Department for Environment, Food and Rural Affairs: Drinking Water Inspectorate

Project WT 1227

Viruses in raw and partially treated water: targeted monitoring using the latest methods

Final report: April 2013
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1 SUMMARY

- A series of analytical methods and associated standard operating procedures were developed to facilitate the isolation of norovirus (NV) and adenovirus (AdV) from raw and partially treated water samples. The identified concentration procedure utilised a conventional adsorption/elution approach using membrane filters and acidified beef extract and, in its present form, appeared to favour the recovery of AdV, although NV was consistently concentrated from spiked samples. The enumeration of the two viruses was effected using quantitative polymerase chain reaction (qPCR) and reverse-transcription qPCR (RT-qPCR) to quantify AdV and NV respectively in water sample concentrates. All of the methods are considered robust and reliable and could reasonably be transferred to competent water utility laboratories for routine use.

- The documented analytical methodologies were used to analyse raw and partially-treated water samples generated from a works monitoring programme undertaken over a nine month period (June 2011 - March 2012 inclusive) at 4 water treatment works across the UK.

- 74% of raw water samples were AdV-positive. AdV was present in raw waters throughout the year and, whilst the water treatment process reduced the level of AdV by between 2 and 4 orders of magnitude, the virus was apparently able to persist through to the pre-chlorination stages. Around 20% of all pre-chlorination (final stage) samples were AdV positive although none of the isolates proved to be infective when assessed by ICC-PCR.

- Removal of AdV occurred mainly at the first stage of treatment (post clarification), thereafter removal was negligible.

- NV was generally not detected in raw waters except during the winter months, December-March, when 94% of the raw water samples were positive. In contrast to AdV, there was apparently no significant effect of treatment on the level of NV. The levels of NV in raw waters were often so low that the demonstration of a significant reduction in numbers was impossible. However, in one instance where NV levels in raw waters were considered significant (Asset M; 14.02.2012: Appendix C), the virus was undetected in post-GAC (pre-chlorination) samples.

- There was strong evidence that, in raw waters where NV was detected, AdV was also detected. However, AdV was frequently detected in the absence of NV. Thus, on the basis of the data presented here, AdV may potentially be considered as a conservative indicator for the presence of NV in raw waters.

- In raw or pre-chlorinated water, there was no evidence to support the use of a chemical or bacteriological parameter to indicate the removal of the target viruses. However, changes in levels of bacteriological and chemical parameters did indicate that water treatment had taken place, with an associated decrease in AdV levels. For example, where there were reductions in levels of overall organic particulate matter (as measured by turbidity, there was also a general reduction in virus numbers).
INTRODUCTION

The recent development of analytical methods to detect and enumerate specific enteric viruses has facilitated the design of focused investigations to determine the levels of these viruses in environmental water samples. Accordingly, the Drinking Water Inspectorate (DWI) has identified a need to re-examine the risk to drinking water supplies presented by enteric viruses, specifically norovirus, by undertaking a targeted monitoring exercise at a number of water treatment facilities to determine the concentration of norovirus and other enteric viruses of significance and interest in this context.

Historically, this type of study has proved extremely difficult to undertake. The low and erratic concentrations of enteric viruses in surface waters which could be detected and enumerated only in cell culture (the enterovirus group), meant that the demonstration of a statistically significant decrease ($\log_{10}$ reduction) in numbers throughout a treatment process was almost impossible. However, the recent emergence of sensitive, reproducible and quantitative molecular technologies which generate manageable, quantitative data for a range of viruses, together with cell culture assays to provide a measure of infectivity for some of these viruses, have provided an opportunity to design and execute studies previously beyond the scope of the water virologist.

The scientific objectives of the study were as follows:

1. Select relevant viruses of importance in public health that are likely to be found in source waters at significant concentrations and for which quantitative methods exist
2. Develop existing methods for routine use in appropriate water utility laboratories including consideration of performance and quality control aspects
3. Consider and develop any possible steps necessary to assess infectivity
4. Make a risk-based selection of at least four water treatment works with significant faecal inputs to the source water and where preliminary treatment barriers focus on removal rather than inactivation of pathogens (i.e. excluding works that use prechlorination) so that removal using flocculation and filtration can be studied
5. Choose other routine water quality parameters including conventional faecal indicators and measures of treatment performance for inclusion in the monitoring phase
6. Conduct a year-long monitoring programme of raw and partially treated water from appropriate points in the treatment streams of the selected works
7. Gather relevant hydrometric data such as rainfall, river flow data and sewage effluent flow data for each sampling occasion
8. Interpret the results obtained based on knowledge of the catchment and treatment conditions at the times of sampling and comment on their relevance to informing the risk assessment process

The study was arranged into two main phases: Phase 1(Objectives 1-5) represented the development component of the work. Progress to the second phase of the project (Objective 6), the works monitoring programme, was wholly dependent on the successful completion of objectives 1-5 and the identification, development and validation of appropriate analytical methods. Objectives 7 and 8 remained incomplete as the database for the incidence and levels of the target viruses was considered insufficiently robust to warrant further segregation according to the hydrodynamics and characteristics of the catchment.

The project was initiated in April 2009 and, following unavoidable delays incurred during the first phase of the project, completed at the end of 2012.
3 METHODOLOGY: Phase 1: Selection of target viruses and development of analytical methods

Achieving the objectives of this project required balancing several priorities, including the identification of the relevant target viruses; the development of a series of robust, quantitative, molecular laboratory methods that could potentially be applied routinely in Water Industry laboratories, and the incorporation of an infectivity assay to assess the risk to public health.

3.1 Selection of target viruses

The selection of target viruses was undertaken at the start of the project using a two tier approach incorporating an objective, specific questionnaire completed by members of the Hyder consortium, and a more conventional literature review based partially on the outcome of the questionnaire.

The eligibility of each candidate virus for possible inclusion in the project was assessed against six criteria:

- Public health relevance and significance, propensity for transmission via drinking water
- Prevalence in sewage and UK source waters in terms of predicted and measured concentrations
- Current method status, volume of additional work required to achieve robustness
- The ability to demonstrate infectivity
- Suitability of methods for potential routine use in Water Industry laboratories, technology transfer aspects
- Complexity and cost of analysis

The literature review (DEFRA, 2009a) was undertaken in parallel with the questionnaire and focused upon those enteric viruses which scored highly in terms of the questionnaire and which have been studied previously in the context of the water environment. It also considered various analytical methodologies and their relevance to the isolation, detection and enumeration of the candidate viruses.

Following collation of all the information and consultation with the DWI, norovirus (with an emphasis on GII norovirus) and adenovirus were selected as target viruses in the study.

3.1.1 Norovirus

Norovirus is the most common microorganism affecting humans of all ages and a major cause of epidemic and endemic gastroenteritis worldwide (Atmar and Estes, 2006). Symptoms of infection include vomiting, diarrhoea and nausea lasting 12-72 hours. Symptoms normally occur 24-48 hours following infection and, although the illness is generally self-limiting and the majority of infected individuals recover without any long term health effects, the high incidence of cases can pose a significant cost on society. Numerous community-based outbreaks in which transmission is thought to take place from person to person have been described, and outbreaks caused by contaminated food, particularly shellfish, are well documented (Atmar et al, 1995). Waterborne outbreaks are generally more difficult to recognise and a very extensive outbreak is usually required for medical personnel and authorities to recognise water as a possible source of infection. However, with the development and availability of specific diagnostic methods, together with epidemiological evidence, outbreaks originating from contaminated drinking water, as well as recreational water, are becoming easier to recognise.
and several waterborne outbreaks have been described (Bon et al, 2005; Maunula et al, 2005, Lysen et al, 2009; Werber et al, 2009; Zhou et al, 2012 and the Lilla Edet Outbreak and Wyoming outbreak 2001, reported in the American Society for Microbiology).

Noroviruses are small round structured viruses which form the genus *Norovirus* within the family Caliciviridae. They are non-enveloped, around 30nm in diameter and contain a positive sense, single stranded RNA genome of approximately 7.6kb. Genetically, noroviruses are a highly diverse group (Atmar and Estes, 2006; Svraka et al, 2007). Based on sequence analysis of the genes encoding the viral RNA-dependent RNA polymerase (RdP) and the capsid protein, the *Norovirus* genus is divided into 5 genogroups (genogroups I-V) that can further be divided into genetic clusters, each represented by a prototype virus. Human illnesses can be caused by GI, GII and GIV genogroups, while GIII and GV cause infections only in animals. Noroviruses GI and GII are the most common agents involved in outbreaks. GI has been shown to account for the majority (up to 92%) of reported norovirus gastroenteritis cases and GII accounts for the large majority of the remainder (Lopman et al, 2004). The diversity of norovirus increases continually due to the generation of new variants.

Infections occur throughout the year, but there is a large annual peak during the cold winter months (Mounts et al, 2002). Infected individuals produce norovirus particles in high numbers, and concentrations in stools may reach $10^{10}$ particles per ml. A low infective dose (10-100 virus particles) and a propensity for genetic mutation means that immunity to norovirus is short-lived. Thus, infection and re-infection of individuals ensure that the virus remains in circulation and that the ‘at risk’ epidemiological cohorts are large.

Unlike many other enteric viruses, human norovirus cannot be cultivated and detected using cell culture. In the past, laboratory detection methods depended primarily on electron microscopy (EM) of faecal preparations to diagnose clinical infections. However, the technique was specialist and laborious and failed either to be applied, or to detect the virus in many instances, leading to the under reporting of confirmed cases. The introduction of immunological assays such as the Enzyme-Linked Immunosorbent Assay (ELISA) proved to be a faster and more user-friendly alternative as a clinical diagnostic tool, but neither EM or ELISA were suitable for the detection of norovirus in environmental samples due to the low levels of the target virus and the matrix complexity.

The advent of molecular analytical techniques, namely direct reverse transcription-PCR (RT-PCR) for the detection of norovirus RNA and, more recently, quantitative reverse transcription-PCR (RT-qPCR), has greatly enhanced our understanding of the virus in terms of epidemiology and occurrence and distribution in the environment and elsewhere. However, whilst RT-qPCR is now used routinely to detect and quantify norovirus in terms of ‘genomic copies’ (gc), the analytical data generated give no indication of the ratio of infective to non-infective virus and provide little information on the risk of transmission to susceptible human populations.

Noroviruses are present in very high numbers in wastewater. Average influent concentrations are estimated at around $10^7$ viruses per litre with significantly higher numbers observed during the winter months. However, whilst wastewater treatment processes are able to physically remove some of the norovirus component in sewage, removal statistics are relatively poor for most waste water treatment types.

In a recent study undertaken on behalf of the Foods Standards Agency (Lowther, 2011) which looked at the levels of norovirus in influent (screened crude wastewater) and treated wastewater (final effluent), both GI and GII norovirus were detected in all of the samples tested. For crude samples, the geometric means for GI and GII norovirus levels were 3691 and 25504 gc/ml respectively whilst equivalent levels for final effluent samples, were 202 and 585 gc/ml respectively. Average log reductions from crude to final effluent were 1.26 (range -0.41-2.57) and 1.64 (range -0.53-3.58) for GI and GII respectively. This represents an average reduction
(in terms of detectable norovirus genomic copies) of 1-2 log\textsubscript{10} (90-99\%) from crude to final effluent. The levels of infective virus remained undetermined because of the inability of the analytical methodology to differentiate between infective and non-infective virus particles. Levels of GII norovirus displayed a distinct winter-spring peak, with highest levels recovered between December and May. GI did not display pronounced seasonability although lowest levels were observed during the summer months.

The UK Department for Environment, Food and Rural Affairs (DEFRA, 2011a) have recently reported on a preliminary study to determine the extent of norovirus removal during sewage treatment. A range of sewage treatment processes were investigated, including activated sludge, biomass support and membrane treatment. Samples were collected from each of 5 sewage treatment works as follows:

- Crude sewage influent downstream of screening and grit removal
- Settled sewage downstream of the primary settlement tanks
- Secondary treated effluent pre disinfection
- Effluent downstream of disinfection (where appropriate)

Samples were analysed for GII norovirus and for a range of faecal indicator organisms together with sanitary determinands, BOD and suspended solids.

The data demonstrated that:

- Norovirus concentrations in influent were between ND (Not Detected) and 6545gc/ml
- Norovirus concentrations in final effluent were between ND and 385gc/ml
- Sewage treatment reduced the norovirus load by between 0.89 log\textsubscript{10} (percolating filter) and 2.57 log\textsubscript{10} (high rate activated sludge including uv disinfection)
- Norovirus remained present in 63\% of samples following secondary treatment and in 36\% samples following tertiary treatment
- The scale of norovirus removal was low compared to the level of removal of indicator bacteria
- None of the treatment processes investigated completely removed norovirus

Noroviruses are thought to have physicochemical stability and are relatively resistant to environmental challenge. They retain their infectivity in cold water for up to a year and are able to tolerate temperatures up to 65\degree C for 30 minutes, pH ranges from 2-9 and free chlorine concentrations of 1mg/litre for 30 minutes. In a human volunteer study where human norovirus was spiked into groundwater and stored in the dark at room temperature, the virus was shown to be infective for at least 61 days and remained detectable for over 3 years (Seitz \textit{et al}, 2011).

Clearly, with a high level of infection in the community, there is the potential for public health concern where treated sewage effluent is discharged to rivers that are subsequently used for the abstraction of water for drinking water purposes. Existing multiple barriers associated with water treatment will mitigate this (Werber \textit{et al}, 2009).
3.1.2 Adenovirus

Human adenoviruses are members of the genus *Mastadenovirus* within the Adenoviridae family, which comprises 53 serotypes classified into 7 subgroups (A-G) based on their haemagglutination properties and DNA homology (Vidovic *et al*., 2011). They have a non-enveloped icosahedral shell, are 90-100nm in diameter and contain double stranded linear DNA.

