GI (○) and NoV GII (□) detected in oysters are expressed as $log_{10}$ genome copies/gram and concentrations of NoV GI (●) and NoV GII (■) in effluent are expressed as $log_{10}$ genome copies/100 ml. Dashed lines indicate the limit of detection for wastewater analysis (upper line) and shellfish (bottom line).**

concentrations ($P < 0.05$) in the winter (January–March) compared with the rest of the year. Mean concentrations of NoV GI and GII detected during this period were 1.31 and 1.65 $log_{10}$ virus genome copies/g greater than concentrations detected during the rest of the year, respectively. Mean $log_{10}$ concentrations of NoV in oysters were significantly correlated with concentrations detected in effluent wastewater on a weekly basis (NoV GI: $r = 0.48$, $P < 0.05$; NoV GII $r = 0.68$, $P < 0.05$).

### *3.1.4 NoV genotype distribution in wastewater and oysters*

Over a 5-week period (3 February–10 March 2010), a wide variety of NoV genotypes (both GI and GII) were detected in weekly samples of influent, effluent and oysters (Fig. 3.2). In total, five GI and seven GII genotypes were detected during the period. NoV GI.7 was the predominant NoV GI genotype present in untreated wastewater during this period and was present in all influent wastewater samples. NoV GI.4 was detected in two untreated wastewater samples. Despite this, NoV GI.4 was the predominant GI genotype detected in oysters and was detected in all five oyster samples, whereas NoV GI.7 was detected in just one oyster sample. NoV GII.4 and GII.12 were the predominant NoV GII genotypes detected in oysters and were both detected in all five oyster samples.

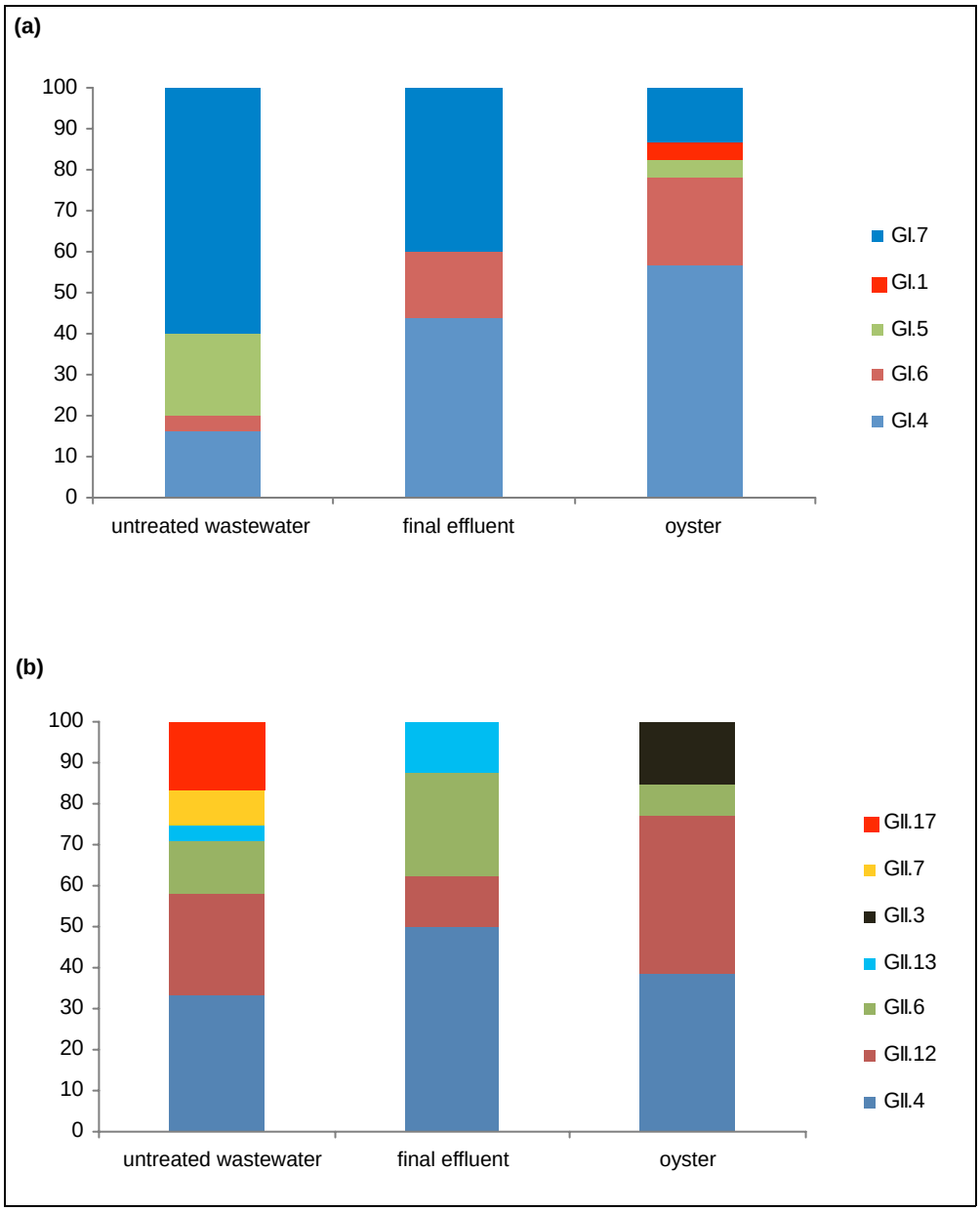


**Figure 3.2. NoV genotypes present in untreated wastewater, final effluent and oysters at WWTP1. A sample of wastewater, final effluent and oysters was taken each week over a 5-week period (3 February–10 March). Between four and six clones were sequenced for NoV GI and GII for each sample. Data presented are the clones characterised as a particular genotype as a percentage of all clones successfully characterised for each sample type for NoV GI (a) or NoV GII (b).**

## 3.2 Monitoring of Secondary-Treated Wastewater with UV Disinfection (WWTP2) January–June 2011

### *3.2.1 Plant operation*

WWTP2 was newly constructed and became fully operational in December 2010. Following this, the plant underwent full commissioning and performance testing in January and February 2011. Physicochemical parameter concentrations and details of CSO discharge events for the plant over the study period (January–June 2011) were collected by the WWTP operators and made available to the project retrospectively to the monitoring period. These data are presented in Table 3.4. Frequent CSO discharges were recorded in January, February and May 2011,

**Table 3.4. Mean monthly levels for physicochemical parameters recorded in influent and final effluent wastewater at WWTP2.**

| Month | No. of overflows (volume $m^3$)[1] | Wastewater | pH | BOD | COD | Suspended solids | Salinity | Total phosphorus | Total nitrogen | Ammonium |
|---|---|---|---|---|---|---|---|---|---|---|
| **January** | 17 | **Influent** | 7.20 | 75.10 | 152.21 | 65.84 | 5382 | 2.36 | 15.98 | 11.87 |
| | (31,572) | **Final effluent** | 7.28 | 23.84[2] | 89.07 | 54.56[2] | n.a. | 1.53 | 14.50 | 10.50 |
| | | **% reduction** | –1.11 | 68.26 | 41.48 | 17.13 | n.a. | 35.17 | 9.26 | 11.54 |
| **February** | 11 | **Influent** | 7.34 | 79.13 | 150.12 | 73.57 | 4651 | 2.26 | 17.83 | 9.90 |
| | (11,106) | **Final effluent** | 7.25 | 27.12[2] | 72.32 | 39.74[2] | 5385 | 1.23 | 12.91 | 8.73 |
| | | **% reduction** | 1.23 | 65.73 | 51.83 | 45.98 | –15.78 | 45.58 | 27.59 | 11.82 |
| **March** | 0 | **Influent** | 7.32 | 155.14 | 241.86 | 127.92 | 4494 | 3.82 | 24.87 | 16.68 |
| | (0) | **Final effluent** | 7.30 | 11.57 | 62.71 | 11.90 | 4753 | 0.60 | 15.07 | 13.90 |
| | | **% reduction** | 0.27 | 92.54 | 74.07 | 90.70 | –5.76 | 84.29 | 39.40 | 16.67 |
| **April** | 3 | **Influent** | 7.32 | 153.67 | 342.15 | 103.00 | 5806 | 3.54 | 26.04 | 17.41 |
| | (716) | **Final effluent** | 7.28 | 8.14 | 84.69 | 20.16 | 5831 | 0.68 | 16.93[2] | 16.06 |
| | | **% reduction** | 0.55 | 94.70 | 75.25 | 80.43 | –0.43 | 80.79 | 34.98 | 7.75 |
| **May** | 8 | **Influent** | 7.29 | 91.75 | 304.00 | 109.15 | 3788 | 2.86 | 21.80 | 16.48 |
| | (19,928) | **Final effluent** | 7.29 | 6.00 | 69.16 | 10.73 | 3740 | 0.44 | 14.64 | 14.32 |
| | | **% reduction** | 0.00 | 93.46 | 77.25 | 90.17 | 1.27 | 84.62 | 32.84 | 13.11 |
| **June** | 2 | **Influent** | 7.22 | 174.20 | 296.23 | 164.21 | 4891 | 4.00 | 26.22 | 21.08 |
| | (1,302) | **Final effluent** | 7.25 | 6.60 | 75.89 | 15.50 | 4410 | 0.50 | 19.00[2] | 17.78 |
| | | **% reduction** | –0.42 | 96.21 | 74.38 | 90.56 | 9.83 | 87.50 | 27.54 | 15.65 |

