Technical aspects of using human adenovirus as a viral water quality indicator

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Primary concentration

Purification (Remove bacteria, protozoa etc.)

Secondary concentration

Culture based detection
A) Uninfected 293 cells and
B) Cytopathic effects induced in 293 cells by Ad41

Nucleic acid based detection
DNA extraction and qPCR
Technical aspects of using human adenovirus as a viral water quality indicator.

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Abstract

Despite dramatic improvements in water treatment technologies in developed countries, waterborne viruses are still associated with many of cases of illness each year. These illnesses include gastroenteritis, meningitis, encephalitis, and respiratory infections. Importantly, outbreaks of viral disease from waters deemed compliant from bacterial indicator testing still occur, which highlights the need to monitor virological quality of water. Human adenoviruses are often used as a viral indicator of water quality (faecal contamination), as this pathogen has high UV-resistance and is prevalent in untreated domestic wastewater all year round, unlike enteroviruses and noroviruses that are often only detected in certain seasons. Standard methods
for recovering and measuring adenovirus numbers in water are lacking, and there are many variations in published methods. Since viral numbers are likely under-estimated when optimal methods are not used, a comprehensive review of these methods is both timely and important. This review critically evaluates how estimates of adenovirus numbers in water are impacted by technical manipulations, such as during adenovirus concentration and detection (including culturing and polymerase-chain reaction). An understanding of the implications of these issues is fundamental to obtaining reliable estimation of adenovirus numbers in water. Reliable estimation of HAdV numbers is critical to enable improved monitoring of the efficacy of water treatment processes, accurate quantitative microbial risk assessment, and to ensure microbiological safety of water.

Keywords: adenovirus; qPCR; ICC-qPCR; water quality; viral culture; viral enumeration

1. Introduction

The microbiological quality of water (e.g., drinking water, recreational water, and wastewater) is usually assessed by testing for bacterial indicators, such as *E. coli* (Power 2010; NHMRC 2011; WHO 2011). However, enteric viruses have been detected in waters determined compliant for bacterial indicators, and subsequently linked to viral-associated outbreaks (Craun 1991; Wyer et al. 2012; Girones and Bofill-Mas 2013). One of the reasons for this, may be because enteric viruses are often more resistant to water treatment/disinfection processes than bacterial indicators. In addition, the high infectivity of these viruses poses an increased risk of transmission compared to bacteria and protozoa, such as *Campylobacter* and *Cryptosporidium* species (WHO 2006). The monitoring of viral disinfection during water treatment is thus becoming a high priority for government regulators (Harwood et al. 2005; Costán-Longares et al.)
A common viral indicator of human faecal pollution in water and wastewater treatment efficacy, is human adenovirus (HAdV) (Harwood et al. 2005; Silva et al. 2011; Girones and Bofill-Mas 2013). The usefulness of HAdV as a virological water quality indicator is attributed to: (1) its stability, persistence, and wide distribution in a range of water matrices, such as wastewater, rivers and drinking water (Pina et al. 1998; Bofill-Mas et al. 2006; Haramoto et al. 2007; Katayama et al. 2008; Dong et al. 2010); (2) continual detection of the virus in sewage, i.e. lack of seasonality in untreated wastewater, although in environmental waters seasonal occurrence has been indicated in some cases (Pina et al. 1998; Haramoto et al. 2007; Fong et al. 2010; Hewitt et al. 2011; Poma et al. 2012; Carducci and Verani 2013); (3) its high resistance to ultraviolet (UV) disinfection (Thurston-Enriquez et al. 2003; Nwachuku et al. 2005; Linden et al. 2007; (4) its higher abundance relative to other enteric viruses (Katayama et al. 2008; La Rosa et al. 2010; Simmons and Xagoraraki 2011; Poma et al. 2012); (5) the availability of culture assays and molecular detection tools (although standard methods for these assays are lacking) and ease of detection compared with ribonucleic acid (RNA) viruses (Jiang 2006; Silva et al. 2011; APHA 2012; Ogorzaly et al. 2013a); and (6) its human host specificity (specifically human faecal contamination) as well as the lack of replication outside of the host (Fong and Lipp 2005; Wong et al. 2012).