Human adenovirus infections occur throughout the world and they have been cited to cause symptoms in several organ systems including the respiratory system (pharyngitis, acute respiratory disease and pneumonia), eye (conjunctivitis), gastrointestinal tract (gastroenteritis), the central nervous system (meningoencephalitis) and genitalia (urethritis and cervicitis).

The most common adenoviruses (adenovirus 1, 2 and 5) infect 40-60% of children. Members of the subgroup F (serotypes 40 and 41) are responsible for most cases of adenovirus-associated gastroenteritis and are considered to be the second most important viral pathogen of childhood gastroenteritis after rotavirus (Crabtree *et al*., 1997).

After a primary infection, lifelong immunity is conferred for the specific causative serotype. Some strains establish persistent infections, and virus particles may be shed for extended periods of time in faeces, urine and respiratory secretions. Transmission includes the faecal oral route and the inhalation of aerosols and high concentrations frequently detected in sewage (Jiang *et al*., 2001).

Adenoviruses have been shown to be up to 60 times more resistant to UV irradiation than RNA viruses such as enteroviruses and Hepatitis A (Gerba *et al*., 2002; Meng and Gerba, 1996) and have been shown to persist longer than other enteric viruses in a range of different environments. It is thought that because they have a double stranded DNA genome, an undamaged strand may serve as a template for repair by host cell enzymes. In addition, their high molecular weight may confer an increased resistance to environmental conditions: infective adenovirus type 2 has been shown to persist for at least 364 days in spiked groundwater (Charles *et al*., 2009). Chlorination appears to be the only effective method to disinfect AdV (Thurston-Enriquez *et al*., 2003), a CT value of 1.5 was required to achieve 2 log10 reduction of AdV40 in treated ground water at pH 8 and 15°C.

The detection and quantification of adenovirus using molecular methods is routinely achieved using Real-Time PCR (qPCR) and this technology has been applied successfully in a number of environmental virology investigations. As for norovirus, the results of these tests offer no information on the viability and infectivity of the virus, and many adenovirus PCR-positive sample concentrates have proved to be non-infectious, producing no cytopathic effect (CPE) in conventional tissue culture (Choi and Jiang, 2005). However, using adenovirus type 2 spiked into groundwater, Charles *et al* (2009), have demonstrated a consistently strong relationship between PCR measurements and infectivity levels (plaque assay) in tissue culture.

The detection of *infective* adenovirus in water and environmental samples is more challenging. Generally, adenoviruses can be isolated and cultured using various cell lines including BGMK, CaCo2, Hela, Hep-2, KB, A549, PLC/PRF5 and HEK 293. However, the efficiency of viral replication on cell cultures varies according to serotype. Thus, whilst some adenovirus serotypes may be detected by CPE in cell culture, the enteric adenoviruses of interest (adenoviruses 40, 41) which are of particular importance in water quality studies, are slow growing and fastidious and produce little or no CPE on most cell lines.

The limited sensitivity and time-consuming procedure of the cell culture assay for the detection of enteric adenoviruses can be improved by optimising the combination of appropriate cell lines
with molecular technology. This approach (Integrated Cell Culture-Polymerase Chain Reaction (ICC-PCR)) relies upon an initial biological amplification of viral nucleic acid in tissue culture, followed by amplification by PCR. Thus, ICC-PCR combines the sensitivity of PCR and the ability of tissue culture to amplify infective virus particles present in water sample concentrates. ICC-PCR has permitted the detection of infectious virus, even where it normally fails to produce CPE or where CPE takes a long time to appear.

Recent advances in ICC-PCR have made this method both rapid and quantitative, and potentially quite routine. It has been applied successfully to detect infective adenoviruses in environmental samples in studies throughout the world.

Human adenoviruses have been found consistently in domestic sewage throughout the world (Puig et al, 1994; Pina et al, 1998; Formiga-Cruz et al, 2005; Irving and Smith, 1981; Pusch et al, 2005; Sedmark et al, 2005) and there is little evidence of seasonal variability (Pina et al, 1998).

Using qPCR, He and Jiang (2005) observed an average concentration of 8.1 x 10^4 gc/100ml in untreated sewage. In Michigan, Fong et al (2009) recorded an average of 1.15 x 10^6 adenoviruses/litre in raw sewage compared with 2 x 10^4/litre in secondary treated effluent. Adenoviruses were consistently present in sewage and the average removal through the wastewater treatment plant was < 2log_{10}. Again, there was no seasonal trend and these viruses could also be found in rivers receiving effluent (average 7.8 x 10^3 adenoviruses/litre). The predominant serotype was 41 followed by types 12, 40, 2 and 3.

The occurrence of adenoviruses in finished drinking water and tap water has been reported in South Korea and South Africa (Grabow et al, 2001; Lee et al, 2005). However, there were no documented waterborne of adenovirus infection in either region.

The number of reports on the occurrence of adenovirus in sewage, environmental waters and drinking waters has escalated in recent years and this is thought to be due on part, to the advent of PCR for the detection and quantification of the virus. However, many of these PCR-positive samples have proved to be negative in terms of the infectivity assay applied (Greening et al, 2002; Choi and Jiang, 2005). Nevertheless, it has been suggested that adenovirus, with its prevalent association with human sewage, environmental stability and resistance to water treatment processes, could serve as a conservative indicator for assessing viral quality and efficiency of water treatment processes.

### 3.2 Method Development

One of the clear objectives of this project was the identification of robust analytical methods for the detection of the selected target viruses, norovirus (NV) and adenovirus (AdV) which could be potentially accessible to water industry laboratories without the requirement for major capital expenditure and highly specialised technical expertise. The methods would ideally balance accessibility, cost and technical feasibility with robustness and reproducibility.

The ability to detect nucleic acid sequences and the application of this technology to detect those fastidious enteric viruses which have previously remained unstudied because of their inability to grow in the laboratory environment, has made it possible to determine levels of viruses in the water environment and to study their removal during, for example, water and sewage treatment processes. However, whilst the molecular biological approach to the quantification of these viruses in water sample concentrates is relatively straightforward in experienced hands, factors such as sample collection, concentration and purification are of equal importance. The concentration of virus particles from large volumes of water has historically proved to be a major challenge in this context. Recovery efficiencies have been shown to be highly variable, even for a prescribed, validated method, within and between
laboratories, samples and replicates. This variability is thought to derive from a number of sources:

- Difference in operator/laboratory staff, subjectivity in sample preparation and concentration procedures
- Difference in water matrix: pH, suspended solids etc
- Difference in virus type and size, their isoelectric point and association with solids

The identification of an analytically robust concentration procedure was thus a prerequisite for the initiation of a field investigation of this type, not only to ensure that a significant proportion of the indigenous target viruses were captured, but also for use in spike and recovery exercises (process controls) to demonstrate that the method was working consistently. This aspect is particularly important in the context of this project, where virus recovery from a range of water matrices encountered across the different works and treatment stages was expected to differ.

### 3.2.1 Concentration of target viruses

A variety of approaches for the concentration of viruses from water samples have been described. These include the use of both positively and negatively charged surfaces such as membrane filters or glass wool columns in combination with chemical flocculation of protein suspensions (beef extract or skimmed milk), and physical procedures e.g. tangential flow-filtration, ultra-centrifugation and ultra-filtration. Most of these methods include a combination of approaches to obtain a final purified virus concentrate.

For this project, it was anticipated that concentrates from large volumes of water (10-100 litres) would be required to detect the expected low numbers of target viruses in partially treated drinking water. Thus, following an initial literature review, three established concentration procedures were considered and briefly evaluated to determine which approach would be most suitable for the project application in terms of the sample volume to be processed, the ability to co-isolate NV and AdV whilst retaining AdV infectivity, recovery efficiency, method complexity and cost.

Two of the candidate methods involved charged membrane filters and elution with beef extract (ABE and HARA), the third used tangential flow filtration followed by ultra-centrifugation (TFF).

**Method 1: Adsorption/Elution on/off a filter membrane using acidified beef extract (ABE)**

This approach generally involves two stages:

- Stage 1 reduces the original volume from 5-100 litres to between 50 and 500ml
- Stage 2 further reduces the volume to between 2 and 10ml

Several types of filters and filtration methods, such as membrane filters, cartridge filters (electropositive and electronegative), glass fibre filters and glass wool filters have been used traditionally to collect and concentrate virus particles from a variety of water types. The capture of the suspended viruses is effected using adsorption/elution of virus particles on/off the filter matrix. This approach is based on ionic charge and involves the manipulation of charges on the surface of the virus, using pH amendment to maximise their adsorption to charged filters (Katzenelson et al., 1976). Briefly, the virus-containing sample is acidified to pH 3.5 and passed under positive pressure or vacuum over a negatively-charged filter. Under acidified conditions, viruses become positively charged and bind to the negatively charged filter by electrostatic attractive forces as the water in which they are suspended is discarded. Bound viruses are then released/displaced from the filter by elution into a small volume of proteinaceous liquid, for
example, beef extract at high pH (pH 9.5). Further concentration is achieved using isoelectric coagulation (floculation) of the protein by reducing the pH to 3.5. The virus adsorbs to, or is trapped within, the floc or precipitate which is then centrifuged to form a pellet before being dissolved in a small volume of buffer (around 2ml) for storage or processing further.

Method 2: Adsorption/elution on/off cation-coated membrane filters using sodium hydroxide without pH amendment (HARA)

This approach, first outlined by Haramoto et al., (2004), also based on adsorption/elution of viruses on/off the filter matrix, involves two stages similar to those described for ABE. However, because the negatively-charged cellulose acetate/cellulose nitrate filter is pre-coated with Aluminium (Al\(^{3+}\)) ions, there is no requirement to manipulate the surface charge on the viruses by reducing the pH of the water sample. As the water sample passes through the filter membrane, negatively-charged viruses in suspension are bound to the positively-charged filter. Elution of the bound viruses is achieved by rinsing with 200ml H\(_2\)SO\(_4\) (pH 3.0) followed by elution with 5ml 1M NaOH (pH 10.8). As there is no requirement for pH amendment of the bulk water sample, it is possible to process (filter) large volume samples (several hundred litres) with relative ease. The matrices tested by Haramoto et al (2009) included bottled water, tap water, river and pond water. Good agreement was noted in performance for all matrices for poliovirus (PCR and plaque assay) and norovirus (PCR), the highest recovery levels were observed for the cleaner matrices.

Method 3: Tangential Flow Filtration (TFF)

TFF is an alternative approach to adsorption/elution techniques and utilises a flow pattern whereby water sample flow is directed tangentially along the surface of a membrane. This has the effect of continually washing the filter surface, avoiding clogging, as the sample volume is gradually reduced. Particles are retained above the filter surface. This approach requires minimal manipulation of the water sample, samples are processed at ambient pH and an elution step is not required.

Evaluation of candidate methods

A series of laboratory trials were undertaken, using the three candidate methods (Methods 1-3) and a range of relevant water matrices, to determine recovery rates for both of the target viruses. The results from these trials have been reported in detail elsewhere, the summary findings are included below.

Results

**TFF** was found to be slow, requiring several hours to process 10 litres of raw water with the Vivaflow 50 and Vivaflow 200 cassettes. The apparent recovery rates for spiked GII NV in environmental water matrices were poor (<5%). However, it should be noted that the requirement for pre-treatment of the NV stocks had not been fully appreciated or investigated at this stage and the presence of RNA molecules may have affected this estimate. The sample concentrates from TFF proved to be highly inhibitory to subsequent PCR detection of the target viruses. This was not expected and it was suspected that it could be specific to the source water. Whist ultra-centrifugation of the TFF concentrate was found to produce a clean suspension, with recoveries improved to ~25%, the TFF method was subsequently considered impractical for routine use. It was rejected on the basis of 1) equipment availability and processing time 2) the limited volumes capable of being processed 3) the elevated costs (£150.00 per unit) associated with the purchase of cassettes and 4) the high capital cost for purchase of an ultra-centrifuge.

**Methods 1 (ABE) and 2 (HARA)** were subsequently taken forward for evaluation in ‘spike/recovery’ experiments using faecal material positive for NV (GII) and AdV (type 2)
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(obtained from the National Collection of Pathogenic Viruses) and a range of water volumes and matrices. Whilst the biological differences between the two viruses may suggest that they may behave differentially in terms of adsorption/elution characteristics, the co-isolation of both viruses using one concentration procedure was the target outcome for the trial.

Early experiments focused on the recovery of spiked NV from water samples using the HARA method. Initial data proved very encouraging in terms of recovery statistics (up to 80% recovery), and several aspects of the procedure (sample flow rates, virus spike concentration, contact time with eluant and virus stability at specific stages of the procedure) were investigated. However, following the depletion of the original stock NV (necessarily derived from faecal material) used at the start of the trials for spike/recovery exercises, and replacement with alternative stocks sourced from further faecal material, recovery rates became apparently significantly reduced and highly variable. This unacceptable variation was initially attributed to the unreliability of the concentration method but, a significant amount of further investigational work using a range of concentration methods, suggested that the cause of at least some of the variability issues could be attributed to the quality and inconsistency of the faecal material used to produce the NV spike suspension. It became increasingly evident that the production of a standard control material was fundamental in the evaluation of the concentration methods and ultimately in the context of the study. Thus, this aspect was thoroughly investigated and resolved before proceeding with further spike/recovery experiments for NV.

Production of standard control material

Human NV is not culturable and cannot be produced in the laboratory as a standard preparation. Most investigations, including this one, rely on NV derived directly from faecal preparations for the generation of control material. Most studies have used a clarified faecal suspension prepared by suspending a sample of faeces in water to produce a 10% (w/v) suspension, followed by centrifuging at various x g to remove particulate matter and produce a stock suspension of NV (in the supernatant). This ‘stock’ suspension will contain both intact and non intact NV particles. The total amount of NV RNA in these preparations, measured using RT-qPCR, does not differentiate between intact and non intact virus particles.