[1]Total volume of dilute untreated effluent discharged from CSO events during the whole month.
[2]Months where mean values failed to meet the target value are highlighted in red. Target values for physicochemical parameters of final effluent: COD (125 mg/l), BOD (20 mg/l), suspended solids (30 mg/l), total nitrogen (15 mg/l), total phosphorus (2 mg/l), pH (6–9), n.a.= not available. It is important to note that the target values are those in the design criteria for the plant and may differ from values prescribed in the Urban Wastewater Treatment Regulations 2001–2010 or EPA licence conditions.

discharging a high volume of untreated diluted wastewater and storm water during those periods. These overflows were not associated with high rainfall events and could be considered an indication of teething problems at the newly commissioned WWTP. In addition, mean monthly levels of physicochemical parameters recorded at the plant failed to meet target values on six occasions (BOD, January and February; suspended solids, January and February; total nitrogen, April and June). It is therefore clear that the WWTP operation was suboptimal during the study period, particularly during the months of January and February, when excessive CSO discharges occurred. The BOD and suspended solid exceedance coincided with the full commissioning and performance testing at the new wastewater treatment plant in January 2011. Subsequently, all physicochemical parameters were within the target limits, apart from a total nitrogen value in June 2011.

### *3.2.2 Concentrations of microbial parameters detected in wastewater*

*E. coli*, FRNA bacteriophage, NoV GI and GII concentrations detected in influent, post-secondary and UV-treated effluents (n = 24) are shown in Table 3.5 and Fig. 3.3. *E. coli* and FRNA bacteriophage were detected in all influent wastewater samples throughout the 6-month study period. The mean $\log_{10}$ *E. coli* concentration in influent samples was 6.18 $\log_{10}$ MPN/100 ml (range: 3.90–7.11 $\log_{10}$ MPN/100 ml). This was reduced to a mean concentration of 2.81 $\log_{10}$ MPN/100 ml in samples of the final effluent, demonstrating an average $\log_{10}$ reduction of 3.37 $\log_{10}$ MPN/100 ml during the entire treatment process. The average reduction of FRNA bacteriophage across the entire treatment process was 2.63 $\log_{10}$ pfu/100 ml, with mean concentrations of 5.32, 3.81 and 2.69 $\log_{10}$ pfu/100 ml detected in influent, secondary-treated and UV-treated effluent, respectively. The $\log_{10}$ reductions for *E. coli* and FRNA bacteriophage achieved by UV treatment alone were 1.92 and 1.12, respectively.

NoV GI and GII were detected in 61% and 95% of influent wastewater samples, respectively. All samples containing NoV GI also contained NoV GII. The mean concentration of NoV GII was 1.56 $\log_{10}$ greater than NoV GI in influent wastewater ($P < 0.001$). NoV GII was detected in 82% of secondary-treated effluent samples, with both NoV GI and GII detected in 66% (n = 14) of samples. NoV GI was not detected in any sample in isolation. In secondary-treated samples, the mean concentration of NoV GII was 1.39 $\log_{10}$ greater than mean concentrations of NoV GI ($P < 0.001$). NoV GII was detected in 90% of UV-disinfected final effluent samples, with both NoV GI and GII detected in 66% of samples. NoV GI was not detected in any secondary-treated sample in isolation. The mean concentrations of NoV GI and GII in influent samples were 2.19 and 3.74 $\log_{10}$ genome copies/100 ml, respectively. In effluent samples, the mean concentrations of NoV GI and GII were reduced by 0.25 and 0.40 $\log_{10}$ to 1.94 and 3.34 $\log_{10}$ genome copies/100 ml, respectively, across the entire treatment process. This reduction was due to the secondary treatment process, with no reduction in NoV concentrations attributable to the UV disinfection process.

### *3.2.3 Oysters*

Mean FRNA bacteriophage and *E. coli* concentrations detected in oysters throughout the study period were 3.30 $\log_{10}$ pfu/100 g and 2.16 $\log_{10}$ MPN/100 g, respectively (Table 3.6). On a sample-by-sample basis, *E. coli* concentrations in oysters were not correlated with concentrations of NoV GI or GII. Similarly, FRNA bacteriophage concentrations did not correlate with NoV GI or GII. Mean NoV GI and GII concentrations detected in oysters over the 6-month monitoring period were 1.68 and 2.93 $\log_{10}$ virus genome copies/g, respectively (Table 3.6). NoV GI concentrations in oysters were low, generally below or close to the LOD of the RT-qPCR assay.

### *3.2.4 Relationship between NoV concentration in effluent and oysters*

In general, NoV GI concentrations were low in wastewater effluent (mean concentration 1.94 ± 0.77 $\log_{10}$ genome copies/100 ml) and oysters (mean concentration 1.68 ± 0.84 $\log_{10}$ genome copies/g), often below or close to the LOD of the assay, and the relationship with NoV GI concentration in wastewater effluent is not considered further. NoV GII concentrations in oysters and final effluent over the study period are presented in Fig. 3.4. NoV GII concentrations in final effluent peaked in March, with the maximum concentration of 4.79 $\log_{10}$ genome

**Table 3.5. Mean $log_{10}$ concentrations of *E. coli*, FRNA bacteriophage, NoV GI and GII during wastewater treatment stages and associated $log_{10}$ reductions at WWTP2.**

| | Wastewater | | | | | | |
|---|---|---|---|---|---|---|---|
| | Influent | Post-secondary treatment | | Post-UV disinfection | | Total $log_{10}$ reduction | Total % reduction |
| | Conc. ± SD (range) | Conc. ± SD (range) | $log_{10}$ reduction | Conc. ± SD (range) | $log_{10}$ reduction | | |
| ***E. coli*** | 6.18 ± 0.91 (3.90–7.11) | 4.73 ± 1.13 (3.54–6.20) | 1.45 | 2.81 ± 1.11 (<LOD–4.73) | 1.92 | 3.37 | 99.9 |
| **FRNA bacteriophage** | 5.32 ± 0.51 (4.59–6.41) | 3.81 ± 0.59 (2.54–4.95) | 1.51 | 2.69 ± 0.72 (<LOD–3.86) | 1.12 | 2.63 | 99.7 |
| **NoV GI** | 2.19 ± 1.03 (<LOD–4.04) | 1.89 ± 0.77 (<LOD–3.57) | 0.30 | 1.94 ± 0.77 (<LOD–3.69) | 0.00 | 0.25 | 43.8 |
| **NoV GII** | 3.74 ± 0.91 (<LOD–5.02) | 3.30 ± 1.19 (<LOD–5.18) | 0.44 | 3.34 ± 0.95 (<LOD–4.80) | 0.00 | 0.40 | 60.2 |

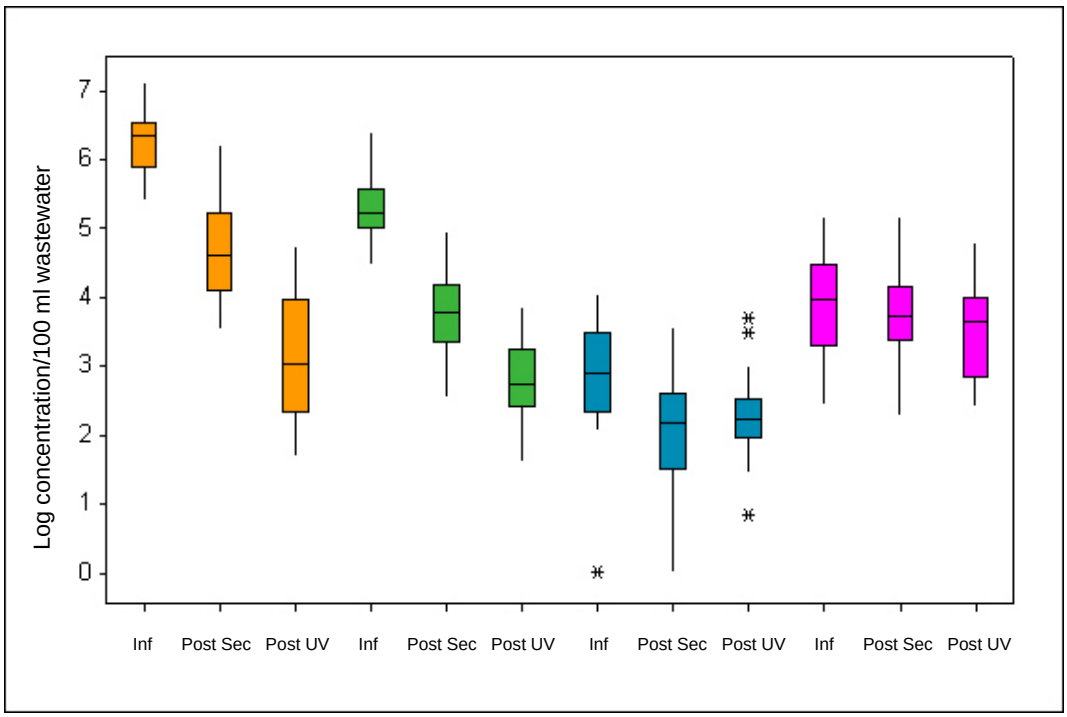


**Figure 3.3. Boxplots of $log_{10}$ concentrations of microbial parameters during wastewater treatment (WWTP2). *E. coli* MPN/100 ml (orange), FRNA bacteriophage pfu/100 ml (green), NoV GI (blue) and NoV GII (pink) genome copies/100 ml. Centre bar is the median value; the box is the lower and upper quartiles, respectively; the whiskers represent the range and asterisks represent outliers.**