The processes used for the concentration and detection of viruses in water samples are shown in Figure 1. Here we critically evaluate the use of these methods for determining HAdV numbers in water matrices, and how these methods are affected by viral properties. In particular, the
concentration method, deoxyribonucleic acid (DNA) extraction method, choice of primer, and cell line used for the culture assays are important variables that can dramatically affect accuracy of recovery and detection (Bofill-Mas et al. 2006; Jiang et al. 2009; Cashdollar and Wymer 2013; Sidhu et al. 2013). For example, poor recovery of HAdV has been associated with filter-based methods for concentration, while enteroviruses have been recovered efficiently using such methods (USEPA-1615 2012; Cashdollar and Wymer 2013; Gibbons et al. 2010). We also consider limitations of the methods, when applying them to analyses of wastewater as an example. Our analysis aims to facilitate estimation levels of viral contamination in water more reliably. Such an estimation is critical for better monitoring of water treatment efficacy and determining suitable re-use options for recycled water.

2. Adenovirus types and their persistence in water matrices.

The most prevalent HAdV serotype detected in wastewater and surface water is Ad41, which is second to rotavirus as a leading cause of childhood gastroenteritis worldwide (Grimwood et al. 1995; Bofill-Mas et al. 2010; Fong et al. 2010; Wyn-Jones et al. 2011; Lee et al. 2012; Ogorzaly et al. 2013b). A wide range of HAdV serotypes has been detected in water matrices (Table 1; Figure 2). This is mainly due to the continual shedding of viruses in high numbers in human excreta, for as long as months following illness (Crabtree et al. 1997; Lee et al. 2012). Diseases associated with different HAdV serotypes, along with population sub-groups likely to be affected by them are presented in Table 2. Respiratory, gastrointestinal and eye infections are the most common (Roche et al. 2002). Illnesses that are prevalent in a given community at a given time are those subsequently detected in community wastewater (Vidovic et al. 2011; Prevost et al. 2015). Hence, an understanding of which HAdV serotypes are present would enable more refined assessments of risks associated with different re-use options for recycled water. For example,
the risk of using water in a manner that creates aerosols (e.g. irrigation and firefighting) is generally higher when the water is contaminated with a respiratory pathogen, transmitted via inhalation such as adenovirus 2 (Ad2), compared to a pathogen associated with gastroenteritis (Ad41).

Persistence of HAdV in water matrices is associated with slow die off (132 days in wastewater and >301 days in sterilised surface water spiked with virus) and high UV resistance (Thurston-Enriquez et al. 2003; Bofill-Mas et al. 2006; Linden et al. 2007; Rigotto et al. 2011). Indeed, HAdV is considered an emerging pathogen due to its high resistance and prevalence in environmental waters, detection of infectious virus in chlorinated drinking water and wastewater (despite its reported sensitivity to chlorine), and the continual description of new HAdV types (Jiang 2006; Robinson et al. 2011).


Human adenoviruses are typically present at high concentrations (10$^3$ to 10$^8$ infectious units/L) in untreated wastewater (Rodriguez et al. 2013b; Ogorzaly et al. 2013a). The monitoring of HAdV disinfection efficiency of wastewater treatment processes is important to assess public health risks associated with discharge of virus-containing effluent into waterways. These waterways often provide sources of raw water for drinking water supplies and/or are used for recreational activities (Wyer et al. 2012; Lin and Ganesh 2013). In addition, treated wastewater is also re-used (recycled water), for example for irrigation (fruits and vegetables, parks and sports fields) and industrial applications, if treatment methods provide required levels of disinfection (Mara 2003; Symonds and Breitbart 2014).
Viral disinfection efficiency is monitored by comparing virus concentration at the inlet and outlet of a given wastewater treatment process, and has traditionally relied upon measurement of the log reduction in levels of viruses, and infectivity of these viruses (Dong et al. 2010; AGWR 2006). Different water re-use options require prescribed log reduction values (Figure 3) to be achieved through treatment processes and are estimated by quantitative microbial risk assessment (QMRA) (AGWR 2006). QMRA is used to estimate the likely burden of disease associated with an illness, caused by exposure to a specific pathogen in a specific re-use scenario. While advances have been made in QMRA methodology, the final risk estimates and therefore our capacity to ensure recycled water is fit for a given purpose is highly dependent on the accuracy of methods used to concentrate and enumerate viruses, such as HAdV (Beaudequin et al. 2015).