The pre-treatment of faecal preparations with RNase following dispersal with glass beads was investigated to determine whether the NV levels in a total faecal preparation were intact (resistant to RNase degradation) or a mixture of intact and non-intact/partial NV particles where the non intact particles are susceptible to RNase digestion. The data obtained clearly demonstrated that, whilst in some of the faecal preparations, up to 90% of the total NV RNA was non-intact and susceptible to RNase digestion (40°C for 1 hour), others were relatively unaffected by RNase treatment. It is thought that the ratio of intact to non-intact virus is variable, depending on the faecal preparation and possibly the phase of patient infection at the point of collection. This finding is significant in the context of this project, since the isolation of NV is highly dependent on the surface charge of the intact virus. Thus, faecal preparations containing a high ratio of intact to non-intact NV particles will demonstrate high recovery rates whereas those with low ratios will demonstrate lower rates, even though the total NV RNA detected is the same. This helps explain the variability observed in the recovery rates obtained for different faecal preparations during the early evaluation of concentration methods. In any event, it was clear that, to obtain a homogenous control material from faecal samples and allow the calculation of accurate recovery rates, the non-intact virus particles must first be removed.

Unlike NV, AdV standards for use in spike/recovery determinations can be produced in laboratory cell culture and standard reference material is readily available from the Health Protection Agency (HPA)(NCPV cat 213: AdV type 2). These standards will inevitably contain intact AdV, cellular debris and free AdV DNA. Following the previous experience with NV and, in order to produce a suspension of intact AdV and pre-empt any potentially spurious recovery
data, on receipt from the HPA, AdV suspensions were initially filtered to remove cell debris and then treated with DNase to remove free nucleic acid.

The Standard Operating Procedures: ‘Preparation of human norovirus standards for use as process controls’ (SOP 1) and ‘Preparation of adenovirus standards for use as process controls’ (SOP 2) are presented in Appendix A.

The recognition that the quality and consistency of the virus standards was crucial, together with the ability to produce and characterise standard suspensions for both target viruses, were key milestones in the identification of the concentration method of choice. A series of spike/recovery experiments were then undertaken using both concentration methods.

The ABE approach, which was already known to be compatible with infectivity assays for enteroviruses, out-performed the HARA method for AdV and for NV. Using spiked raw water samples, the qPCR levels for AdV following concentration using the ABE method were consistently >x32 more sensitive (>5 Ct values earlier) than the equivalent levels using the HARA concentration method. (Ct is defined as the cycle number at which the increase in fluorescence (and therefore cDNA) is exponential. This is known as the “threshold” and is set by the analyst. The point at which the fluorescence generated crosses the threshold is known as the Ct). In addition, whilst indigenous AdV and NV were detected in raw water samples following concentration using ABE, they remained not detected using the HARA method. It was also noted that the HARA method employs extreme pH conditions during the elution/concentration of adsorbed viruses (pH 3.0-11.00), which could potentially compromise AdV in terms of the ability to infect host cells (infectivity assay). Thus, the ABE method, optimised for the concentration of AdV rather than NV, was subsequently taken forward as the method of choice, and a preliminary exercise was undertaken to evaluate the performance for both target viruses using raw and pre-chlorinated water matrices from one of the identified water treatment works (WTW). The results from this trial are summarised in Table 3.1.

Table 3.1 Recovery of adenovirus and GII norovirus using the acidified beef extract concentration method for water from a single water treatment works. Data values are the mean % recovery of 3 observations.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Matrix</th>
<th>Volume of water (L)</th>
<th>Spike concentration</th>
<th>Recovery %</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdV</td>
<td>Raw</td>
<td>5</td>
<td>2.E+05</td>
<td>16</td>
<td></td>
<td>10.2</td>
<td>20.6</td>
</tr>
<tr>
<td>AdV</td>
<td>Pre-chlorinated</td>
<td>20</td>
<td>2.E+05</td>
<td>55.3</td>
<td>44.6</td>
<td>64.1</td>
<td></td>
</tr>
<tr>
<td>NV</td>
<td>Raw</td>
<td>5</td>
<td>8.E+03</td>
<td>17.4</td>
<td>0.1</td>
<td>30.2</td>
<td></td>
</tr>
<tr>
<td>NV</td>
<td>Pre-chlorinated</td>
<td>20</td>
<td>6.E+03</td>
<td>45.5</td>
<td>38.5</td>
<td>49.9</td>
<td></td>
</tr>
</tbody>
</table>

Differences in recoveries were noted between raw and pre-chlorinated water matrices for both viruses (Table 3.1), with more effective recovery from the cleaner water. This was thought to be due to a combination of factors, including the presence of particulate matter and the co-extraction of inhibitory compounds from raw waters. These recovery data were considered acceptable and were consistent with previous recoveries for this type of method. The ABE concentration method was adopted for the works monitoring programme and is documented fully in Appendix A: ‘Concentration of norovirus and adenovirus from raw and partially treated water’.
3.2.2 Process Controls

In addition to their importance in the evaluation and selection of the virus concentration method (Section 3.2.1), the standard virus preparations were also required for use as Quality Control (QC) material to determine recovery rates for each of the viruses during the Works Monitoring Programme. These ‘Process Control’ samples (1 spiked water sample for each water matrix) were used to evaluate whether or not the concentration method was performing within acceptable recovery rates and also to define the method performance statistics generally.

Virus stocks for use as Process Controls were produced for both NV and AdV following particle removal and RNase/DNase treatment respectively (SOP 1 and 2: Appendix A). Volumes of material sufficient for the duration of the monitoring programme were produced by combining multiple tissue culture supernatants (AdV) or faecal preparations (GII NV) and storing at -70°C in volumes containing between $10^5$ and $10^6$ total target virus particles.

All Process Control samples were processed in parallel to the water samples (collected during the works monitoring programme), but in a physically separate laboratory. Process Control recovery data were used to confirm that the method was functioning acceptably but not to adjust virus concentrations in the final data.

3.2.3 Nucleic acid extraction and purification

One of the main problems associated with the concentration of enteric viruses from large volumes of water into a smaller sample extract, is the simultaneous concentration of substances inhibitory to the analytical detection procedure (real time RT-PCR). In terms of fresh/drinking waters, these substances may include metals, flocculants and humic acids and, where concentrations are high, their presence may result in toxic effects on both the mammalian cells used to detect infective viruses and on RT-PCR amplification either during the reverse transcription stage or during the DNA amplification stage (enzyme inhibition will result in a delay in detection of the target nucleic acid which, in turn, will give an underestimation of the nucleic acid concentration in the sample concentrate). In addition, the beef extract used as eluant to detach viruses from the filter matrix during the adsorption/elution stage, contains organic and inorganic compounds which are also associated with inhibitory effects. Whilst several approaches have been identified to overcome this effect, inhibition remains an obstacle to the isolation and detection of viruses in environmental samples.

Accordingly, a series of trials were undertaken with a range of commercially available extraction kits designed to purify target nucleic acids from sample concentrates.

AdV detection/quantification was achieved directly from sample concentrates. Although the extraction of AdV DNA prior to amplification did remove low levels of inhibitors present in some of the water matrices tested, a portion of the nucleic acid was lost for all of the kits evaluated. This was considered unacceptable, and the inhibition was subsequently overcome by dilution of the sample concentrate: neat and one-tenth diluted concentrates were used directly (addition of 2µl concentrate into a 25µl qPCR reaction volume) according to the documented method ‘Quantification of adenovirus in water sample concentrates using quantitative polymerase chain reaction’ (Appendix A).

Unlike AdV, nucleic acid (RNA) purification prior to NV estimation was essential to overcome inhibition of RT-qPCR. Six commercially available RNA extraction kits were evaluated for their efficacy in the removal/reduction of inhibitory substances.

The kits were selected as already in use (NucliSENS and Promega) or as alternatives using different methods of lysis and concentration.
1. **NucliSENS**: validated by Cefas for shellfish work; their preferred method and used for initial work in this project.

2. **Pure Link**: Allows extraction of both RNA and DNA viruses

3. **Dynabeads Silane**: Allows extraction of both RNA and DNA viruses using magnetic beads rather than spin column

4. **Trizol**: alternative lysis/extraction approach; solvent extraction rather than binding to a silica matrix

5. **Qiagen QIAmp**: uses additional carrier RNA for effective extraction/concentration, suggested carrier RNA may reduce non-specific inhibition

6. **Promega**: used at the NLS-Starcross laboratory for initial work

Water samples (raw, pre-chlorinated and pre-clarified) were collected from one of the identified WTW (14, 40 and 7.5 litres respectively), spiked with GII NV standard preparation and concentrated using the ABE method. Aliquots of each concentrate were then extracted using each of the six kits under evaluation. The percentage recovery was calculated by comparing the level of NV added to the concentrate with the level recovered following extraction. The results are summarised in Table 3.2.

### Table 3.2 Evaluation of RNA extraction kits for detection of GII norovirus using RT-qPCR. Data values are the mean % recovery of 3 observations.

<table>
<thead>
<tr>
<th>RNA extraction Kit</th>
<th>Raw</th>
<th>Pre-chlorinated</th>
<th>Pre-clarified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promega</td>
<td>3.5</td>
<td>81.8</td>
<td>33.2</td>
</tr>
<tr>
<td>QIAmp</td>
<td>0</td>
<td>29.4</td>
<td>0</td>
</tr>
<tr>
<td>NucliSENS</td>
<td>0.6</td>
<td>62.1</td>
<td>29.9</td>
</tr>
<tr>
<td>Dynabeads</td>
<td>0</td>
<td>4.4</td>
<td>0</td>
</tr>
<tr>
<td>Purelink</td>
<td>0</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>Trizol</td>
<td>32.5</td>
<td>92.4</td>
<td>64.1</td>
</tr>
</tbody>
</table>

The Trizol kit clearly showed effective recovery when compared to any of the other kits and specifically demonstrated superior performance for raw water concentrates (Table 3.2). This kit was subsequently used throughout the monitoring programme to extract NV RNA from sample concentrates.

### 3.2.4 Determination of the Limit of Detection for target viruses using the acidified beef extract (ABE) concentration procedure

Following the selection of the ABE method to concentrate the target viruses simultaneously, the Limits of Detection (LOD) for each of the water matrices relevant to the study were determined theoretically and practically for pre-chlorinated water samples.
Determination of the theoretical LOD

The theoretical LOD (tLOD) calculations considered are based on the detection of 1 viral gene copy within a qPCR well (although it is recognised that several factors may affect the ability to place target nucleic acids in to the [RT]qPCR well). Arguments exist to justify the detection of less than a single copy (amplification may be adversely affected by inhibition etc., resulting in amplification efficiencies of less than 100%), but such scenarios are neither considered nor discussed here.

Following the successful amplification of a single target viral gene copy, the LOD is influenced by:

- The volume of nucleic acid preparation used per amplification well (including dilutions)
- The number of wells used per sample
- The proportion of the total sample concentrate that is represented by each amplification (including extraction and purification if appropriate)
- The original sample volume: volume of water concentrated

For this project, the following protocols were most commonly used:

**Norovirus**

5µl RNA extract per amplification well

3 amplification wells per sample

Neat (x1) and one-tenth (0.1) dilutions

100µl volume of extracted RNA

100µl of sample concentrate extracted

Approximately 4 ml of sample concentrate (actual volume determined by weighing)

Thus, assuming a tLOD of 1 gene copy (gc) in any one amplification well:

For undiluted RNA: 1 gc x (100µl RNA prep/15µl RNA template x 1 dilution) x (100µl RNA prep/100µl extracted concentrate) x (4000 µl total concentrate/100µl extracted concentrate)

\[ = 1 \times 6.67 \times 1 \times 40 = 267 \text{gc in total sample concentrate} \]

If this sample concentrate derived from 5 litres of water, this would equate to 53gc/litre in the original water sample.

The 4ml sample concentrates obtained during the monitoring programme typically represented 5 litres of raw water, 10 litres of partially treated water or 20 litres of pre-chlorinated water. Thus, the tLOD values for NV for each water matrix were 53gc/litre (raw water); 27gc/litre (partially-treated water) and 13gc/litre (pre-chlorinated water).

If one tenth dilutions of the RNA preparation are used to calculate data points (to help overcome inhibition of the RT-qPCR process), this would raise the tLOD by a factor of ten.

**Adenovirus**

2µl sample concentrate per amplification well
3 amplification wells per sample

Neat (x1) and one-tenth (0.1) dilutions

Approximately 4 ml of sample concentrate (actual volume determined by weighing)

Using the same approach and assumptions as for NV, the tLOD values were determined for AdV as: 133 gc/litre (raw water); 67 gc/litre (partially-treated water) and 34 gc/litre (pre-chlorinated water).

The tLOD can be amended in each case by increasing the volume of sample concentrate analysed by i) increasing the volume of RNA or DNA template analysed or ii) by increasing the volume of water concentrated. However, with the second approach, there is a risk of increasing the concentration of chemicals inhibitory to enzyme activity which will ultimately compromise the copying/amplification process. Thus, to optimise the method for routine application in the context of water treatment and public health information, consideration may be given to increasing the number of qPCR/RT-qPCR wells used per sample, to effectively increase the volume of sample analysed and optimise the detection of any viruses present.

**Determination of the practical LOD**

Practically-determined limits of detection (pLOD) are of most use for an analytical method which is in routine use as they assess performance of the method within an individual laboratory. For this project, a series of pre-chlorination volumes of water were challenged (spiked) with measured numbers of target virus particles (AdV and NV: GI and GII) and concentrated and analysed using the adopted method. The procedure followed the standard approach used throughout the monitoring phase, with 5 replicate volumes of 20 litres pre-chlorinated water being processed for each of 3 concentrations (spike levels) of each virus type.