**Table 3.6. Mean concentrations of microbial parameters in oysters at WWTP2.**

| | ***E. coli*** ($log_{10}$ MPN/100 g) | FRNA bacteriophage ($log_{10}$ pfu/100 g) | NoV GI ($log_{10}$ virus genome copies/g) | NoV GII ($log_{10}$ virus genome copies/g) |
|---|---|---|---|---|
| **Mean ± SD** | 2.16 ± 0.71 (<LOD–3.54) | 3.30 ± 0.39 (2.48–4.00) | 1.68 ± 0.84 (<LOD– 3.70) | 2.93 ± 1.08 (<LOD–4.10) |

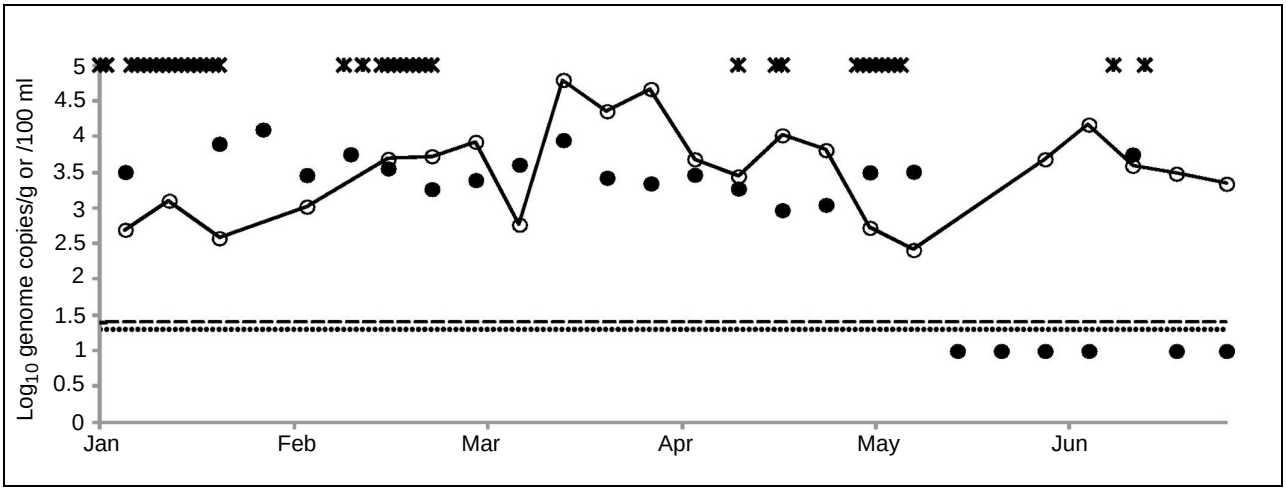


**Figure 3.4. Combined sewer overflow events and concentrations of NoV GII in wastewater effluent and shellfish from January to June 2011 $log_{10}$ concentrations of NoV GII in oysters (●) and wastewater effluent (○) and CSO discharge events (asterisk) are shown. LOD for NoV in oysters (dotted line) and wastewater (broken line) are also included.**

copies/100 ml detected on 16 March. NoV GII concentrations in oysters showed no temporal trend over the period from January to May. In May, NoV GII concentrations in oysters dropped to below the LOD for the assay and remained below this limit throughout June until monitoring ceased, apart from one sample. On 15 June, one oyster sample contained an NoV GII concentration of 3.75 $log_{10}$ genome copies/g. This was preceded on 12 June by a CSO discharge (total of 997 $m^3$). The next oyster sample was collected on 22 June and the NoV GII concentration had returned to below the LOD for the method. Overall, no correlation was detected between the NoV GII concentrations in wastewater effluent samples and oyster samples analysed during the monitoring period. However, any relationship may have been masked by the high frequency of CSO discharges from the WWTP during the period (Fig. 3.4). In addition, it is possible that other diffuse and point sources of human faecal pollution may have impacted on the NoV concentration in the oysters.

### *3.2.5 Comparison of FRNA bacteriophage GA concentrations (infectious and RT-qPCR assays) with NoV GII concentrations*

Mean concentrations of infectious GA FRNA bacteriophage (plaque hybridisation assay), total GA FRNA bacteriophage (RT-qPCR assay) and NoV GII concentrations (RT-qPCR assay) in influent secondary-treated and final effluent (n = 10) are shown in Table 3.7 and Fig. 3.5. In influent wastewater, concentrations of FRNA GA bacteriophage detected using the RT-qPCR assay (5.14 $log_{10}$ pfu/100 ml) were not significantly different to those detected using the infectivity/hybridisation assay (5.29 $log_{10}$ pfu/100 ml, P = 0.59). In secondary-treated wastewater, mean concentrations of GA bacteriophage detected using the RT-qPCR assay were 1.15 $log_{10}$ greater than those determined using the infectivity/hybridisation assay and this was found to be significantly different (P = 0.03) using the Mann–Whitney Rank Sum Test. Similarly, in UV-treated wastewater, mean concentrations of GA bacteriophage detected using the RT-qPCR assay were 1.69 $log_{10}$ greater than those determined using the infectivity/hybridisation assay and this was found to be significantly different ($P \leq 0.001$). NoV GII concentrations in wastewater as determined with the RT-qPCR followed a similar pattern of reduction as observed for FRNA bacteriophage and determined using the RT-qPCR assay.

In oysters, mean concentrations of GA bacteriophage detected using the RT-qPCR assay were 0.89 $log_{10}$ greater than those determined using the infectivity/hybridisation assay and this was found to be significantly different ($P \leq 0.001$).

**Table 3.7. Mean $log_{10}$ concentrations and $log_{10}$ reductions for NoV GII and FRNA bacteriophage GA determined by RT-qPCR or infectivity assay at WWTP2.**

| n = 10 | Stage of wastewater treatment process | | | | | | | Oysters |
|---|---|---|---|---|---|---|---|---|
| | Influent | Post-secondary treatment | | Post-UV disinfection | | Total mean $log_{10}$ reduction | Total percentage reduction | Concentration |
| | Concentration | Concentration | Mean $log_{10}$ reduction | Concentration | Additional $log_{10}$ reduction | | | |
| **Infectious bacteriophage GA (plaque hybridisation)** | 5.29 ± 0.453 (4.88–6.32) | 3.57 ± 0.56 (2.81–4.45) | 1.72 | 2.97 ± 0.61 (1.70–3.74) | 0.60 | 2.32 | 99.52 | 3.17 ± 0.36 (2.48–3.50) |
| **Total bacteriophage GA (real-time RT-qPCR)** | 5.14 ± 0.86 (3.96–6.58) | 4.72 ± 1.13 (2.78–6.49) | 0.42 | 4.66 ± 0.96 (3.36–6.16) | 0.06 | 0.48 | 67 | 4.06 ± 0.69 (2.5–5.06) |
| **NoV GII (real-time RT-qPCR)** | 3.87 ± 0.70 (2.93–4.96) | 3.46± 1.22 (<LOD–5.18) | 0.40 | 3.61 ± 0.81 (2.41–4.80) | 0.00 | 0.26 | 45 | 3.04 ± 1.05 (<LOD–3.95) |

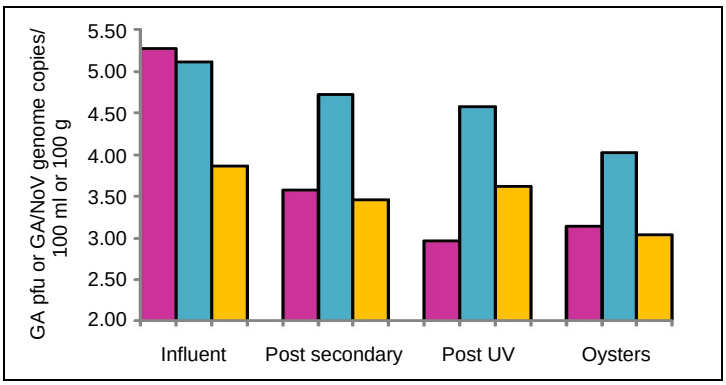


**Figure 3.5. Mean $\log_{10}$ concentrations (n = 10) of FRNA GA bacteriophage using infectivity assay (pink bar) and RT-qPCR (blue bar), and NoV GII (yellow bar) in influent, secondary-treated effluent, UV-treated effluent and oysters.**