4. Recovery of HAdV from water and concentration methods.

Large sample volumes are required for viral analysis of water, to maximise the chances of detecting potentially dispersed particles in the sample (APHA 2012; Brinkman et al. 2013). The analysis of large volume water samples requires the use of concentration techniques to reduce sample volume (Dong et al. 2010; Poma et al. 2012). Without concentration, the numbers of viruses in water samples are often below the limit of detection (LOD) using conventional methods. However, these low levels of virus can still lead to illness, and thus represent a significant level of pollution (APHA 2012; Brinkman et al. 2013). Current methods either extract viruses from the sample for resuspension in a smaller volume, or extract water from the sample in the case of ultrafiltration, and are still being optimized (APHA 2012). Here we consider the sampling volume, recovery estimation, and different concentration methods as applied to analysis of waterborne HAdV.
4.1 Sampling volume

The volume of water regarded as being “meaningful” for the extraction of viruses is dependent on the properties of the water matrix being analysed, the density of the viruses present and the exposure scenario (Dong et al. 2010; APHA 2012; Liu et al. 2012; USEPA-1615 2012; USEPA-Guidelines for Water Reuse 2012). For sewage influent, a volume of ~1 L may be sufficient for a meaningful volume as a high density of viruses is present and the processable volume is limited by high levels of organic material (Pepper et al. 2014). For treated wastewater, the volume processed depends on whether the water is re-used, and the type of re-use purpose and exposure scenario (Table 3). Re-use purposes involving human contact or contact with potable water supplies are associated with a higher level of exposure compared to other uses of recycled water, and larger volumes of water need to be assessed (APHA 2012; AGWR 2008). The volumes listed in Table 3 may vary according to national or state guidelines or legislation (AGWR 2006; AGWR 2008; USEPA-Water Reuse Guidelines 2012; WHO 2006; EPA-464.2 2003). In fact, it would be useful if recommended analysis volumes for different re-use purposes were more clearly defined in the guidelines assessed, but this may be due to the lack of standard methods for concentration and dependency on capabilities of accredited testing laboratories. Variable volumes of wastewater (100 mL to 100 L) are also analysed in the literature, which contributes to a difficulty in comparing concentration methods across different reports.

4.2 Measuring recovery to assess concentration efficiency.

The performance of a concentration method is determined by estimating recovery as a percentage. Comparing viral titres in the original and concentrated sample is feasible where the viral titre is high enough to be determined from the original sample, as in the case of coliphages. The recovery of coliphages can infer the recovery of HAdV, however, more accurate recovery
estimates may be obtained by spiking a control sample with the target virus (Francy et al. 2011).

Petterson et al. (2015) further discuss inadequacy of currently available surrogates used as process controls. HAdV recovery levels as low as 0.09% and 0.2% (Table 4) indicates the need to monitor the efficiency of concentration methods, as a first step to estimating viral numbers in water reliably. Unfortunately, this reporting of recovery is lacking in many analyses (Table 4 and 5).

Efficiency of a concentration method can be affected by differences in the physicochemical characteristics of the water sample, such as the pH, ionic strength/composition, and concentration and type of organic carbon present (e.g., soil or faecal; Liu et al. 2012; Cashdollar and Wymer 2013). Presence of organic materials and other microorganisms (particularly algae) can also limit the processable volume, for example before filter clogging occurs (Francy et al. 2013). Effect of viral properties on efficiency of different concentration methods is increasingly recognised. Such viral properties include physical structure and surface chemistry (hydrophobicity and electrical charge) (Gibbons et al. 2010; Liu et al. 2012; Cashdollar and Wymer 2013). The large protruding fibres of Ad41/Ad40 have been associated with physical entrapment of the virus in different types of filters used for concentration, resulting in poor elution and recovery (Gibbons et al. 2010; Darr et al. 2009; McMinn 2013). In contrast, noroviruses and enteroviruses, which lack long protruding fibres have been more efficiently recovered using existing filter methods for concentration (USEPA-1615 2012). Thus, determining the performance of a given concentration method as applied to a specific application is critical.
4.3 Concentration methods.