The concentrations of the virus stocks for use as spike material were estimated prior to preparation and use, but were also measured in parallel to the LOD samples as a confirmation of actual numbers. While the estimated and measured virus numbers were in good agreement for AdV spikes, there were detectable differences for both GI and GII NV (Table 3.3). The measured GI spike was considerably higher than the expected measurement and consequently, the full pLOD range could not be determined. The measured GII spike was lower than expected, but the pLOD range could still be determined (Table 3.3).

<table>
<thead>
<tr>
<th>Expected</th>
<th>Measured</th>
<th>AdV</th>
<th>NV:GI</th>
<th>NV:GII</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^3</td>
<td>5.07 x 10^3</td>
<td>1.29 x 10^4</td>
<td>2.13 x 10^3</td>
<td></td>
</tr>
<tr>
<td>1 x 10^3</td>
<td>1.07 x 10^3</td>
<td>4.30 x 10^3</td>
<td>3.40 x 10^2</td>
<td></td>
</tr>
<tr>
<td>5 x 10^2</td>
<td>2.78 x 10^2</td>
<td>3.62 x 10^3</td>
<td>1.74 x 10^2</td>
<td></td>
</tr>
</tbody>
</table>

The frequency of replicates at which inoculation, recovery and detection was achieved for each spike level is summarised in Table 3.4, with limits determined for AdV and for GII NV. The qPCR data from the upper limit of the range enabled quantification; the data from the lower limit of the range did not. The practical LOD for the method as a whole was higher than the theoretical LOD (Table 3.4).
Table 3.4  
Practical determination of detection limits for the three target viruses (gc/litre)

<table>
<thead>
<tr>
<th>Expected</th>
<th>AdV</th>
<th>NV:GI</th>
<th>NV:GII</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5 \times 10^3$</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>$1 \times 10^3$</td>
<td>4/5</td>
<td>5/5</td>
<td>4/5</td>
</tr>
<tr>
<td>$5 \times 10^2$</td>
<td>1/5</td>
<td>5/5</td>
<td>1/5</td>
</tr>
<tr>
<td>pLOD</td>
<td>280&lt;pLOD&lt;1000</td>
<td>0&lt;pLOD&lt;3600</td>
<td>175&lt;pLOD&lt;340</td>
</tr>
<tr>
<td>tLOD</td>
<td>34</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

The ABE concentration procedure results in virus concentrates of around 4ml volumes. In this study, according to the documented method, only a fraction (generally <150µl or <4%) is actually analysed for virus content. Thus, it follows that the bulk (around 96%) of the original concentrated water sample is never actually analysed for the target viruses. Thus, if the virus concentrate is non-homogenous, it is conceivable that both the detection rate and the LOD determined may be inaccurate and potentially misleading.

The standard methodology used throughout this project involves the analysis of 3 replicate wells of 5µl (NV) or 2µl (AdV) nucleic acid preparation generating the detection limits summarised in Table 3.4. It was considered likely that increasing the volume of sample concentrate analysed would increase the probability of detecting each virus at the practical LOD. Accordingly, a series of trials was undertaken using the prescribed documented volumes and replicates and a further set of the same sample concentrates using increased volumes and number of replicates. The data generated were subsequently processed according to the approach described by Cannon (2001) to determine the number of qPCR wells required to be 95% confident of detecting any of the three virus types at the practical LOD. Thus, the number of replicate qPCR wells required for a 95% certainty of detecting each virus type at the practical LOD are 9, 5 and 9 for AdV, GI and GII NV respectively. As noted previously, the pLOD for NV GI was not fully determined, which explains why the recommended number for NV is reduced; practically, it is assumed that 9 wells should be considered for all viruses. Although this approach was not used during the monitoring programme, it is recommended that these replicate numbers are adopted in a routine laboratory context as feasible and achievable.

3.2.5 Reproducibility: Inter-laboratory comparison of qPCR data for the quantification of NV in a range of sample concentrates

As part of the method evaluation procedure, a variety of sample types (sample concentrates) were processed in both the Cefas and Starcross laboratories to determine whether or not the qPCR stage of the method for NV could be reliably reproduced. Sample concentrates were processed in parallel on four separate occasions throughout the duration of the project, and included nucleic acid standards, faecal preparations, concentrates from spiked water samples (from one of the treatment assets), and spiked concentrates generated during the works monitoring programme (process controls). Although as a matter of interest, some samples were measured for GI norovirus, the majority (98%) of the parallel samples targeted GII norovirus.

Data generated from the qPCR assays from both laboratories were expressed in the same units and transformed ($\log_{10}X$) prior to comparison.

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A significant correlation was obtained for all of the comparisons, including the dataset containing all of the data representing a variety of sample types and analytical approaches. As the full dataset contained samples processed using different extraction procedures etc, relevant subsets of the data were compared to minimise the variables.

\[ y = 0.92x + 0.14 \]
\[ R^2 = 0.92 \]

Figure 3.1 NUCLEIC ACID STANDARDS: Relationship between RT-qPCR standards between the two laboratories (Cefas and Starcross) Solid markers represent DNA standards (N=18); open markers represent RNA standards (N=4). Solid line represents the data regression equation; dashed line represents an exact data match.

The most relevant comparisons; nucleic standards only and environmental/works water sample concentrates are presented in Figures 3.1 and 3.2 respectively. It is clear from Figure 3.1 (R-sq = 0.92 n=22) that the standards used during RT-qPCR for estimating NV concentrations in works samples concentrates perform consistently well in both laboratories (a relationship close to 1:1, and an intercept value suggesting a difference of only a few molecules from zero: Figure 3.1). This is significant and encouraging as the method used to produce the standards was different in each laboratory: The production of DNA standards at Cefas utilises plasmid preparations of cloned sequences, extracted, purified and quantified in-house. In contrast, DNA standards used at Starcross are artificially synthesised DNA Ultramers. Likewise, the method of production of the RNA standards differed between laboratories, with RNA transcripts produced by *in vitro* transcription from linearised plasmid using RNA promoter sites upstream of the insert (Cefas) or RNA transcripts produced by *in vitro* transcription from DNA Ultramer standards using a hybridised T7 promoter oligonucleotide to initiate transcription (Starcross).

The close agreement between standards is encouraging as, in addition to the differences in standards, there were small differences in the qRT-PCR amplification conditions (Appendix C).
Figure 3.2  WORKS SAMPLES CONCENTRATES: Relationship between estimates of norovirus concentrations in environmental/works water sample concentrates between the two laboratories (N=45). Solid line represents the data regression equation; dashed line represents an exact data match.

Quantitative data from the works samples concentrates only, demonstrated a weaker relationship (R-sq = 0.77 n=45; Figure 3.2). Samples were exchanged between the two laboratories on three separate occasions (at the start of the project, during the monitoring phase and at the end of the monitoring phase). The data presented here represent all three occasions and compare the greatest number of samples. The data for sample concentrates generated during the works monitoring phase varied between laboratories the most, but no explanation for the difference in performance was identified. Accordingly, there was no reason to exclude this sub-set of the data but the discrepancy is noted.

3.2.6 Assessment of infectivity: The detection of infective AdV particles using Integrated Cell Culture-Polymerase Chain Reaction (ICC-PCR)

PCR methods, like many other genome-based technologies, report the presence of genetic material in the target organism, but offer no information on the viability and infectivity of the organism.

Currently, the combination of PCR and cell culture offers the best approach to assess virus infectivity, including the detection of slow growing viruses such as human adenoviruses. This technique, Integrated Cell Culture-Polymerase Chain Reaction (ICC-PCR) permits the detection
of infectious virus, even where it normally fails produce cytopathic effect (CPE) or where CPE
takes a long time to appear, and relies upon an initial biological amplification of viral nucleic acid
in cell culture, followed by amplification and detection via PCR.

ICC-PCR may be used to assess the virus content of an inoculum or sample concentrate either
in terms of presence-absence or as a semi-quantitative measure of the number of infective
viruses present. Although the development of a quantitative method was not prescribed or
pursued as a major objective of this project, the opportunity to explore the possibility was
presented and explored accordingly. The approach adopted here is based on the protocol used
in the Virobathe study for the estimation of presence or absence of infective AdV in fresh and
recreational waters (Virobathe, 2007).

A semi-quantitative estimate of infective virus numbers in water sample concentrates was
achieved using a standard curve constructed by inoculating tissue culture flasks containing
confluent A549 cells with a range of AdV concentrations (10^2-10^5 HPA stock: AdV Type 2
NCPV:213) measured by qPCR. These concentrations were designated T0: initial inoculum.

Following incubation at 37°C for 1 hour to allow the attachment of infective AdV particles, the
inocula were removed and replaced with maintenance medium. The flasks were then incubated
for a further 5 days to allow the infective AdV to replicate within the host cells, and then,
following lysis of the cell monolayer and release of the virus, the level of virus in each flask was
measured by qPCR and recorded (T5: final concentration). The difference in estimated virus
concentration (T5-T0) following incubation for each inoculum level was then plotted against the
initial inoculum level (measured by qPCR of HPA virus stock) (T0). This relationship is presented
in Figure 3.3. Thus, the concentration of AdV in a water sample concentrate may be determined
using the linear regression equation: y= 1.12x + 4.53. The concentration of AdV following
incubation in cell culture after 5 days (estimated by PCR)( y) is imputed into the equation to
obtain a value for x, the concentration of AdV in the original inoculum (sample concentrate).

This linear regression line (R-sq = 0.74, n=9) was also used to estimate the concentration of the
control AdV stock (measured by direct qPCR of the virus suspension) inoculated into each of 5
process control (spiked) water samples from the observed increase in virus number following
infection (Table 3.5). The data demonstrate comparable estimates of AdV numbers, and provide
circumstantial evidence that the stock virus, prepared as described previously (SOP 2 ,
Appendix A) consisted of only intact virus particles. If the outlier data point (Table 3.5:
E/07.03.12, Raw) of 0.12 is excluded, an even closer agreement of mean adenovirus numbers
is observed: 2.92 log_{10} (estimated by direct PCR) and 2.88 log_{10} (estimated by infectivity).
Figure 3.3  The relationship between increase in AdV genome number after infection of A549 cells and 5 days incubation, and the initial inoculum density.

Table 3.5  Estimates of adenovirus number in process control spikes by direct PCR and using the infectivity assay (ICC-PCR) (log_{10} total number of virus particles)

<table>
<thead>
<tr>
<th>Sample origin (Date, matrix) Process control spike</th>
<th>AdV numbers (direct PCR)</th>
<th>AdV numbers (infectivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E (15.06.11, Raw)</td>
<td>3.20</td>
<td>2.38</td>
</tr>
<tr>
<td>E (15.06.11, Raw)</td>
<td>3.45</td>
<td>3.00</td>
</tr>
<tr>
<td>E (07.03.12, Raw)</td>
<td>2.63</td>
<td>0.12</td>
</tr>
<tr>
<td>M (20.07.11, Raw)</td>
<td>2.37</td>
<td>3.14</td>
</tr>
<tr>
<td>M (20.07.11, Post RGF)</td>
<td>2.96</td>
<td>3.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean</th>
<th>2.92</th>
<th>2.33</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>0.43</td>
<td>1.27</td>
</tr>
</tbody>
</table>

The ICC-PCR method is documented fully in Appendix A ‘The detection of infective adenovirus particles using Integrated Cell Culture-Polymerase Chain Reaction (ICC-PCR).’
4 METHODOLOGY: Phase 2 Works Monitoring Programme

The principal purpose of the works monitoring programme was to evaluate virus removal throughout the overall treatment process and across each treatment stage. Secondary purposes were to:

- Assess correlations between virus removal and other measurements
- Assess dependence of virus removal on operational performance parameters

To achieve these purposes, the works monitoring programme was designed to:

- Estimate ‘average’ or ‘typical’ removal rates with adequate precision to inform risk management
- Evaluate removal rates incorporating a time-to-time variability in those rates
- Address the secondary purposes: removal of viruses at different location:time combinations will be compared with other measurements and parameters at those location:times

4.1 Selection of Works

The four Water Treatment Works (WTWs) for inclusion in the monitoring programme were selected at the start of the project to represent a range of water treatment processes incorporating flocculation and filtration. The rationale for the identification of Water Utilities to be approached for possible participation in the project was based primarily on the requirement for representation of a range of geographies and catchment types in both England and Wales. Using these criteria, three utilities were identified and the appropriate individuals were approached to formalise the agreement to participate.

Each utility representative was asked to identify at least one works within their region that was considered ‘high risk’ in terms of the microbiological quality of the source water. Thus, the selection of works was risked-based and considered:

- Historical microbiological water quality data from archive to determine faecal loading in source waters and other potential inputs
- Area of catchment and population density
- Catchment characteristics in terms of human activity and land use parameters
- Proximity of human point sources to abstraction points and volume of input
- Operational and performance statistics
- Drinking Water Safety Plans

In addition, they were asked to supply details of the treatment process, and operational information as appropriate.