### *3.2.6 Impact of combined sewer overflow discharges*

During the period from 30 September to 15 December, four CSO discharges occurred at the WWTP (Fig. 3.6). These occurred on 9 September (413 $m^3$), 23 October (534 $m^3$), 29 November (1,160 $m^3$) and 8 December (1,882 $m^3$). Throughout this period, NoV GI concentrations in both wastewater and oyster samples were less than or close to the LOD and are not considered further. Total NoV GII concentrations in the CSO effluent (genome copies/100 ml × discharge volume) from two of the events are shown in Fig. 3.6, along with NoV GII concentrations in oysters sampled in response to the discharges. On 30 September, a total of $8.79 \times 10^9$ genome copies of NoV GII were released during the overflow period. In anticipation of a CSO discharge event, the WWTP operator informed the laboratory. An oyster sample was taken approximately 2 h prior to the overflow commencing. This sample was below the LOD for NoV GII. A further oyster sample was taken approximately 12 h after the CSO discharge event commenced and the NoV GII concentration was 2.54 $\log_{10}$ genome copies/g (346 genomes/g). A further four oysters samples were taken over the next 96 h, with NoV GII concentrations ranging from 1.95 to 2.70 $\log_{10}$ genome copies/g, with no apparent temporal trend observed. On 29 November, $5.99 \times 10^9$ genome copies were released following a CSO discharge event. An oyster sample was taken immediately 3 h after the start of the discharge event. The NoV concentration in this sample was 3.43 $\log_{10}$ genome copies/g (2,691 genomes/g). A further six oyster samples were taken over the next 48 h, with NoV GII concentrations ranging from 3.02 to 3.49 $\log_{10}$ genome copies/g (1,047–3,090 genomes/g), with no apparent temporal trend. The NoV GII concentration in an oyster sample taken on 6 December (7 days post-event) had an NoV GII concentration of 3.33 $\log_{10}$ genome copies/g. The NoV GII concentration in an oyster sample taken 14 days prior to the event on 15 November was 2.73 $\log_{10}$ genome copies/g.

## 3.3 Comparison of Concentrations of FRNA Bacteriophage GA in CSO and UV-Treated Wastewater Effluent

CSO discharge and UV-treated wastewater samples were analysed for infectious and total GA FRNA bacteriophage concentrations using the plaque hybridisation and RT-qPCR assays. $\text{Log}_{10}$ concentrations of GA FRNA bacteriophage obtained by both assays are shown in Fig. 3.7. In all cases, the RT-qPCR detected a higher concentration of GA FRNA bacteriophage in UV-treated wastewater samples than the plaque hybridisation assay. In four CSO discharge samples, RT-qPCR detected higher concentrations of GA FRNA bacteriophage on two occasions, almost equal concentrations on one occasion, and lower concentrations in the final sample. The difference between the concentrations of infectious FRNA bacteriophage GA, as determined by the plaque hybridisation assay, and total FRNA bacteriophage GA, as determined by the real-time RT-qPCR method, are shown in Fig. 3.8. The mean

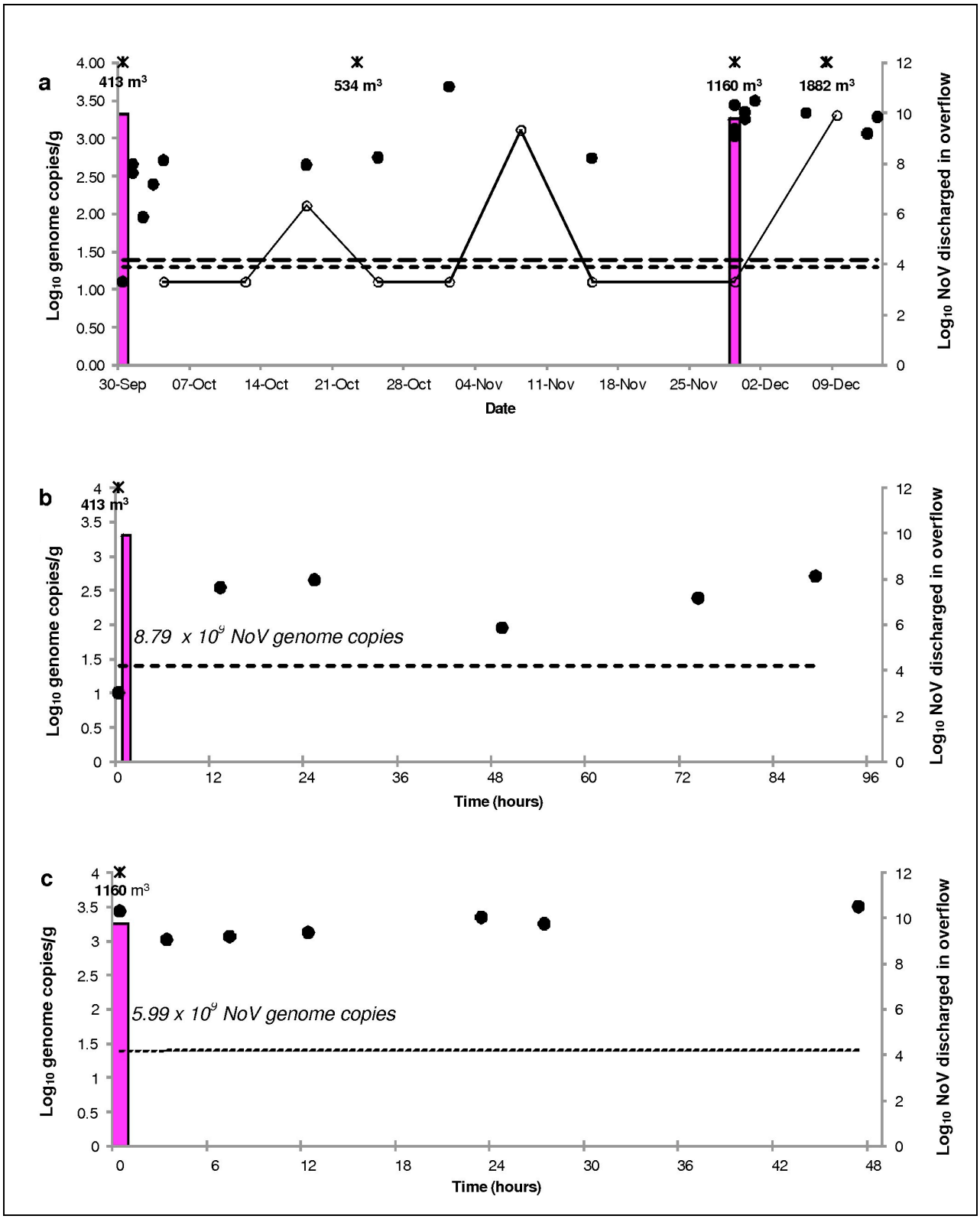


**Figure 3.6. $Log_{10}$ concentrations of NoV GII in oysters (●) and wastewater effluent (○) from September to December 2011 at WWTP2 (a), during the September event (b) and the November event (c). CSO discharges are indicated (asterisks) in addition to the total volume of the discharge and the $log_{10}$ quantities of NoV GII released during each discharge (pink bars). The LODs for the NoV GII assays are indicated for wastewater (dashed line) and oysters (dotted line).**

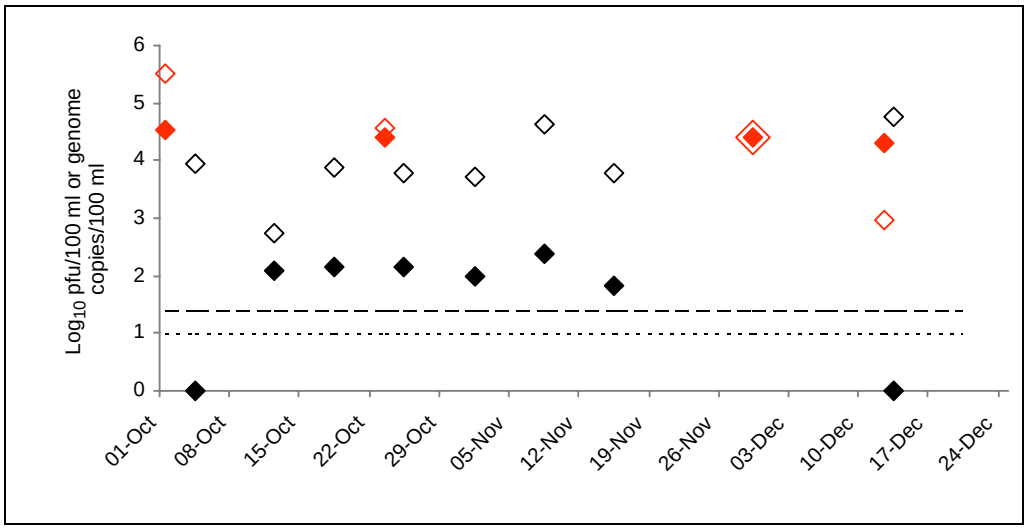


**Figure 3.7. $Log_{10}$ concentrations of FRNA bacteriophage GA using an infectivity assay (closed diamond) and a real-time RT-qPCR assay (open diamond) in effluent wastewater and CSO wastewater (CSO samples shown in red). The LOD for the real-time RT-qPCR assay (dashed line) and the GA infectivity assay (dotted line) are indicated.**

difference between the concentration of GA FRNA bacteriophage determined using the two assays in UV-treated wastewater samples was $log_{10}$ 2.51 (n = 8). Therefore, on average, the real time RT-qPCR method detected $log_{10}$ 2.51 more GA FRNA bacteriophage than the plaque hybridisation method. For the CSO discharge samples no significant difference was observed in the concentration of GA FRNA bacteriophage determined using the two methods.