Choice of concentration method is determined by water characteristics, as well as volume of water that needs to be analysed and effects of virus properties needs to be assessed. A series of concentration techniques is often used consecutively to reduce sample volume (Figure 1). Primary concentration is used to reduce the bulk of the water sample volume, usually by at least 100X. When processing large volume water samples (10s to 100s L) microporous filters or hollow fibre ultrafiltration (HFUF) are commonly used for primary concentration. In such cases, the primary eluate is typically 300 mL to 1.6 L. The sample volume is reduced further using a secondary concentration step (usually by at least ~10X), so that more of the sample can be analysed, which improves sample representation and accuracy of estimating virus numbers (APHA 2012; Brinkman et al. 2013; Ikner et al. 2012). A tertiary concentration step can be used to reduce sample volume even more, particularly for molecular analysis where less of the sample is analysed compared to culture methods (USEPA-1615 2012). Methods used for secondary and tertiary concentration include organic flocculation, celite concentration, PEG precipitation, ultracentrifugal filters and ultracentrifugation. These methods are also used as the primary concentration method when smaller volumes of untreated wastewater (≤1 L) and wastewater effluents (1-10 L) are analysed (Table 4 and 5).

Different concentration methods are outlined in Figure 4, and described in more detail elsewhere (Ikner et al. 2012; Cashdollar and Wymer 2013). This review discusses application of these methods for recovery of HAdV from wastewater and examples of recent optimisations using other water types, which might also be applied to wastewater.
4.3.1 Primary concentration methods

4.3.1.1 Non-filter based methods for primary concentration.

Non-filter based methods including PEG precipitation, organic flocculation, celite concentration and ultracentrifugation and are often used as the first or sole concentration procedure for wastewater analysis (Table 4 and 5), and are therefore discussed here in the context of primary concentration. These methods are limited to analysis of smaller volume samples compared to filter-based methods.

Use of PEG precipitation (Figure 4C) for viral concentration is often reported for turbid wastewater samples (Tables 4 and 5). Use of the method resulted in ≥10% recovery of HAdV from 1L wastewater influent and effluent samples in studies by Hewitt et al. (2011), while reporting of recovery was lacking in other examples.

Viral concentration using celites is increasingly reported, following changes in the manufacturing of beef extract used for organic flocculation (McMinn et al. 2012). Celites (diatomaceous earth) act as a binding agent, similarly to flocs formed by beef extract (Figure 4B). Celite concentration was shown to be effective as the primary concentration method for 1 L untreated wastewater, where HAdV recovery was 55% (Table 4; Brinkman et al. 2013). This recovery was obtained using 10 mL of the concentrate for maxiprep DNA extraction, and was much higher compared to recovery when centrifugal ultrafilters were used for secondary concentration (Table 4). This procedure might also be useful for 10-20 L samples.

The use of ultracentrifugation (Figure 4E) was associated with recovery of HAdV up to 70% from 40 mL wastewater samples (Pina et al. 1998). Interestingly, Bofill-Mas et al. (2006) reported
higher estimates of HAdV concentration from small volume wastewater samples concentrated using ultracentrifugation alone, compared to larger volume samples processed using 1MDS filters and ultracentrifugation. This illustrates that the use of larger sample volumes for improving sample representation can be at the concession of viral loss during concentration, and favours minimising sample processing where possible (Petterson et al. 2015).

4.3.1.2 Electronegative filters.

Concentration of large volume water samples (10s to 100s L) generally requires use of filtration techniques. Viral adsorption to- and elution- from electronegative (EN) filters is one such technique, and usually requires lowering the sample pH and/or adding multivalent cations (Figure 4A, Wallis et al. 1979; Gerba 1984). Filterite cartridge filters, which have a larger surface area, are one example of an EN filter used for large volume samples. EN small disk filters, such as Millipore HA disk filters (variety of pore sizes), can also be used to recover viruses from smaller volume samples (up to ~10L).