The assets subsequently identified for inclusion in the study were one in Wales, company one (Asset ‘C’), company two (Asset ‘M’) company three where two works were studied (Assets ‘E’ and ‘T’) (Table 4.1).
### Table 4.1 Works monitoring programme: Water Treatment assets included in the study

<table>
<thead>
<tr>
<th>COMPANY</th>
<th>ASSET CODE</th>
<th>TREATMENT SCHEDULE</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>C</td>
<td>Impounding reservoir Coagulation Clarification Filtration GAC adsorption</td>
</tr>
<tr>
<td>Two</td>
<td>E</td>
<td>River abstraction Coagulation/Flocculation Clarification Filtration GAC absorption</td>
</tr>
<tr>
<td>Three</td>
<td>M</td>
<td>River abstraction Coagulation/Flocculation Clarification Filtration Ozonation GAC adsorption</td>
</tr>
<tr>
<td>Four</td>
<td>T</td>
<td>River abstraction Ozonation Coagulation DAF GAC adsorption</td>
</tr>
</tbody>
</table>

### 4.2 Monitoring programme

Each WTW was sampled on 5 occasions on alternate months during the period June 2011 to March 2012 (inclusive) (Table 4.2) and, on each sampling occasion, water samples were taken from each of the sample points (treatment stages) identified as relevant for each works (Table 4.3). For the purpose of the subsequent data analysis, the sampling dates were divided into 5 periods (A,B,C,D and E) within which, each works was represented (Table 4.2).
Table 4.2 Works Monitoring Programme: Sampling timetable

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Period</th>
<th>C</th>
<th>E</th>
<th>M</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>07/06/2011</td>
<td></td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15/06/2011</td>
<td></td>
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Table 4.3 Works Monitoring Programme: Sample point locations for each works

<table>
<thead>
<tr>
<th>ASSET (WTW)</th>
<th>TREATMENT STAGE/SAMPLE POINT</th>
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<tr>
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<td>RAW</td>
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</tbody>
</table>

On each of the sampling occasions, samples were taken over an approximate 3 hour period, raw waters (sample point 1) followed by the others in chronological order to pre-chlorination (sample point 4).

At each sample point and, on each sampling occasion, samples of raw and partially treated water were taken for subsequent NV and AdV analysis (triplicate samples) (Table 4.4). The water volumes processed varied according to treatment stage: 5 litres (raw water), between 10 and 20 litres (depending on the chemical nature of the water in terms of compatibility with the filter matrix) for mid-process samples, and 20 litres for pre-chlorination samples (sample point 4). Samples were also taken for F-specific coliphage analysis (1 sample), and for a range of bacteriological and chemical parameters as measure of treatment performance and to establish any possible relationship between the removal of the target viruses and the removal of other analytes (1 sample; Table 4.4).
Table 4.4 Works monitoring programme: Sample details for each sampling point

<table>
<thead>
<tr>
<th>WATER MATRIX</th>
<th>SAMPLE DETAILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW WATER (Sample Point 1)</td>
<td>NV/AdV: 3 x 5 litres</td>
</tr>
<tr>
<td></td>
<td>NV/AdV Process (positive) Control: 1 x 5 litres</td>
</tr>
<tr>
<td></td>
<td>F-specific coliphage: 1 x 5 litres</td>
</tr>
<tr>
<td></td>
<td>*Chemistry: 1 sample</td>
</tr>
<tr>
<td></td>
<td>**Bacteriology: 1 sample</td>
</tr>
<tr>
<td>PARTIALLY TREATED WATER (Sample Points 2, 3 and 4)</td>
<td>NV/AdV: 3 x 20 litres</td>
</tr>
<tr>
<td></td>
<td>NV/AdV Process (positive) Control: 1 x 20 litres</td>
</tr>
<tr>
<td></td>
<td>F-specific coliphage: 1 x 5 litres</td>
</tr>
<tr>
<td></td>
<td>*Chemistry: 1 sample</td>
</tr>
<tr>
<td></td>
<td>**Bacteriology: 1 sample</td>
</tr>
</tbody>
</table>

*CHEMISTRY PARAMETERS

- Colour (Hazen)
- Conductivity (µS/cm)
- pH (pH units)
- Turbidity (FTU)
- Suspended solids (mg/l)

**BACTERIOLOGY PARAMETERS

- Total coliforms (cfu/100ml)
- E.coli (cfu/100ml)
- Enterococci (cfu/100ml)
- Clostridium perfringens (cfu/100ml)
- Total viable counts 3 days @ 22 degrees C
- Total viable counts 1 day @ 37 degrees C

The samples were transported under the appropriate conditions, at ambient temperature by dedicated courier, to the Environment Agency National Laboratory Service (NLS) Starcross laboratory in Exeter for arrival either the same day (Assets C and M) or next day (am) (Assets E and T) delivery, depending on the geographical location of the water treatment works. All of the samples were analysed for all of the analytical parameters within 24 hours of sampling, irrespective of geographical location. Specifically, for virus analysis, the storage conditions and elapsed time between sampling and sample processing were in accordance with Blue Book recommendations (Anon, 1995, 2010).

4.3 Analysis of water samples

4.3.1 Adenovirus and Norovirus: Concentration of water samples

Each of the replicate samples (generally triplicate, but occasionally more) from each identified sample point-stage destined for NV and AdV analysis, were concentrated using the ABE adsorption/elution method which is described in detail in Analytical Method 1: Concentration of norovirus and adenovirus from raw and partially treated waters (Appendix A). Briefly, each water sample was adjusted to pH 3.2 using 1M HCl and 1M NaOH and filtered, under pressure (1 Bar) through a 0.45µm porosity cellulose nitrate membrane to allow the viruses to adsorb to the filter matrix. The adsorbed viruses were then eluted from the filter using 3% beef extract (pH 9.5) and further concentrated by adjusting the pH of the eluant to 3.3. Following centrifugation at 7000g for 20 minutes, the supernatant was carefully removed and the pellet redissolved in 4 ml of 0.15M disodium hydrogen orthophosphate.
The resulting sample concentrate (approximately 4ml) from each replicate was then divided into four equal aliquots:

1. For NV and AdV determination by qRT-PCR and qPCR respectively
2. For retrospective AdV determination by ICC-PCR where qPCR analysis indicated high levels of AdV DNA
3. For repeat analysis where required
4. As reserve and/or archive

Each aliquot was frozen and stored at -70°C until further processing.

**Quality Control samples**

**Process Controls**

One Process Control (positive control) sample was included on each sampling occasion and for each water matrix (sample point/treatment stage) to demonstrate that the analytical procedure was performing within acceptable limits in terms of recovery statistics. The appropriate volume (5 litres raw waters; 20 litres partially treated waters) of each water matrix (Table 4.4) was spiked with between 10^5 and 10^6 each of NV and AdV particles prepared as described in Appendix A: SOP1; SOP2. These samples were concentrated and stored as described above for the works samples, in a separate laboratory and at the end of the sample processing day.

**Negative Controls**

A negative control or laboratory blank sample, using 5 litres of RO water, was processed at the start of each sample processing run. These samples were processed using the same filtration equipment in advance of the works samples and the process control samples.

**4.3.2 Quantification of Adenovirus and Norovirus using qPCR and RT-qPCR**

**Principle**

Quantitative polymerase chain reaction (qPCR) and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) are two similar techniques used to quantify target virus nucleic acids to enable an estimate of the amount of virus present in a sample. The assays use a polymerase enzyme to ‘read’ target DNA sequences and produce copies. The new copies can then be read again by the enzyme to produce yet more copies (a polymerase chain reaction, PCR). The data are made quantitative (q) by comparing how many cycles of the chain reaction are needed to copy a detectable amount of DNA.

In qPCR, the DNA polymerase enzyme produces DNA copies of DNA target. For AdV (a DNA virus), this is achieved directly from the viral DNA. For NV (an RNA virus), the RNA genome is first copied to DNA before proceeding to the quantitative assay. The RNA to DNA step uses a DNA polymerase enzyme (reverse transcriptase) to reverse-transcribe the RNA to make DNA.

TagMan® qPCR and RT-qPCR use fluorescently labelled artificial DNA probes that bind to the target nucleic acids. These probes have 2 labels, one at each end. When the probe is intact (at the beginning of the amplification process), excitation light shone into the well containing the PCR mixture causes fluorescent light to be emitted from one of the labels, but this light is captured and quenched by the other label, therefore no fluorescence is emitted overall. During the copying process, the polymerase enzyme degrades the probe, so that excitation light shone into the well containing the PCR mixture does result in emitted (fluorescent) light. This amount
of light is then measured; the more light produced, the more copied DNA is present. By cross-referencing the light emitted from DNA in the unknown (sample concentrate) reactions to light from known (control) reactions with known different amounts of DNA added (standard curve), it is possible to quantify the amount of virus in the original water sample.

Norovirus

The detailed procedure for the quantification of NV in water sample concentrates using RT-qPCR is described in detail elsewhere (‘Quantification of NV in water sample concentrates using reverse transcriptase chain reaction’, Appendix A). Briefly, samples for subsequent qRT-PCR analysis were prepared by RNA extraction of 100µl of the water sample concentrates using TRizol Plus RNA Purification kit (Life Technologies). For both NV genogroup-specific qRT-PCR primer/probe sets, triplicate aliquots of 5µl neat extracted RNA and triplicate aliquots of 5µl1/10 diluted RNA were made up in 25µl final volume with one-step reaction mix (TaqMan® One-Step RNA kit, Life Technologies) in a 96-well optical reaction plate. Wells containing negative extraction controls (water only), RNA and DNA spikes and laboratory blanks were included with each set of samples. NV thermal cycling conditions were 15 minutes at 48°C for one cycle (RT step), 95°C for 10 minutes (activation of ‘Hot Start’ enzyme mix and initial sample dissociation) followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C for both GI and GII genogroups.

The copy number (genomic copies) of NV in the original water sample was determined by comparison of the Ct (Critical Threshold) values obtained for sample concentrates to those of the standard curve. The calculation of the number of genomic copies in the original sample took account of the volume of RNA template and dilution factor, the volume of sample concentrate analysed and the initial volume of water processed.

Adenovirus

The procedure for the quantification of AdV in water sample concentrates using qPCR is described in detail elsewhere (‘Quantification of adenovirus in water sample concentrates using quantitative polymerase chain reaction’, Appendix A). Adenovirus detection by qPCR utilised the PCR primers and TaqMan® probe set of Hernroth et al (2002). There was no requirement for nucleic acid extraction, and AdV DNA was amplified directly from triplicate aliquots of 2µl of neat sample concentrates and ten-fold diluted sample concentrates. Real-time amplification was achieved using standard qPCR buffers and enzyme (TaqMan® Universal PCR Master Mix, Applied Biosystems) in 25µl final reaction volumes (in triplicate) in a 96-well optical reaction plate. The appropriate controls were included for each set of samples. Adenovirus thermal cycling conditions were 2 minutes at 50°C for one cycle (activation of the uracil N-glycosylase), 10 minutes at 95°C (activation of ‘Hot Start’ enzyme mix and initial sample dissociation), 45 cycles of 15 seconds at 95°C and 1 minute at 60°C.

The copy number (genomic copies) of AdV in the original water sample was determined by comparison of the Ct values obtained for sample concentrates to those of the standard curve.

Primer and probe sequences for both NV and AdV assays are provided in Table 4.5.

<table>
<thead>
<tr>
<th>Primer/probe name</th>
<th>Sequence (5’ – 3’)</th>
<th>Original reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI assay</td>
<td></td>
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</tr>
<tr>
<td>QNIF4 (FW)</td>
<td>CGYTGGATGCGNTTCCAT</td>
<td>(based on da Silva et al., 2007)</td>
</tr>
<tr>
<td>NV1LCR (REV)</td>
<td>CCTTAGACGCCATCATTTTAC</td>
<td>(Svraka et al, 2007)</td>
</tr>
</tbody>
</table>
4.3.3 Enumeration of F-specific coliphage

Bacteriophages are viruses that can only infect and replicate within bacteria. Male-specific (F+) RNA bacteriophages infect those members of the Enterobacteriaceae (including coliform bacteria) which possess F pilus. These coliform bacteria generally only produce F pilus if the environment in which they develop is >30°C. Thus, in a temperate climate, coliphages that utilise the F-pili binding sites are often assumed to have originated in faecal material have been used as indirect indicators of faecal pollution in both environmental waters and shellfish. Environmental samples may be analysed for the presence of F-specific bacteriophage either directly from the sample or following concentration, depending on the level of contamination. The concentration procedure is similar to that for human viruses and relies upon pH amendment of the water sample to facilitate adsorption/elution of the virus on/off a cellulose nitrate membrane.

Aliquots of samples, sample concentrates or dilutions were mixed with ‘soft agar’ and the host bacterial culture (Salmonella typhimurium WG49) before being poured onto a basal nutrient plate. As the host bacterial lawn grows to confluence, the male specific (F+) coliphages present in the sample will bind to the developing pilus, causing areas of cell lysis which appear as circular plaques in the bacterial lawn. The plaques were counted and each plaque was taken to be the product of one coliphage. Thus, the number of coliphages in the original sample was readily calculated. RNAse is included in the agar in a parallel set of plates to eliminate the RNA phages, thus acting as a confirmatory step.

4.3.4 Bacteriological analysis

Bacteriological analysis of water samples was undertaken using standard procedures and UKAS accredited methodologies. For reference, method summaries are included in Appendix B for the enumeration of total coliforms, E.coli, enterococci*, Clostridium perfringens** and total viable counts (TVC: following incubation at 22°C for 3 days and at 37°C for 1 day).

* used in initial phase of works monitoring programme ** used subsequently as a more appropriate analyte
4.3.5 Chemical analysis

Chemical analysis of water samples was also undertaken using UKAS accredited methodologies. An outline of the methodologies used to measure pH, conductivity and turbidity (automated analysis), colour by spectrophotometry and suspended solids by filtration is included in Appendix B.
5 RESULTS

The raw analytical data generated during the Works Monitoring Programme are presented in Appendix C.

5.1 Virus Assays: Adenovirus and Norovirus

5.1.1 Presence/Absence of Viruses: seasonal distribution

Across all WTWs and water matrices, 34% (81/239) and 19% (43/225) of the total number of samples submitted throughout the monitoring programme were positive for AdV and NV (GII) respectively. Thus, in a high proportion of samples submitted for virus analysis, both AdV and NV remained undetected.