## 3.4 UV Disinfection Studies at Model WWTP

NoV GI concentrations in secondary-treated effluent prior to UV disinfection at the model WWTP were either below or close to the LOD and are not considered further here. Mean concentrations of GA FRNA bacteriophage in secondary-treated effluent were reduced from 5.73 to 4.96 $log_{10}$ genome copies/100 ml, as determined using the real-time RT-qPCR method (Table 3.8). By

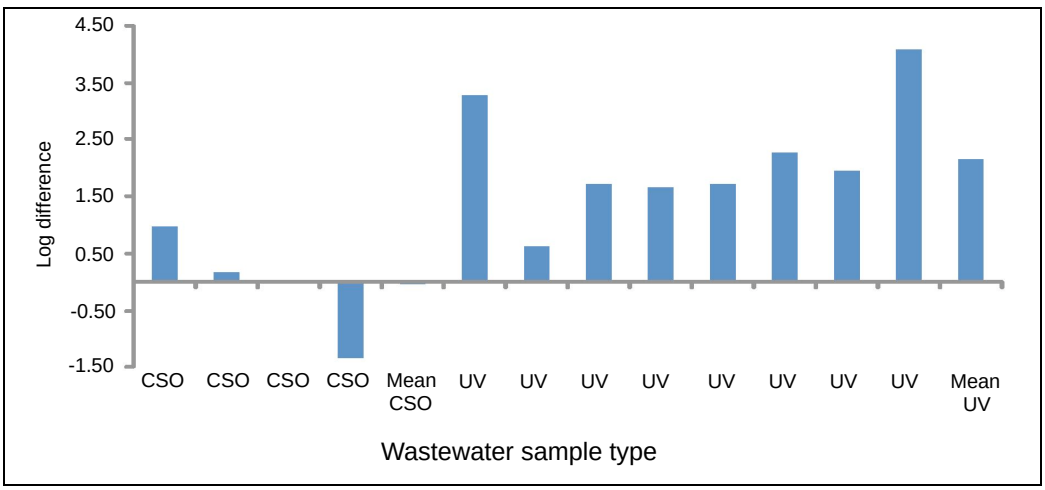


**Figure 3.8. $Log_{10}$ differences between the GA infectivity assay and the real-time RT-qPCR assay in CSO discharge and post-UV disinfection samples during the CSO study. Mean $log_{10}$ differences are also included for both sample types.**

**Table 3.8. Mean $log_{10}$ concentrations and $log_{10}$ reductions for FRNA bacteriophage GA using the infectivity assay and RT-qPCR assay and NoV in wastewater samples before and after UV treatment at the model WWTP.**

| | Wastewater treatment process | | |
|---|---|---|---|
| | **Secondary effluent concentration** | **Post-UV disinfection concentration** | **$Log_{10}$ (percentage) reduction** |
| **Infectious bacteriophage GA (plaque hybridisation)** | 4.07 ± 0.71 (2.94–5.00) | 2.27 ± 1.24 (<LOD–3.64) | 1.80 (98.42) |
| **Total bacteriophage GA (real-time RT-qPCR)** | 5.73 ± 0.40 (4.89–6.34) | 4.96 ± 0.63 (3.53–5.55) | 0.77 (83.02) |
| **NoV GII (real-time RT-qPCR)** | 4.32 ± 0.55 (3.90–5.95) | 3.80 ± 0.53 (2.99–4.78) | 0.52 (69.80) |

**Mean values were determined from 12 independent observations. The log10 reduction was calculated by subtracting mean values of post-UV from the secondary treated effluent.**

comparison, a greater reduction was achieved through UV disinfection when measured using the plaque hybridisation infectivity assay, with mean concentrations of GA FRNA bacteriophage in secondary-treated effluent reduced from 4.07 to 2.27 $log_{10}$ pfu/100 ml. Mean concentrations of NoV GII in secondary-treated effluent were reduced from 4.32 to 3.80 $log_{10}$ genome copies/100 ml, as determined by the real time RT-qPCR method.

## 3.5 Virus Survival in Sea Water

Initial NoV survival studies using sea water seeded with wastewater gave variable and inconclusive results. This was principally because it was not possible to seed the sea water with high enough concentrations of NoV using wastewater as representative turbidity (those collected from sea water above the outfall from WWTP1) could only be achieved using wastewater diluted greater than 1 in 100. Using faecal material, no significant difference was found between the median values in irradiated samples and control samples using the Mann–Whitney Rank Sum Test under summer (P = 0.33) and winter conditions (P = 0.33). Results indicated that there was no appreciable reduction in NoV concentrations as determined using real-time RT-qPCR over 2- to 7-day periods. Therefore, it was not possible to determine $T_{90}$ values for NoV in sea water under summer and winter conditions.

FRNA bacteriophage were not inactivated under control (dark) conditions and no significant difference was found between initial and final concentrations (Mann–Whitney Rank Sum Test, P = 0.54 and P = 0.84 for summer and winter temperatures, respectively). Under summer temperature and sunlight conditions, FRNA bacteriophage underwent a linear inactivation ($R^2$ = 0.99) and a $T_{90}$ value of 14.57 min was calculated from the regression line. Under winter temperature and sunlight conditions, FRNA bacteriophage were reduced at a lesser rate than under summer conditions; however, again a linear inactivation was observed ($R^2$ = 0.94) and a $T_{90}$ value of 2.16 h was calculated. In contrast to results obtained using the infectivity assay, the real-time RT-qPCR assay showed no significant reduction (Mann–Whitney Rank Sum Test) in concentrations of FRNA bacteriophage under either the summer (a total of 2 days; P = 0.505) or winter (a total of 7 days: P = 0.49) conditions.

# 4 Discussion and Conclusions

NoV GI and GII were detected in all WWTP1 influent wastewater samples investigated in this study, demonstrating the widespread distribution of NoV in the general population even during the summer months when symptomatic infection in the population is low. However, NoV GI and GII were only detected in 61% and 96%, respectively, of influent wastewater samples taken at WWTP2. This may be related to the size of the population served by the two WWTPs (PE of approx. 90,000 compared with 10,000). The majority of previous studies investigating the occurrence of NoV in wastewater indicate that the virus was often absent, particularly during the summer period (van den Berg et al., 2005; da Silva et al., 2008; Katayama et al., 2008). However, NoV was detected year round from a WWTP serving a PE in excess of 800,000 in Sweden (Nordgren et al., 2009). In the UK, NoV GI and GII were detected in all influent and effluent samples at a WWTP with secondary treatment serving a PE in excess of 100,000 over a 2-year period (Lowther, 2011). Given the relative size of the two WWTPs investigated in this study and data from previous studies, it would appear that the year-round detection of NoV in wastewater may be related to the size of the population served by the WWTP. Alternatively, differences between NoV GI detection rates observed in influent samples at the two WWTPs studied may represent geographical differences between the two populations investigated or an annual variation associated with differing GI circulation in the community in 2009/2010 and 2011. In this study, a variety of NoV genotypes (both NoV GI and GII) in both untreated wastewater and treated final effluent at WWTP1 were detected. In total, four NoV GI and six NoV GII genotypes were detected in wastewater. NoV GII.4 variants are reported as responsible for the majority of clinical cases of NoV-associated gastroenteritis in the community. The diversity of NoV genotypes detected in wastewater in this study demonstrates that significant numbers of non-NoV GII.4 genotypes are circulating in the general community. It may be that non-NoV GII.4 genotypes are responsible for asymptomatic infections in the community or, alternatively, that symptomatic non-NoV GII.4 genotype infections are under-reported

The mean reduction of *E. coli* at WWTP1 during the study was 1.49 $\log_{10}$ (96.8%). This is approximately 0.5 $\log_{10}$ less than the median value reported for faecal coliforms from a study investigating six WWTPs employing activated sludge treatment but is still within the range presented (Weatherley, 1999). The average reduction for FRNA bacteriophage at the site was 2.13 $\log_{10}$ (99.2%), which is consistent with the median value of 2.04 $\log_{10}$ reported previously (Weatherley, 1999). Concentrations of NoV GI and GII were reduced on average by 0.8 $\log_{10}$ (83.8%) and 0.92 $\log_{10}$ (87.9%), respectively, at this site. This reduction is within the previously reported range (Ottoson et al., 2006; Nordgren et al., 2009) but is slightly lower than that published in a recent report from the UK, which demonstrated 1.26 $\log_{10}$ (94.5%) and 1.64 $\log_{10}$ (97.7%) reductions for NoV GI and GII, respectively, in an activated sludge WWTP serving a population >100,000 PE (Lowther, 2011). Therefore, the reductions in *E. coli* and FRNA bacteriophage achieved by this WWTP are within the range observed internationally and it is likely that the NoV reductions presented here are representative of those achieved at other well-operated WWTPs using activated sludge treatment. However, they may not represent the optimal NoV reductions that may be achieved by this treatment process and further viral monitoring of additional WWTPs to demonstrate high performing systems is warranted.