For all WTWs combined, 73.5% (50/68) and 20.3% (13/64) of raw (sample point 1) and final (pre-chlorinated) (sample point 4) water samples respectively were positive for AdV (Table 5.1 and Table 5.2) Similarly, 24.2% (16/66) of raw and 13.3% (8/60) of final waters were positive for NV (Table 5.3 and Table 5.4).

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<td>16/16</td>
<td>8/19</td>
<td>50/68 = 73.5%</td>
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### Table 5.2  Seasonal (monthly) distribution of Adenovirus positive water samples across all WTWs: Final (pre-chlorinated) waters: sample point 4 (positive samples/total number of samples)

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<td>3/17</td>
<td>3/15</td>
<td>13/64 = 20.3%</td>
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### Table 5.3  Seasonal (monthly) distribution of Norovirus positive samples across all WTWs: Raw waters: sample point 1 (positive samples/total number of samples)

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<tr>
<td>All</td>
<td>3/16</td>
<td>2/15</td>
<td>8/16</td>
<td>3/19</td>
<td>16/66 = 24.2%</td>
</tr>
</tbody>
</table>
Table 5.4  Seasonal (monthly) distribution of Norovirus positive samples across all WTWs: Final (prechlorinated) waters: sample point 4 (positive samples/total number of samples)

<table>
<thead>
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<td>8/60  = 13.3%</td>
</tr>
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</table>

Although there was apparently little evidence of seasonality in the incidence of AdV-positive raw water samples, AdV was consistently detected in raw waters throughout the monitoring programme and at each of the 4 WTWs (Table 5.1).

In contrast, there was clear evidence of seasonality in the incidence of NV- positive raw water samples (Table 5.3). For all WTWs combined, 24.2 and 13.3% of raw and final (prechlorinated: sample point 4) water samples respectively were positive for NV (Table 5.3 and Table 5.4). Of the 16 raw water samples positive for NV throughout the period of investigation, 15 (94%) of these occurred between December and March (Table 5.4). Interestingly, some evidence of seasonality was also demonstrated for NV in partially treated waters and, of the 8 samples positive for NV throughout the monitoring programme, 5 (62%) of these occurred during the same period in Jan- Mar (Table 5.4).

The data relating to the percentage positive AdV and NV samples in terms of treatment stage and sampling date are presented in Figure 5.1.
There was statistically significant evidence (Fishers exact probability test, \( p < 0.001 \)) that the proportion of AdV positive assays was greater in raw water (Stage=1; \( 50/68=74\% \)) than at later stages (\( 31/171=18\% \)). However, there was no statistically significant evidence of differences between stages 2, 3, and 4 (Figure 5.1).

For raw waters, there was statistically significant evidence (Fishers exact probability test, \( p=0.01 \)) that the proportion of NV positive assays was greater in period E (28 Feb to 27 Mar, \( 7/13=53.8\% \)) than in earlier periods (7 Jun to 14 Feb, \( 9/53=17.0\% \)) (Figure 5.1).

Data presented in Figure 5.1 suggest that the proportion of NV positive samples for raw water (Stage 1) is greater than for later stages, at least for the winter months. However, the evidence...
for difference between stages does not reach statistical significance. Over all periods, the overall proportion of NV positives in raw water (Stage=1; 16/66=24%) is reduced by the low incidence recorded in earlier periods (June-February), so that the difference between raw and treated waters (27/159=17%) does not reach statistical significance (Fisher's exact test, p=0.26). In considering period E (Feb-March) alone, the proportion of NV positive assays in raw water is greater (7/13=54%) than the proportion at later stages (11/33=33%), but the total numbers of assays are fewer and again, the evidence does not reach statistical significance (p=0.315).

Failure to find statistically significant evidence for differences (between dates and/or between stages) does not prove there are no differences. Random “noise” in the data set will be substantial, so that large numbers of observations may be required to demonstrate small differences. If the incidence in raw water is low, then any reductions at later stages must be correspondingly small.

5.1.2 Quantification: the reduction of virus numbers during the water treatment process

There is a substantial loss of information in regarding the virus assay results as simply positive/negative. The overall effect of a water treatment process may be better described by considering numbers (average virus concentrations) and differences in concentrations in water samples taken at different points within a specific water treatment regime. The application of qPCR and RT-qPCR to quantify the levels of AdV and NV respectively in water sample concentrates allows an estimation of the numbers of each virus in terms of genomic copies (gc) at each stage during the water treatment process. A comparison of numbers (gc) recorded for samples at each treatment stage may then be used to estimate the change/reduction in virus concentrations attributed to the water treatment process.

Figure 5.2 demonstrates the effect of each individual water treatment process (works C,E,M,T) on the concentration of AdV and NV in terms of treatment stage. For each works, the levels of AdV were generally reduced from between 2 log10 and 4 log10 (raw waters: stage 1) to below the limit of detection for waters pre-disinfection (stage 4).

For NV, there appeared to be no significant effect of treatment (Figure 5.2). The levels of NV in raw waters were often so low that the demonstration of a significant reduction in numbers was impossible. However, in consideration of the limited database, the apparent failure to detect a treatment effect in the context of this monitoring programme is not evidence enough to conclude that there is no treatment effect. There is no evidence to suggest that Norovirus would react differently to Adenovirus in terms of its ability to be physically removed (by coagulation stages etc) by the treatment process.
Figure 5.2  Observed levels (gc/litre) of ADV and NV in relation to treatment stage within each WTW (C,E,M,T). Not Detected (ND) values treated as LoD (Limit of Detection)/√2 for plotting and calculation of means.
5.1.3 Model building: Maximum likelihood fit

The primary objective of this project was to determine the effect of the water treatment process on the levels of AdV and NV. The factors influencing the concentration of both target viruses at any point are potentially:

**Site** (1 of 4 WTWs);

**Treatment stage** (1, 2, 3 or 4) within each site and

**Date** (time of year).

It was considered likely that there was an interdependency of these factors, the effect of each one being dependent on the other(s). However, the factor of principal interest in this context was treatment stage.

In order to assess the influence of each of the factors involved in the reduction of virus numbers and specifically to determine whether or not there was a significant reduction in virus numbers attributable to the water treatment process (treatment stage), it was appropriate to construct a model, which considered the interdependency of the identified influencing factors, and apply it to the database:

\[ \text{Virus level} \sim \text{Date} + \text{Site} + \text{Site:Stage} + \text{Date:Site} + \text{Date:Site:Stage} \quad \text{Equation 1} \]

Conventionally, ANOVA would be used to fit this model, choosing coefficients (group means) to minimise the sum of the squared errors between fitted and observed values (“least squares” fit). However, in this database, substantial numbers of observed values were “not detected” (Section 5.1.1) i.e. the concentration of viruses was unknown but below the Limit of Detection (LOD); \( x < \text{LOD} \);

Although these censored data do not have exact values (or associated errors), it is possible to calculate the likelihood of seeing the data, conditional on a set of coefficients. A model can be fitted by choosing coefficients (group means) to maximise the likelihood of the observed data (maximum likelihood fit). The ‘survreg’ function in the survival package\(^1\) in R version 2.15.0\(^2\) was used to fit Equation 1 to the data. Virus LOD values were taken as the minimum reported numerical values, 6gc/litre for AdV; 2gc/litre for NV which are broadly similar to the estimated pLOD values (Section 3.2.4).

Maximum likelihood fitting requires the assumption of a frequency distribution associated with the variation between replicates, within a Date:Site:Stage combination. Probability plots of the residuals from the model (for uncensored data where residuals can be calculated) demonstrated that the majority of residuals fell within the 95% confidence intervals and the database was thus consistent with a lognormal distribution. It was therefore appropriate to apply the maximum likelihood fit. This was then used to test the statistical significance of the evidence for the effects of the identified influencing factors (Table 5.5).

---

\(^1\) Terry Therneau (2012). A Package for Survival Analysis in S. R package version 2.36-12

The analysis of data using the maximum likelihood fit indicates:

- The concentration of both AdV and NV in water sampled throughout the monitoring programme was dependent on Date (time of year). The effect of Date on virus concentration was much stronger for NV \( (p<0.001) \) than for AdV \( (p=0.026) \)
- There was no significant evidence for the effect of Site (WTW) on the concentration of either AdV \( (p=0.369) \) or NV \( (p=0.614) \)
- It was clear that the levels of AdV were significantly reduced during the water treatment process and the effect of water treatment stage within a WTW \( (\text{Site:Stage}) \) on AdV concentrations was highly significant \( (p<0.001) \)
- The effect of treatment stage within a WTW \( (\text{Site:Stage}) \) on the levels of NV \( (p=0.017) \) was weaker than that for AdV \( (p<0.001) \)
- For both viruses, there was no statistical evidence to suggest that the effect of treatment stage was dependent on the time of year \( (\text{Date:Site:Stage}) \) \( (\text{AdV} \ p=0.6; \ \text{NV} \ p=1.0) \)

The maximum likelihood fit model was used to estimate fitted means and appropriate standard errors for each Date:Site:Stage combination. Because the data are lognormally distributed, the mean number of viruses and associated standard errors have been expressed as \( \log_{10} \) (gc/litre). Thus, for those Date:Site:Stage combinations where all of the replicate samples returned a result of ‘ND’, the estimated \( \log_{10} \) (gc/litre) is arbitrarily low with very large standard errors, typically between 3 and 4. For those combinations where at least one replicate gave a numerical result, the estimated \( \log_{10} \) (gc/litre) was much better determined with much smaller standard errors, typically 0.13 for AdV and 0.4 for NV.

Median fitted values across all sites for each treatment stage and period are presented in Table 5.6.

- AdV levels in raw water were approximately \( 2 \log_{10} \) (gc/litre) in all periods except D (Jan/Feb)\( (\text{Table 5.6}) \). The low median in period D (0.79) is heavily influenced by the “ND” results from Asset ‘E’ and it is considered inappropriate to conclude that there were substantial differences in the levels of AdV in raw waters between periods.
• NV levels in raw water were essentially non-detectable in all periods except Feb/March when levels reached about 1 log_{10}(gc/litre); Table 5.5 indicates that this evidence is statistically significant, the p-value for the effect of Date is < 0.001.

• AdV levels in treated water samples were essentially non-detectable. There was some suggestion of variation between periods (Table 5.6), but the evidence was not statistically significant, the p-value for an interaction between Date and the effect of Stage (within Site) was 0.6 (Table 5.5).

Table 5.6 Median values for AdV and NV across all sites of maximum likelihood fitted mean log_{10} (gc/litre)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Period</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7/6/11-19/7/11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Raw</td>
<td></td>
<td>2.54</td>
<td>2.32</td>
<td>2.85</td>
<td>0.79</td>
<td>3.72</td>
<td>2.68</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1.48</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-2.40</td>
<td>-5.26</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.09</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
</tr>
<tr>
<td>4 Pre-disinfect</td>
<td></td>
<td>1.12</td>
<td>-2.08</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>1.87</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
<td>-5.26</td>
</tr>
</tbody>
</table>

|               | 2/8/11-13/9/11     |    |    |    |    |    |     |
| 1 Raw         |                    | -9.46| -9.39| -4.52| -4.58| 1.04| -9.30 |
| 2             |                    | -9.51| -9.51| -9.24| -0.03| -0.02| -9.24 |
| 3             |                    | -9.40| -9.41| -4.92| -0.03| -9.41| -9.40 |
| 4 Pre-disinfect|                  | -9.52| -9.51| -4.58| -9.40| -0.06| -9.42 |
| All           |                    | -9.51| -9.51| -9.24| -0.39| 0.08| -9.37 |

• The data presented in Table 5.6 suggest that in Feb/March (period E), when NV concentrations in raw waters were highest (median 1.04 log_{10} gc/litre), equivalent concentrations in partially treated waters were lower at about 0. However, the evidence for an effect of treatment is quite weak. Table 5.5 shows a p value for Stage within Site = 0.017 and there is no evidence that this effect depends on Date (p = 1.0). However, the absence of statistically significant evidence for an effect of treatment on NV concentrations should not be interpreted as evidence of no effect. There was generally no detectable NV in raw water (Table 5.6), making it practically impossible to detect any effect of treatment.

5.1.4 Process (Positive) Controls

Process Control samples were included on each sampling occasion to confirm that the concentration procedure was performing acceptably. It was considered that consistently poor recovery rates (<10%) would potentially invalidate the associated works monitoring data.

The overall recovery rates (and data distribution) for NV and AdV are presented in Figures 5.3 and 5.4 respectively and in Appendix C. Thus, whilst recovery rates for process control samples were acceptable for both viruses throughout the monitoring programme and the results for the works samples were accepted as valid, it was clear that the overall recoveries for AdV exceeded those for NV, for all sites, time and matrices.
The percentage recoveries for NV and AdV process control samples in relation to site, time of year and sample matrix were used to determine whether high recoveries of one virus were associated with high recoveries of the other.

![Boxplot of Norovirus, overall recoveries](image)

**Figure 5.3** Boxplot representing the median percentage recovery for norovirus process control spikes according to sample site, time of year and matrix

The data were also analysed to determine whether or not recovery rates were influenced by site, date or matrix. Figures 5.3 and 5.4 suggest that there was no relationship either overall or when the data were subdivided by site, date or matrix. Correlation coefficients were calculated following an arcsine square-root transformation of the data. In addition, the significance of the overall correlation coefficient was calculated using a likelihood ratio (the difference between the probability of obtaining the observed results under the logistic model and the probability of obtaining the observed results in a model with no relationship between the independent and dependent variables).

No significant correlations were observed for the data, either using logistic regression on the overall dataset (P=0.97, odds ratio), or from linear regression of the transformed data, subdivided into different categories. The highest R-square values were noted when data were subdivided by matrix, for the first and second stages of the treatment process (R-sq=0.31, n=19; R-sq=0.38, n=14 respectively). Overall, there was no clear evidence to suggest that the spike recovery rates for the two virus types were related. Thus, the recovery of each virus was not influenced by date, site or water matrix.
5.1.5 Assessment of infectivity: Quantification of infectious adenovirus particles in sample concentrates using ICC-PCR

A selection of water sample concentrates generated from the works monitoring programme which demonstrated high levels of AdV with qPCR, were submitted for ICC-PCR analysis (Table 5.7) to determine whether or not the AdV particles detected were infective.

<table>
<thead>
<tr>
<th>ASSET CODE</th>
<th>SAMPLE DATE</th>
<th>SAMPLE MATRIX</th>
<th>qPCR measurement+ gc/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>29.02.2012</td>
<td>Raw (2)</td>
<td>5413</td>
</tr>
<tr>
<td>E</td>
<td>15.06.2011</td>
<td>Raw (2)</td>
<td>873</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post Actiflow (2)</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post RGF (2)</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post GAC (2)</td>
<td>25</td>
</tr>
<tr>
<td>E</td>
<td>07.03.2012</td>
<td>Raw (1)</td>
<td>6479</td>
</tr>
<tr>
<td>M</td>
<td>20.07.2011</td>
<td>Raw (3)</td>
<td>4285</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post ozonation (3)</td>
<td>1966</td>
</tr>
</tbody>
</table>

Figure 5.4  Boxplot representing the median percentage recovery for adenovirus process control spikes according to sample site, time of year and matrix
* sample concentrate from 1 of the replicate volumes of water

+ obtained previously using qPCR analysis

None of the sample concentrates which had previously recorded the presence of AdV using qPCR (up to $10^3$ genomic copies per litre) demonstrated the presence of infective virus. However, the process control samples concentrated alongside these samples (Table 3.1) demonstrated the presence of infective AdV, proving that the ICC-PCR assay was compatible with the detection of AdV in concentrates from different water matrices.

5.2 Microbiological analysis

5.2.1 F specific coliphage

F specific coliphage were generally only detected in raw waters and, many of the results obtained for subsequent treatment stages, were at or below the limit of detection (Figure 5.5), with the exception of asset ‘M’ where they were detected in low concentrations before the final treatment stage (post GAC).

![Figure 5.5](image)

**Figure 5.5** The concentration of F specific coliphage in relation to treatment stage; pfu/litre; “<” values plotted at 10 pfu/litre (principal LOD)

F specific coliphage remained undetected in the final (pre-chlorination) waters in all of the works throughout the monitoring period.
5.2.2 Bacteriological parameters

The number of enteric bacteria decreased as the water progressed through the treatment process for all of the works and during each period (A-E). They were only rarely detected in final (pre-chlorination) waters and in very low concentrations (Figure 5.6).

Total Viable Counts (TVC) of heterotrophic bacteria following incubation at 22 and 37°C were, as expected, well above the detection limits (Figure 5.7). Whilst concentrations were relatively high in raw waters, their numbers were generally significantly reduced with treatment stage. The effect of treatment on the levels of TVC varied between WTW, but significant numbers were invariably detected from time to time in pre-chlorination samples at all of the WTW (Figure 5.7).

Figure 5.6 Concentrations of Coliforms, E.coli, Enterococci, and Clostridia in relation to treatment stage; cfu/100ml; “<” values plotted at principal LOD of 1cfu/100ml
Figure 5.7  The concentration of total viable bacteria following incubation at 22°C and 37°C (TVC) in relation to treatment stage; cfu/ml; “<” values plotted at 1 (LOD)

5.3 Chemical parameters

The levels of colour, turbidity and solids were lower at asset ‘T’ than the other sites, and generally below detection limits, even for raw water (Figure 5.8). At the other sites, colour, turbidity and solids measurements were relatively high in raw water, reducing to below the limit of detection in water taken from sampling points further down the treatment process (Figure 5.8).
The data for pH and conductivity are presented in Figure 5.9.

Conductivity measurements for raw waters were characteristic of each site and appeared to be related to time of year (period) or conceivably and simplistically to different days and fluctuating rainfall/environmental conditions. Generally, conductivity measurements were unchanged during the water treatment process (Figure 5.9).
Figure 5.9 The measurements of Conductivity and pH in relation to treatment stage

5.4 Correlations: Relationships between target virus concentrations and of microbiological/chemical parameters

Any potential relationships between the levels of chemical and microbiological parameters and concentrations of the target viruses at different stages of the water treatment processes were explored as follows:

- Correlations between virus concentrations and chemical/microbiological parameters across 73 Site:Date:Stage combinations.
- Correlations between virus concentrations and chemical/microbiological parameters at different sample points (treatment stages).

Virus concentrations were taken as mean values from the maximum likelihood fit (Table 5.6)

Many of the variables and the virus values had distributions which deviated strongly from a Normal distribution so that data analysis using conventional (Pearson product moment) correlation coefficients would be misleading. Accordingly, rank (Spearman) correlation coefficients were used based on the relative order of results without regard to the exact values. The use of this test was also beneficial in that the correlations were unaffected by the choice of values assigned to “not detected” results, which were treated as zero.
5.4.1 Correlations between virus concentrations and microbiological/chemical parameters across 73 Site:Date:Stage combinations

Correlations between virus concentrations and the chemical/bacteriological variables across those 73 combinations are summarised in Table 5.8.

Table 5.8 Correlations between virus assays and chemical/bacteriological parameters. Spearman rho (p value).

<table>
<thead>
<tr>
<th></th>
<th>AdV</th>
<th>NV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV</td>
<td>0.122 (0.303)</td>
<td>0.100 (0.400)</td>
</tr>
<tr>
<td>COLOUR</td>
<td>0.604 (0.000)</td>
<td>0.100 (0.400)</td>
</tr>
<tr>
<td>CONDUCT</td>
<td>0.117 (0.326)</td>
<td>-0.012 (0.920)</td>
</tr>
<tr>
<td>PH</td>
<td>0.463 (0.000)</td>
<td>-0.026 (0.825)</td>
</tr>
<tr>
<td>TURBIDITY</td>
<td>0.468 (0.000)</td>
<td>0.167 (0.159)</td>
</tr>
<tr>
<td>SOLIDS</td>
<td>0.520 (0.000)</td>
<td>0.049 (0.679)</td>
</tr>
<tr>
<td>COLIF</td>
<td>0.534 (0.000)</td>
<td>0.190 (0.108)</td>
</tr>
<tr>
<td>ECOLI</td>
<td>0.490 (0.000)</td>
<td>0.199 (0.092)</td>
</tr>
<tr>
<td>ENTERO</td>
<td>0.179 (0.344)</td>
<td>-0.165 (0.383)</td>
</tr>
<tr>
<td>CLOST</td>
<td>0.693 (0.000)</td>
<td>0.306 (0.046)</td>
</tr>
<tr>
<td>TVC.22</td>
<td>0.408 (0.000)</td>
<td>0.135 (0.253)</td>
</tr>
<tr>
<td>TVC.37</td>
<td>0.481 (0.000)</td>
<td>0.014 (0.909)</td>
</tr>
<tr>
<td>F+PHAGE</td>
<td>0.335 (0.004)</td>
<td>0.178 (0.132)</td>
</tr>
</tbody>
</table>

- There were no statistically significant correlations between NV and chemical and bacteriological parameters; the p value of 0.046 for Clost is regarded as weak evidence in the context of the number of tests (Table 5.8).
- There were many highly significant correlations between AdV and chemical and bacteriological parameters. However, as both AdV levels and chemical/bacteriological concentrations change (reduce) between Stage 1 (Raw water) and subsequent sampling points (Stages 2-4: Sections 5.2 and 5.3), such correlations may be anticipated as a treatment, but not a biological, effect.

5.4.2 Correlations between virus concentrations and microbiological/chemical parameters at different sample points (treatment stages)

The data presented in Table 5.9 (correlations at each stage) show no strong evidence of correlations between AdV and chemical/bacteriological parameters within a stage.
Table 5.9: Correlations between virus assays and chemical/bacteriological parameters at different sample points (Sequence). Spearman rho (p value).

<table>
<thead>
<tr>
<th></th>
<th>ADV NV</th>
<th>ADV Stage = 1 (Raw)</th>
<th>NV Stage = 2</th>
<th>ADV Stage = 2</th>
<th>NV Stage = 3</th>
<th>ADV Stage = 3</th>
<th>NV Stage = 4 (Final)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NV</td>
<td>0.694 (0.001)</td>
<td>-0.271 (0.263)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLOUR</td>
<td>0.294 (0.209)</td>
<td>0.052 (0.826)</td>
<td>0.244 (0.314)</td>
<td>-0.538 (0.018)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONDUCT</td>
<td>0.110 (0.645)</td>
<td>0.121 (0.613)</td>
<td>0.429 (0.067)</td>
<td>-0.175 (0.473)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>-0.383 (0.096)</td>
<td>0.082 (0.732)</td>
<td>0.057 (0.816)</td>
<td>-0.101 (0.681)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TURBIDITY</td>
<td>0.435 (0.055)</td>
<td>0.147 (0.535)</td>
<td>0.048 (0.845)</td>
<td>0.393 (0.096)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOLIDS</td>
<td>0.430 (0.059)</td>
<td>0.008 (0.972)</td>
<td>0.398 (0.091)</td>
<td>-0.081 (0.742)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLIF</td>
<td>0.502 (0.024)</td>
<td>0.385 (0.094)</td>
<td>0.434 (0.063)</td>
<td>-0.033 (0.893)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECOLI</td>
<td>0.417 (0.067)</td>
<td>0.329 (0.157)</td>
<td>0.333 (0.164)</td>
<td>-0.033 (0.894)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENTERO</td>
<td>-0.114 (0.788)</td>
<td>-0.321 (0.438)</td>
<td>0.132 (0.756)</td>
<td>-0.490 (0.218)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLOST</td>
<td>0.630 (0.028)</td>
<td>0.462 (0.134)</td>
<td>0.461 (0.154)</td>
<td>0.382 (0.246)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVC.22</td>
<td>0.498 (0.025)</td>
<td>0.262 (0.264)</td>
<td>0.311 (0.195)</td>
<td>-0.096 (0.697)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVC.37</td>
<td>0.408 (0.074)</td>
<td>0.056 (0.813)</td>
<td>0.389 (0.099)</td>
<td>-0.127 (0.606)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.PHAGE</td>
<td>0.235 (0.318)</td>
<td>0.375 (0.104)</td>
<td>-0.117 (0.634)</td>
<td>-0.103 (0.676)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stage = 3</td>
<td>Stage = 4 (Final)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NV</td>
<td>0.181 (0.537)</td>
<td>-0.147 (0.536)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLOUR</td>
<td>0.114 (0.697)</td>
<td>0.241 (0.406)</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONDUCT</td>
<td>0.297 (0.303)</td>
<td>0.150 (0.609)</td>
<td>0.273 (0.243)</td>
<td>-0.032 (0.894)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>0.163 (0.577)</td>
<td>-0.696 (0.006)</td>
<td>0.072 (0.762)</td>
<td>0.110 (0.643)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TURBIDITY</td>
<td>-0.229 (0.432)</td>
<td>-0.379 (0.181)</td>
<td>-0.237 (0.314)</td>
<td>0.099 (0.679)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOLIDS</td>
<td>0.267 (0.357)</td>
<td>0.000 (1.000)</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLIF</td>
<td>0.185 (0.526)</td>
<td>0.513 (0.061)</td>
<td>0.077 (0.748)</td>
<td>-0.571 (0.009)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECOLI</td>
<td>0.115 (0.696)</td>
<td>0.444 (0.111)</td>
<td>0.014 (0.952)</td>
<td>-0.378 (0.100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENTERO</td>
<td>-0.198 (0.707)</td>
<td>0.198 (0.707)</td>
<td>0.085 (0.842)</td>
<td>-0.439 (0.276)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLOST</td>
<td>0.540 (0.167)</td>
<td>0.247 (0.555)</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVC.22</td>
<td>0.069 (0.816)</td>
<td>0.415 (0.140)</td>
<td>0.118 (0.619)</td>
<td>-0.246 (0.295)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVC.37</td>
<td>0.227 (0.436)</td>
<td>0.475 (0.086)</td>
<td>-0.066 (0.781)</td>
<td>-0.434 (0.056)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.PHAGE</td>
<td>-0.163 (0.579)</td>
<td>-0.009 (0.975)</td>
<td>0.020 (0.932)</td>
<td>-0.188 (0.426)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All values identical

- None of the chemical and microbiological parameter measurements gave useful indications of the levels of viruses (NV and AdV) in raw or final waters, although the changes in levels/concentrations do indicate that treatment has taken place, with an associated decrease in AdV levels.
There was no strong evidence of correlations between AdV and microbiological/chemical parameters within a treatment stage.

In Stage 4 (Final) there appears to be a single statistically significant correlation between COLIF (coliforms) and NV ($p = 0.009$). However, there were only six positive NV results from the 20 samples in stage 4, and all those had COLIF < 1, so the correlation is based upon small numbers of data. Furthermore, Table 5.9 represents a large number of correlations so some spurious low $p$ values are to be expected; thus, in the context of the other correlations for Stage 4, the value of $p = 0.009$ is consistent with this correlation being a ‘false alarm’.

There was strong evidence of an association between NV and AdV in raw water; in all eight Date:Site combinations where NV was detected in raw water, AdV was also detected in raw water. However, as an indicator of NV presence, AdV presence gave some “false positives”; ie AdV was detected in raw water in 18 Date:Site combinations, but in 10 of these, NV remained not detected (Table 5.10). However, it is conceivable that NV may have been present at levels undetectable by the analytical methods available.

Table 5.10  The relationship between the presence of NV and AdV in raw water samples

<table>
<thead>
<tr>
<th>NV</th>
<th>-ve</th>
<th>+ve</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-ve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NV</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>ADV</td>
<td>10</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>All</td>
<td>12</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>
5.5 Works Monitoring Programme: Summary of key findings

The key findings from the works monitoring programme are summarised in Table 5.11.