WWTP2 was a newly commissioned secondary WWTP with UV disinfection of the final effluent. NoV GI and GII concentrations were reduced on average by just 0.30 $\log_{10}$ (49.9%) and 0.44 $\log_{10}$ (63.7%), respectively, following secondary treatment over the 6-month study period. This reduction is less than has been reported previously (Nordgren et al., 2009; Lowther, 2011) and observed at WWTP1 during this study. UV disinfection did not achieve any further reduction of the mean concentration of NoV GI and GII in the final effluent. However, the NoV reductions at

this site compared with WWTP2 must be considered in the context of the general performance of this WWTP which was still undergoing full commissioning during the early part of the monitoring period. WWTP2 became fully operational in December 2010 and microbiological monitoring as part of this study of the system commenced in January 2011. Subsequent to completion of this monitoring programme, it became apparent that a number of problems were experienced with the general operation of WWTP2 during this commissioning phase in January and February 2011. This was identified in particular by exceedance of prescribed standards for BOD and suspended solids in January and February 2011 and excessive CSO discharge events that were not related to excessive rainfall. Therefore, the NoV reduction values determined at WWTP2 during this period cannot be considered to be representative of NoV reductions that may be achieved through UV disinfection in other WWTPs or this one when operating correctly following full commissioning. Results from monitoring at WWTP2 highlight the importance of achieving good-quality biologically treated effluent before further treatment by UV disinfection. Further investigation at this and alternative WWTPs with UV disinfection to establish the extent of virus removal would be beneficial.

At both WWTPs investigated, there was a difference between the extent of NoV reduction compared with the reduction of the surrogate virus indicator FRNA bacteriophage. FRNA bacteriophage can be considered to be an appropriate virus indicator in this context. FRNA bacteriophage are ubiquitous in wastewater and are easily quantified using standardised ISO methods (Anonymous, 1995) based on a simple plaque assay that detects only infectious virus particles. In direct comparison with relevant pathogenic human viruses that can be cultured, such as poliovirus, they have been demonstrated to be a conservative indicator of virus behaviour (Simonet and Gantzer, 2006). Therefore, it is likely that FRNA bacteriophage reductions presented in this report probably represent the minimum that would have been achieved for infectious NoV. During this study, FRNA bacteriophage reductions were significantly greater than for NoV. However, in comparing these reductions, it must be remembered that the analytical methods used are not comparable. FRNA bacteriophage levels were determined using the plaque agar overlay assay which exclusively detects infectious bacteriophage whereas the real-time RT-qPCR method employed for NoV detection may detect both infectious and non-infectious virus particles (Bhattacharya et al., 2004; Rodriguez et al., 2009). In this study, a specific infectivity assay for FRNA bacteriophage GA was directly compared with a real-time RT-qPCR assay for the same agent. In influent wastewater samples, there was good agreement between the two methods, with no statistically significant difference between concentrations detected by both assays. However, in treated effluent (particularly following UV disinfection) and in oyster samples, there were significant differences, with higher concentrations detected using the real-time RT-qPCR method on all such occasions. This indicates that the real-time RT-qPCR method detected both infectious and non-infectious GA FRNA bacteriophage. The reduction of GA FRNA bacteriophage and NoV when using the real-time RT-qPCR method for both viruses demonstrates a similar pattern. If, as seems likely, the real-time RT-qPCR method for NoV detects non-infectious virus particles in a similar way, it questions the value of testing by real-time RT-qPCR to determine NoV reduction during WWT. NoV testing will likely overestimate the concentration of infectious NoV in treated effluent and, therefore, underestimate the true extent of the reduction of infectious NoV during treatment. Evidence from using FRNA bacteriophage GA would suggest that this would be particularly exaggerated in UV-disinfected wastewater. The principal mechanism of inactivation associated with UV disinfection is genome damage through the generation of different types of photoproducts such as pyrimidine dimers (Cadet and Vigny, 1991) causing nucleic acid strand breaks which render the virus non-infectious, although it should be noted that, at higher UV doses, protein integrity or conformation may also be affected by breaking disulphur bonds (Cadet and Vigny, 1991). It is likely, therefore, that, after UV disinfection, a high proportion of virus particles with intact viral capsids (proteins) but damaged and non-infectious genomes are detected by the real-time RT-qPCR procedure. This is likely to be particularly exaggerated because of the short RNA fragments targeted by real-time primer sets

(approx. 90 nucleotides for NoV and 111 nucleotides for GA). Therefore, this study demonstrated a clear requirement to develop test methodologies that will distinguish between infectious and non-infectious NoV particles in environmental samples. Although recent progress has been made towards the development of a culture system for NoV (Straub et al., 2007, 2011), this has not been reproduced reliably elsewhere and, because of its complexity, is unlikely to be used in a routine context. Various alternative molecular approaches have been proposed, including enzyme pre-treatment (Nuanualsuwan and Cliver, 2002) and the use of long-range RT-qPCR (Wolf et al., 2009), and applied in a laboratory setting. These and other potential approaches merit further investigation to establish an assay for distinguishing between infectious and non-infectious NoV in environmental samples.

Currently, there is no regulatory requirement to set a parametric limit for virus reduction during WWT in Ireland or under European regulation. In general, compliance monitoring of WWTPs is set around monitoring physicochemical parameters, although some limited microbiological (bacterial) monitoring is also carried out at specific WWTPs. Given the potential public health and economic impact of NoV contamination in bivalve shellfish production areas and the key step that WWT plays as a control point, it would be appropriate to examine further the options for establishing operational performance criteria that may be used to monitor the effectiveness of viral reductions at WWTPs discharging in the vicinity of shellfish production areas. Such criteria could be set on an individual basis for each WWTP following trials or applied more generally on the basis of the level and type of treatment used. These criteria could be used to determine the efficiency of the virus reduction and operational management at the WWTP rather than providing a compulsory standard as other considerations such as dilution and environmental die-off should also be taken into account when considering the impact on the receiving waters. This project demonstrates that NoV monitoring by the real-time RT-qPCR procedure is an inappropriate measure of infectious virus reduction. Therefore, it would be more appropriate to establish any such operational management criteria on the basis of FRNA bacteriophage removal which would more accurately reflect the removal of infectious virus. Ongoing compliance with such criteria could be assessed through FRNA bacteriophage monitoring before and after treatment, undertaken at a frequency determined on a risk-based approach. In addition to the fact that they can be detected using an infectivity assay, FRNA bacteriophage fulfil a number of conditions that make them ideal candidates for indicators of virus removal during WWT. They are universally present in consistent numbers in influent wastewater whereas pathogen levels fluctuate and can be absent for much of the time at some WWTPs. Therefore the use of FRNA bacteriophage would demonstrate a consistent level of virus removal, indicating maintenance of the effectiveness of the WWT process. Critically, FRNA bacteriophage are relatively cheap and easy to enumerate and an ISO method for their detection in wastewater exists (Anonymous, 1995). FRNA bacteriophage analysis requires no specialist equipment and could be undertaken by commercial microbiology laboratories with minimal set-up fees.

During year-long monitoring at WWTP1, peak levels of NoV were detected during the winter period (January–March), which is consistent with the peak number of NoV infections observed in the general community. Furthermore, this increase was most pronounced for NoV GII, which again is consistent with the fact that most community infections are associated with NoV GII. NoV was detected in all samples from oysters adjacent to the discharge from this WWTP. NoV concentrations in oysters were clearly correlated to NoV concentration in the effluent with peak concentrations again detected during the winter period. In addition, these oysters contained a range of NoV genotypes within each of the NoV GI and GII genogroups. The predominant NoV GI genotype associated with oysters was NoV GI.4, despite the fact that it was not the predominant genotype detected in the wastewater. Recently, NoV GI genotypes, in general, and NoV GI.4, in particular, have been identified as being responsible for causing a relatively high frequency of food-borne outbreaks of gastroenteritis whereas most community outbreaks based on person-to-person spread are caused by NoV GII.4 genotypes (Verhoef et al., 2010). In previous studies, NoV GI.4 was frequently detected in influent

and effluent wastewaters in Japan (Iwai et al., 2009; Kitajima et al., 2012). It has also been reported that NoV GI.4 was the most frequently identified genotype in a large gastroenteritis outbreak of suspected water-borne transmission in Northern Italy (Di Bartoloa et al., 2011) and in a large food-borne outbreak in which more than 200 people were affected by frozen raspberries in Finland (Maunula et al., 2009). It is possible that NoV GI.4 has some properties that allow for better persistence in the environment and possibly better accumulation in shellfish. Previous studies have demonstrated that some NoV GI genotypes can bind specifically to histo-blood group antigens such as those structures present in oyster digestive tissues and may account for preferential concentration of NoV GI genotypes (Maalouf et al., 2010). The current study further demonstrates that oysters are readily impacted by wastewater discharges and can provide a vector for the spread of NoV in the population. The fact that several NoV genotypes were detected simultaneously in the oysters demonstrates that the potential exists for infection with multiple genotypes leading to novel recombinant genotypes of NoV. This highlights the importance of WWT to control the impact of discharges into existing shellfish production areas and the need to optimise treatment processes for virus removal. It also highlights the importance of locating new shellfish growing areas away from existing potential sources of NoV contamination. UV disinfection at the model WWTP investigated in this study achieved an average $\log_{10}$ reduction of infectious GA FRNA bacteriophage of 1.80 (98.4%) and indicates the extent of infectious virus reduction that could be achieved by the addition of UV disinfection to a fully operating secondary WWTP. By comparison, an average reduction of just 0.52 $\log_{10}$ (69.8%) was observed for NoV GII as determined using the real-time RT-qPCR method. However, given that FRNA bacteriophage are considered to be a conservative indicator of virus inactivation, it is likely that at least this level of reduction observed for GA FRNA bacteriophage (1.80 $\log_{10}$, 98.4%) could be achieved for NoV and would be observed if an infectivity assay for NoV was available. In addition, in a laboratory study using a long-range RT-PCR method, NoV was considered to be relatively susceptible to inactivation by UV disinfection (Wolf et al., 2009). It is therefore likely that the addition of UV disinfection to WWTPs, where discharges impact on receiving waters with shellfish production areas, would significantly reduce the concentration of infectious NoV present in shellfish. This could have a potentially significant impact on the public health risk associated with the consumption of raw bivalve shellfish.

A recent opinion issued by the EFSA in response to questions from the Food Safety Authority of Ireland indicated that risk managers should consider setting an acceptable standard for NoV concentrations in oysters (EFSA, 2012). The recommendation was given that the current standardised real-time RT-qPCR is suitable for this purpose. The panel recognised that this procedure was likely to detect non-infectious NoV particles and that no threshold of infection could be identified. Despite this shortcoming, it was concluded that NoV illness was dose related and that NoV genome copy concentration was related to the risk of illness with increasing concentrations associated with the increasing risk of illness. In this study, NoV genome copies in oysters at the two sites investigated increased significantly during the high-risk winter period. In addition, the concentration of NoV was significantly higher in oysters at WWTP1 than WWTP2, probably because of the difference in PE served and volume of wastewater discharged. Therefore, NoV detection in oysters by real-time RT-qPCR provides an index of virus risk and has a role to play in identifying high-risk production sites and periods of peak NoV contamination. Furthermore, during this study, NoV was detected in oysters following a CSO discharge event when untreated dilute wastewater and other potential diffuse pollution sources were discharged into the harvest area. Before this CSO discharge event, NoV concentrations in oysters at the site were below the LOD. Despite the fact that the real-time RT-qPCR detects non-infectious virus, NoV monitoring by this method remains a useful risk management tool for assessing both risk and the effectiveness of intervention strategies (Doré et al., 2010). The qualities that determine the utility of FRNA bacteriophage as an indicator of virus removal during WWT and direct detection NoV by RT-qPCR in oysters as an index of virus risk are summarised in Table 4.1.

There is no legislative requirement to set a parametric limit for virus reduction during WWT. Neither are there

**Table 4.1. Comparison of FRNA bacteriophage and NoV detection as indicators of virus removal during wastewater treatment or index of virus risk in oysters.**

| | FRNA bacteriophage (plaque assay) | NoV (real-time RT-qPCR) |
|---|---|---|
| **Indicator of virus reduction during wastewater treatment – desirable criteria** | | |
| Ubiquitous in wastewater allowing continuous assessment of virus reduction at all times | Yes | No. Absent in some WWTPs in summer |
| Detects infectious virus only, allowing extent of true reduction of infectious virus to be determined | Yes | No |
| **Index of virus risk in bivalve shellfish – desirable criteria** | | |
| Concentration in shellfish elevated in locations of higher risk | Yes/No. Related to size of impacts but may be influenced by animal sources | Yes. Related to extent of human impact sources |
| Concentration elevated at times of higher risk | No. Constant year round | Yes. Concentration related to current infections in population |
| Concentration in shellfish directly related to risk of infection | No | Yes (EFSA, 2012; Lowther et al., 2012a) |
| **General criteria** | | |
| Cheap and easy to analyse | Yes. Approx. €30 | No. Approx. €200+. Requires specialised equipment |
| Standard methods available | Yes (ISO 10705-1) | Yes (ISO/TS 15216-1) |

viral parametric limits in current water quality legislation. The introduction of an NoV standard in shellfish could be significant for WWTP operation. Such a standard would effectively set a target for NoV concentrations in oysters which WWT should endeavour to meet. Whilst UV disinfection (and potentially other treatments) could reduce the extent of environmental contamination with infectious NoV and other human pathogenic viruses, from the data presented in this study it is clear that non-infectious NoV particles would continue to be detected in shellfish by the current real-time RT-qPCR methods. Therefore, even at WWTPs where effective disinfection processes are employed, there would be the potential for safe oysters to fail the standard because of the detection of non-infectious viruses. This further highlights the need to develop methods that can distinguish between infectious and non-infectious NoV. In addition, it may be worth investigating the use of alternative technologies, including membrane bioreactors (MBRs), which might be used to remove the virus particles, the potential of which has been highlighted in a recent study in France (Sima et al., 2011).

Limited studies were carried out on the survival of NoV in sea water as it proved difficult to obtain NoV-positive faecal material and attempts to determine $T_{90}$ values for NoV in sea water under summer and winter conditions were unsuccessful. Initially, this was because of difficulty in seeding sea water with sufficiently high concentrations of NoV. In addition, NoV concentrations in sea water showed little or no reduction during extended periods of up to 7 days as determined by real-time RT-qPCR detection. However, as real-time RT-qPCR detects non-infectious as well as infectious virus particles, the reductions determined must be considered unrepresentative. This is further supported by the fact that a similar low level of reduction was observed for FRNA bacteriophage using RT-qPCR, whereas significant reductions were observed using the infectivity assay. Sunlight consists of a variety of wavelengths of light, with the shorter wavelengths (such as germicidal 254 nm) being absorbed by the atmosphere with UVB (290–315 nm)

reported to be the most damaging portion of sunlight for indicators of water-borne pathogens (Sinton et al., 2002). Sea water attenuates sunlight with longer wavelengths of light being present (UVA 315–400 nm) and these wavelengths have been shown to specifically affect RNA viruses (Fisher et al., 2011). It has been suggested that the reduction in infectivity of viruses in sea water is as a result of photo-oxidative mechanisms that are directed towards the capsid host-binding receptors, potentially leaving intact regions of the viral genome (Romero et al., 2011) and may explain the persistence of virus as determined by real-time RT-qPCR.

In the absence of the technical ability to determine the reduction of infectious NoV, $T_{90}$ values were determined for FRNA bacteriophage. FRNA bacteriophage have been recommended as a conservative indicator of enteric virus (poliovirus) survival in marine environments (Love et al., 2010). Loss of infectivity for FRNA bacteriophage occurred at a greater rate during summer compared with winter conditions. While the $T_{90}$ values obtained during the summer experiments (14.57 min) are similar to the range reported of 0.6–1.02 h (Love et al., 2010; Romero et al., 2011), few data are available concerning survival during winter conditions representative of those in Ireland. However, it took approximately nine times longer ($T_{90}$ 2.16 h) to reduce FRNA bacteriophage to the same extent as under summer conditions. As this study's experiments were performed using low-turbidity natural sea water, the results may represent a best-case scenario for viral solar disinfection in sea water and may overestimate the contribution that sunlight and temperature make in the natural environment. The survival characteristics of FRNA bacteriophage in sea water are likely to represent the best estimate of NoV reduction under environmental conditions and may provide information on the likely impact of WWTP discharges on shellfisheries in the vicinity.

## 4.1 Summary of Findings

- Currently there are no regulatory limits or national guidelines for NoV concentrations in shellfish flesh or in waterbodies. The absence of such regulatory standards or guidelines makes it difficult to determine and apply appropriate NoV reduction criteria for WWTPs.
- NoV was detected in wastewater throughout the year and the average $\log_{10}$ reduction as determined by RT-qPCR during secondary WWT (activated sludge) was 0.92 (87.9%) and 0.79 (83.8%) for NoV GI and GII, respectively.
- Real-time RT-qPCR detects infectious and non-infectious viruses and, therefore, is likely to overestimate the number of infectious NoV particles present in both wastewater and oysters. This leads to an underestimation of the extent of infectious NoV reduction during wastewater treatment. This underestimation was most pronounced during UV disinfection as determined using the comparison of RT-qPCR and infectivity assays for GA FRNA bacteriophage as a human virus surrogate.
- Given the inability of real-time RT-qPCR procedures to distinguish between infectious and non-infectious NoV, it should be considered an inappropriate method for measuring NoV reduction during wastewater treatment.
- In this study, NoV concentrations in oysters were correlated with NoV concentrations in wastewater effluent and clear peaks were observed during the winter period when community infections also peaked. Previous studies have demonstrated that the risk of NoV infection from consuming oysters increases with increasing concentrations of NoV genome copies as determined by RT-qPCR. Therefore, NoV concentrations in oysters, as determined by RT-qPCR, provide an appropriate index of the relative risk to consumers from ingesting such oysters. Monitoring of oyster production areas for NoV using RT-qPCR is therefore a valuable tool to identify 'at risk' production areas and periods of increased risk that may require additional management intervention by producers and/or regulators to reduce the risk.
- FRNA bacteriophage are a useful indicator of the extent of infectious pathogenic virus reduction during wastewater treatment. They have the

potential to provide an economically viable ongoing assessment of the extent of virus removal at WWTPs through regular monitoring before and after treatment.