<table>
<thead>
<tr>
<th>CRITERIA</th>
<th>NOROVIRUS</th>
<th>ADENOVIRUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Raw water samples positive</td>
<td>Detected in 16/66 (24%) of raw water samples (all works)</td>
<td>Detected in most of the raw water samples 50/68 (74%) (all works)</td>
</tr>
<tr>
<td>Comparative incidence</td>
<td>Substantial correlation between the presence of AdV and NV in raw waters: wherever NV was detected, AdV was also detected. However, the converse was not true. AdV conservative indicator for NV?</td>
<td></td>
</tr>
<tr>
<td>Levels in raw waters</td>
<td>Essentially non detectable in all periods except March when levels are around 1 log_{10} gc/litre</td>
<td>Median levels approximately 2 log_{10} gc/litre in all periods</td>
</tr>
<tr>
<td>Seasonality</td>
<td>Strong evidence that incidence in raw waters was higher during Dec to March. 15/16 (94%) occurred in this period</td>
<td>Little evidence of seasonality</td>
</tr>
<tr>
<td>Effect of water treatment process</td>
<td>Evidence for effect of treatment is weak as little detected in raw waters 8/60 (13.3%) positive in pre disinfection waters</td>
<td>Most of the treated water samples were negative (below LOD) 13/64 (20.3%) positive in pre disinfection waters</td>
</tr>
<tr>
<td>Effect of water treatment stage</td>
<td>The proportion of NV +ve samples for raw waters is greater than at later treatment stages for winter months 7/23 (54%); 11/33 (33%). However, the difference between treatment stages is not statistically significant</td>
<td>The proportion of AdV +ve assays in raw waters (50/68=74%) was statistically significantly greater than at later stages (31/171=18%) (20.3% final water). There was no evidence of significant differences between stages 2, 3 and 4 (all sites)</td>
</tr>
<tr>
<td>Correlation with microbiological/chemical parameters</td>
<td>No demonstrable correlation with chemical and microbiological parameters</td>
<td>Several correlations between levels of chemical and microbiological parameters. None are indicators of AdV directly, but do indicate that treatment has taken place</td>
</tr>
</tbody>
</table>
6 CONCLUSIONS

One of the main objectives of this study was to develop and document existing analytical methods for potentially routine use in water utility laboratories (Phase 1). Accordingly and necessarily, the preliminary focus for the investigation was the identification and validation of the most appropriate methodologies that were robust, reliable, fit for purpose and readily adaptable for routine use. A series of analytical methods and standard operating procedures are included in Appendix A.

- The development of Standard Operating Procedures for the preparation of NV and AdV standards was a key milestone in the progression of this project. The importance of a reliable standard preparation of control material cannot be overstated in the context of this type of investigation.
- All of the methods identified could reasonably be transferred to competent water utility laboratories, and the enumeration of NV and AdV could be incorporated either routinely as an additional operational parameter to measure treatment performance and/or to inform risk assessments for Drinking Water Safety Plans, or as additional analytical determinand in investigational work following/during an incident.
- It is suggested that the ABE concentration procedure can be used with confidence to isolate both NV and AdV from raw and partially treated waters. Whilst this method currently appears to be optimised for the recovery of AdV (as suggested by the superior process control recovery data), the recovery data for NV, as assessed by RT-qPCR, remained satisfactory throughout the monitoring programme for all of the water matrices examined.
- Although the AdV isolated and quantified by q-PCR in samples from the works monitoring programme proved to be non-infective when subsequently assessed by ICC-PCR, infective AdV was recovered from spiked (process control) samples, which suggests that the concentration process is compatible with the recovery of infective AdV particles.
- Inhibition of the PCR amplification process caused by metals, flocculants, humic acids and a number of other organic and inorganic substances concentrated along with the target viruses may be overcome using Trizol extraction. This solvent extraction procedure was successfully used to extract NV RNA and remove inhibitory substances from the water sample concentrates, irrespective of sample matrix.
- The recovery rates for the combined concentration/quantification procedure for both target viruses were acceptable and reproducible throughout the works monitoring programme for all of the water matrices examined. The recovery of both viruses was not influenced by date, site or water matrix and there was no clear evidence to suggest that the spike recovery rates for the two virus types were related. The differential recovery rates may be attributed to the difference in size, physiology and surface charge of the two viruses.
- The practical limit of detection (pLOD) for AdV and NV(GII) was 34gc and 13gc respectively.
- The detection and quantification of NV in water sample concentrates using RT-qPCR was shown to be reproducible between competent laboratories.

The Works Monitoring Programme (Phase 2) was originally projected to run over a 12 month period. However, the unpredicted additional work and costs associated with the method development phase of the project meant that an abridged monitoring exercise had to be designed and undertaken within the constraints of a reduced budget. The monitoring programme subsequently ran over 9 months and generated only around half of the data...
originally anticipated to ensure a robust database. Nevertheless, following rigorous analysis, the data were considered sufficiently robust to draw some definitive conclusions:

- Most of the 68 raw water samples (74%) were positive for AdV indicating the persistence and ubiquitous nature of this virus in environmental/abstraction waters. AdV was present in raw waters throughout the year and, whilst the treatment process reduced numbers by between 2 and 4 log_{10}, the virus was able to persist through to the pre-chlorination stage and 20.3% of all samples going forward to disinfection remained AdV positive.

- Removal of AdV occurred mainly at the first stage of treatment (post clarification), thereafter removal was negligible. This is perhaps not surprising, since the coagulation/flocculation stage will facilitate the adsorption of charged viruses on to suspended matter in the water which will then be removed during clarification.

- The AdV isolated and quantified by q-PCR in both raw and pre-chlorination samples subsequently proved to be non-infective when assessed by semi-quantitative ICC-PCR. The apparent incompatibility of the data is discussed further in Section 7.

- In contrast, NV was generally absent from raw waters except during the winter months, December –March when 94% of the samples were positive. This winter ‘peak’ presumably reflects a high prevalence of NV in the community which is consistent with epidemiological evidence. For NV, there appeared to be no significant effect of treatment. However, the levels of NV in raw waters were often below the LOD, and the demonstration of a significant reduction in numbers was impossible. However, in consideration of the database, the apparent failure to detect a treatment effect in the context of this monitoring programme is not evidence enough to conclude that there is no treatment effect.

- There was strong evidence of an association between the presence of NV and AdV in raw water. In the 8 Date:Site combinations where NV was detected, AdV was also detected. However, as an indicator of NV presence, AdV presence gave some false ‘positives’ ie AdV was detected in raw water in 18 Date:Site combinations, NV was not detected in 10 of these. Thus, on the basis of the data presented here, AdV may potentially be considered a conservative indicator for the presence of NV in raw waters, although this conclusion does not consider the following aspects: the concentration procedure adopted was shown to favour the isolation of AdV (Section 3.2.1); the seasonal prevalence of NV in the winter months compared with the apparent year round presence of AdV in raw waters resulted in a comparatively small database for NV; only around 4% of the total sample concentrate was analysed (Section 3.2.4), processing a higher proportion of this could significantly increase both the probability of detection of NV and the numbers of genomic copies enumerated.

- None of the chemical or microbiological parameters (measures of performance) gave a useful indication of the levels of viruses in raw or final (pre-chlorinated waters), although the changes in level/concentrations did indicate that treatment had taken place, with an associated decrease in AdV levels.
DISCUSSION

One of the major limitations of this work is the low concentrations of target viruses observed and a paucity of data, particularly for NV. The protracted amount of time and money necessarily spent on the development and validation of credible methods in Phase 1 resulted in a major amendment in the scope and timescale for the second phase of the study, the works monitoring programme. Nevertheless, we have been able to demonstrate that AdV is consistently removed from raw water during the course of the water treatment process. Whilst the virus was sometimes detected in low numbers by qPCR in pre-chlorinated waters, none of the isolates proved to be infective when transferred to tissue culture.

In contrast, the equivalent dataset for NV proved less convincing in that the virus was only infrequently detected in raw water samples. NV-positive raw water samples with relatively low concentrations were detected exclusively during the winter months and, whilst it was clear that the virus was present, too few samples were positive to demonstrate a reduction in numbers following treatment. It is likely that NV is removed during the water treatment process in the same manner as AdV and other enteric viruses but, given the levels and incidence, large numbers of observations may be required to demonstrate an effect.

One of the key objectives of this project was to determine whether or not the target viruses isolated were of public health significance in terms of their potential to infect human cells. Accordingly, sample concentrates from the works monitoring programme that displayed high concentrations of AdV, as assessed by qPCR, were submitted to an infectivity assay (ICC-PCR) in laboratory tissue culture. Whist the infectivity assay was shown to be highly successful for the detection and quantification of infective laboratory-derived AdV (process control sample concentrates), infective AdV remained undetected in all of the sample concentrates from the monitoring programme, suggesting that the isolates positive by qPCR were non-infective.

The AdV detected by qPCR represents either intact (infective or non-infective) virus particles or fragments of non-infective DNA. It is considered unlikely that DNA fragments would be captured effectively during the sample concentration step since the adsorption/elution of viruses on/off the filter matrix is dependent on the surface charge of the intact virus. If we accept that the isolated viruses are intact, then their apparent lack of viability as measured by their ability to infect cells in culture may be attributed to:

- The inability of the environmentally stressed/damaged AdV isolated from the works samples to illicit an infection of the host cells in culture
- The apparently intact virus isolated from the works samples being damaged e.g. during the concentration procedure and losing the ability to initiate infection in tissue culture. These viruses may be fragile and more susceptible to damage than the AdV type 2 used as the process control which proved to be consistently infective in the ICC-PCR assay.
- An incompatibility with the host cell culture (cell line) – the A549 cells used in this study were not susceptible to infection with the AdV serotypes isolated, either inherently or because the passage number of the culture was inappropriate. The primer/probe combinations used in this study target the AdV hexon gene which is present in many of the human AdV serotypes. It is well established that the efficiency of replication on tissue culture varies with serotypes, and susceptibility of cell lines may vary depending on sample sources (Jiang, 2006). Whilst it was clear that the A549 cells used in the ICC-PCR assay were susceptible to infection by the HPA standard (AdV type 2, NCPV 213), it was unclear (undetermined) which AdV types had been isolated and quantified by qPCR during the monitoring programme. Thus, it is conceivable that the AdV isolates from the monitoring programme were incompatible with the A549 cells and failed to initiate an
infection. A preliminary database search indicated that the amplicon targeted by the qPCR assay would cover multiple AdV types.

- The level of AdV in the sample concentrates being below the pLOD for the ICC-PCR assay
- An inherent flaw in the ICC-PCR method. It is possible that minor method amendments could enhance the infection potential of AdV isolates:
  - Increase in exposure time (length of time the virus remains in contact with the cells prior to removal of the inoculum (sample concentrate)) from 1 hour to several hours
  - Increase in incubation time of exposed cell culture (before qPCR assay) from 5 to 10 days
  - Consideration of an alternative cell line

We have noted previously (Section 3.2.3) that, for AdV, the extraction of sample concentrates to remove inhibitory substances was unnecessary in the preparation of sample concentrates for qPCR analysis. It is therefore conceivable that the inocula (sample concentrates) added to the cell culture at the start of the assay, whilst non inhibitory to the PCR amplification process, may contain substances which are toxic for the host cells in culture. This would result in destruction of the cell monolayer and prevent adsorption of any infective viruses. Although the cell monolayer appeared microscopically to be intact for the duration of the infectivity assay, it is conceivable that the surface of the cells may have been damaged during exposure to the water sample concentrate.

Clearly, it is important to establish whether the viruses isolated from treated water are infective or not. Whilst others have failed to establish a high infectivity rate for AdV isolated in the drinking water context, we would recommend that this aspect is worthy of further investigation.

Irrespective of the capacity to infect human cells, it is considered that AdV is potentially a most useful indicator in the context of measuring the efficacy of a water treatment process. It is prevalent and persistent in raw waters throughout the year, amenable for concentration, and may be quantified using routine qPCR technology without the need for extraction.

On the basis of the data collated and presented here, it is acknowledged that several other areas also warrant further investigation:

- Optimisation of the ABE concentration procedure for the isolation and co-concentration of NV to maximize recovery rates.
- Consideration of the optimum volume of water required for concentration. Currently only around 4% of the final sample concentrate is analysed by PCR which equates to 200, 400 and 800ml of the total of 5, 10 and 20 litres of water concentrated. It is possible that the concentration process could be miniaturised to involve much smaller volumes of water and user-friendly filtration equipment which, in turn could maximize recovery rates, potentially increase the proportion of the sample analysed and reduce costs. To maintain the same LOD, a reduction in water sample volume concentrated would have to be associated with an increase in the volume of sample concentrate analysed.
- Determination of the optimum number of qPCR wells (volume of sample concentrate) required to optimise the probability of detection of concentrated viruses. We have noted previously that, whilst the standard methodology employed in the investigation used only 3 qPCR wells per sample concentrate, the theoretical requirement, to be 95% confident of detecting an of the 3 viruses at the practical LOD, is 9,5,9 for AdV, GI and GII NV respectively (Section 3.2.4). This aspect of the work is worthy of evaluation, particularly if
the methodology is to be used to inform risk assessments and/or in targeted investigations.

- Extension of the works monitoring programme to gather additional data for NV. The effect of the water treatment process on the removal of NV remains unclear. A further monitoring programme, focusing on the period of highest NV concentration in raw waters (November-March), could be undertaken at a limited number of water treatment works. This study could be designed to optimise the analytical methodologies incorporating, for example, an optimised (possibly miniaturised) concentration procedure alongside processing an increased proportion of the water sample concentrate.
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