- In this study, the mean concentration of FRNA bacteriophage in influent was reduced by an average of approximately 2 $\log_{10}$ (approx. 99%) following activated sludge secondary treatment (WWTP1). The mean concentration of FRNA bacteriophage was reduced by a further 1.80 $\log_{10}$ (98.42%) following UV disinfection (model treatment plant). This demonstrates the potential for a well-operated secondary-treated WWTP with UV disinfection to achieve approximately 4 $\log_{10}$ reduction of infectious virus. This highlights the potential reduction in the extent of NoV contamination in shellfish production areas that may be achieved by the use of secondary WWT followed by UV disinfection in a well-operated WWTP.
- CSO discharges containing a combination of dilute untreated wastewater and storm water contain a greater ratio of infectious to non-infectious virus than treated effluent (as determined using the FRNA infectivity assay).
- This study demonstrated that oysters containing concentrations of NoV below the LOD prior to a CSO discharge tested positive for NoV after a high rainfall event that resulted in a CSO discharge. It was not possible to determine the relative contribution of the CSO discharge compared with other potential diffuse and point sources of human faecal contamination to the waterbody that may also have contributed to the oyster NoV contamination during the high rainfall event. However, given the high concentration of infectious NoV present in untreated wastewater, CSO discharge of dilute untreated wastewater mixed with storm water has the potential to lead to increased concentrations of infectious NoV in oysters where such discharges impact on waterbodies containing shellfish production areas. This has the potential to impact on public health where such oysters are consumed.
- FRNA bacteriophage when used as an indicator of virus survival in sea water was demonstrated to have a $T_{90}$ value of 2.16 h during winter conditions compared with 14.57 min for the summer conditions.

# 5 Recommendations

The focus of this study was to assess the fate of NoV during municipal WWT and to investigate the impact of wastewater discharges on NoV concentrations in shellfish. Recommendations are presented here based on the research and conclusions presented in this report. However, it must be recognised that the discharge of municipal wastewater effluent into the marine environment is a cost-effective practice that is widely used throughout the world. In Ireland, extensive procedures already exist to mitigate the impact of wastewater discharges into the marine environments. These include measures to control the impact of CSO discharges provided in the Department of the Environment and Local Government[2] (DELG) 1995 document *Procedures and Criteria in Relation to Storm Water Overflows*. The effectiveness of these measures for controlling NoV contamination in shellfisheries is unknown. However, in the first instance, CSOs should continue to be designed and operated in accordance with the DELG guidance, as required by the EPA in Wastewater Discharge Licences and Permits. It must therefore be accepted that while every practicable effort should be made by the State to endeavour to reduce the impact of wastewater discharges in shellfish production areas, it remains a fact that it is not possible to prevent an impact entirely. Therefore, even under the most stringent treatment procedures, on occasion, oysters will become contaminated with NoV. There is, therefore, a general requirement to establish risk management procedures to reduce the risk from the consumption of shellfish harvested from areas that are impacted by municipal wastewater and other sources of human faecal material that may cause NoV contamination. As the consumption of raw oysters represents the most significant risk of illness to consumers, management procedures should focus on areas where they are produced. Effective risk management in such areas requires a holistic approach involving all stakeholders, including WWTP operators, regulatory authorities, state agencies and shellfish producers alike. An effective solution would be to locate shellfish growing areas, including proposed new areas, away from waterbodies impacted by urban wastewater discharges and other sources of contamination that could lead to NoV contamination and a risk to public health.

- In addition to endeavouring to limit the extent of NoV contamination of oyster harvest areas arising from municipal wastewater discharges, a wider risk-based management approach to controlling the public health risks, involving all relevant stakeholders, is required. Guidelines for the control of the public health risks associated with NoV-contaminated oysters should be developed by relevant state agencies.

- RT-qPCR methods currently used for the quantification of NoV detect both non-infectious and infectious virus particles. Further development of methods that distinguish between infectious and non-infectious NoV particles in bivalve shellfish and wastewater should be undertaken as a priority.

- Further research is required to determine the relationship between the concentration of NoV in oysters determined using RT qPCR and the associated risk of infection in order to establish acceptable limits and to evaluate the potential risk to consumers.

- NoV monitoring using RT-qPCR of ‘at risk’ oyster harvest areas should be conducted to identify high-risk locations and periods when NoV contamination is elevated. In identified ‘at risk’ locations, specific intervention strategies should be developed and employed by producers during high-risk periods to reduce NoV exposure to consumers. NoV monitoring to assess the effectiveness of such interventions should be conducted.

- Further studies should be conducted to establish the impact of discharges from CSOs on receiving

2. Now the Department of the Environment, Community and Local Government. (DECLG)

waters in which shellfish are produced and the subsequent impact on the concentration of NoV in oysters.

- Given the evidence in this report that untreated wastewater contains higher ratios of infectious viruses than treated effluent (as demonstrated using FRNA bacteriophage GA), appropriate guidelines should be developed to limit the impact of CSO discharges that contain dilute untreated wastewater mixed with storm water in shellfish production areas.

- NoV monitoring using currently available RT-qPCR methods that do not distinguish between infectious and non-infectious virus particles is not an appropriate means of assessing the effectiveness of wastewater treatment processes to reduce infectious NoV in wastewater. Instead, FRNA bacteriophage should be used as an indicator organism for NoV removal during wastewater treatment processes.

- Guideline operational criteria for virus reduction during wastewater treatment that includes disinfection should be established in WWTPs that may impact waterbodies containing shellfisheries. The operational guideline criteria should be based on FRNA bacteriophage reductions and would provide a metric for WWTP operators to measure the efficiency of virus reduction in individual WWTPs. An ongoing programme of FRNA bacteriophage monitoring should be developed in such WWTPs to ensure ongoing compliance with the operational standard criteria.

- Alternative wastewater treatment processes that may remove, rather than inactivate, viruses should be investigated to establish their ability and cost-effectiveness to reduce viruses. An assessment of the capital and associated running costs of the treatment method should also be made to determine their cost-effectiveness.

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# Acronyms

| | |
|---|---|
| **BOD** | Biological oxygen demand |
| **CEN** | European Committee for Standardisation (Comité Européen de Normalisation) |
| **cfu** | Colony-forming unit |
| **COD** | Chemical oxygen demand |
| **CSO** | Combined sewer overflow |
| **$C_T$ value** | Cycle-threshold |
| **DECLG** | Department of the Environment, Community and Local Government. |
| **DELG** | Department of the Environment and Local Government. |
| **DIG** | Digoxigenin |
| **DNA** | Deoxyribonucleic acid |
| **DNase** | Deoxyribonuclease |
| **dNTP** | Deoxyribonucleotide triphosphate |
| **dsDNA** | Double-stranded DNA |
| ***E. coli*** | *Escherichia coli* |
| **EC RNA** | External control RNA |
| **EFSA** | European Food Safety Authority |
| **EXO IPC** | Exogenous internal positive control |
| **FRNA** | F-specific RNA |
| **HAV** | Hepatitis A virus |
| **HCl** | Hydrochloric acid |
| **IPC** | Internal positive control |
| **ISO** | International organisation for standardisation |
| **KCl** | Potassium chloride |
| **LOD** | Limit of detection |
| **MBR** | Membrane bioreactor |
| **$MgCl_2$** | Magnesium chloride |
| **MMGB** | Minerals modified glutamate broth |
| **MPN** | Most Probable Number |
| **NaCl** | Sodium chloride |
| **NaOH** | Sodium hydroxide |
| **NoV** | Norovirus |
| **NTU** | Nephelometric turbidity unit |

| | |
|---|---|
| **PCR** | Polymerase chain reaction |
| **PE** | Population equivalent |
| **pfu** | Plaque forming unit |
| **RNA** | Ribonucleic acid |
| **RNase** | Ribonuclease |
| **RT** | Reverse transcription |
| **RT-qPCR** | Reverse transcription quantitative polymerase chain reaction |
| **SD** | Standard deviation |
| **$T_{90}$ value** | The amount of time required to reduce microorganism concentrations by 1 log unit |
| **TBX** | Tryptone bile X-glucuronide |
| **Tris–HCl** | Tris(hydroxymethyl)aminomethane hydrochloride |
| **UV** | Ultraviolet |
| **WWT** | Wastewater treatment |
| **WWTP** | Wastewater treatment plant |

# An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil, Pobal agus Rialtais Áitiúil.

## ÁR bhFREAGRACHTAÍ

### CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail;
- scardadh dramhuisce;
- dumpáil mara.

### FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

### MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

### RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

### TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

### MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

### PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

### BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

### STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

**Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013**

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.

Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

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**Comhshaol, Pobal agus Rialtas Áitiúil**
Environment, Community and Local Government