Inefficient elution of HAdV from EN filters can be associated with poor recovery (e.g. 3-5%, Ahmed et al. 2015). Elution of HAdV from EN disk filters has been improved using optimised elution buffers and methods, in analyses using various water types. The optimal elution buffer reported by Wu et al. (2011) was 1.5% beef extract + 0.75% glycerol and resulted in 55% recovery of Ad41 from 1L source water, which was higher than recovery using 1mM NaOH for elution. Use of glycerol in the elution buffer might protect viral integrity during rapid changes in pH and/or better covered the membrane surface during elution (Wu et al. 2011). Sun et al. (2016) reported that elution with 1mM NaOH resulted in higher recovery compared to beef extract buffers assessed. An optimal elution method, involving stirring the filter with a magnetic
bar for 30 minutes, resulted in HAdV recovery of up to 79% (Table 6). This elution method was much more efficient compared to others that are commonly used, such as filtration or brief vortexing. The aforementioned studies used 4 or 8 µM pore filters. Limited analysis suggests that these filters might be associated with higher recovery compared to 0.45 µM pore filters, and that 8 µM filters may enable adequate recovery and much faster filtration times (Wu et al. 2011; Ahmed et al. 2015; Sun et al. 2016). The use of larger diameter 90 mm disk filters provides a 4.5X larger surface that can also assist with sample processing (Ahmed et al. 2015). Use of filters directly for DNA extraction was associated with recovery of ~32% from 1 L river water, and may be a suitable option where virus viability does not need to be assessed (Table 6, Ahmed et al. 2015).

Use of EN Filterite cartridges for large volume water samples was assessed by Enriquez and Gerba (1995). Recoveries of Ad40 were 22.5%, 37% and 38% for wastewater, tap water and seawater respectively, and this includes losses that might have occurred during secondary concentration (Table 6). Beef extract + glycine elution buffer was used in this study. Evaluation of optimal elution buffers described above might be of value for improving elution from EN cartridge filters.

In summary, the various optimised conditions for EN disk filters above have provided high levels of HAdV recovery and might also be evaluated for wastewater. Use of EN cartridge filters might also provide adequate HAdV recovery from wastewater. The need to lower sample pH for adsorption of viruses to EN filters, which can be difficult for large volume samples, is potentially a major reason the use of other filter types have been more evaluated for virus recovery from water (Ikner et al. 2012; Cashdollar and Wymer 2013).
4.3.1.3 Electropositive filters.

Use of electropositive (EP) filters for viral concentration generally does not require sample preconditioning when sample pH is near neutral (Cashdollar and Wymer 2013; Ikner et al. 2012). Commonly used EP filters include 1MDS and NanoCeram disk or cartridge filters. 1MDS cartridges are associated with much higher cost (~US $250) compared to NanoCeram (~US $50) cartridges (Cashdollar and Wymer 2013). The inexpensive small disk filters are made from the same material as the cartridge filters, and provide a cost effective means to evaluate conditions that might be assessed using cartridge filters, as well as being useful for processing samples up to ~10 L (McMinn 2013).

Use of 1MDS cartridge filters was associated with low and variable recovery (2-25%) of HAdV from wastewater (Table 4, Bofill-Mas et al. 2006). Enriquez and Gerba (1995) reported lower recovery of Ad40 from tap water using 1MDS cartridges compared with Filterite cartridges (Table 6). However, these results might be improved if recently optimised elution buffers/methods for either filter type were assessed.

Similarly to EN filters, optimisation of experimental parameters has been required for improving HAdV recovery from EP filters. Use of optimal conditions for EP 1MDS disk filters enabled recovery of AdF of 52-64% from small volume tap water samples, when used with celite secondary concentration (McMinn 2013). Subtle differences in the pH of the eluting buffer (e.g. pH 9.5 compared to 10) were shown to significantly affect recovery (McMinn 2013). Additionally, recovery was greatly reduced for sample pH < 8. AdF recovery was higher using 1MDS disk filters compared to NanoCeram disk filters (Table 6). Interestingly, serotype also affected recovery using...
NanoCeram disk filters (Ad41 20% and Ad40 39%). Differences in capsid fibre shafts (Darr et al. 2009) might explain the disparity in recovery between different serotypes. Similar recovery of Ad41 (~20%) was reported by Pang et al. (2012), using 90 mm NanoCeram disk filters for 10L water samples and FeCl₃ flocculation for secondary concentration (Table 6).

Variable recovery of HAdV using NanoCeram cartridge filters has been reported. Ikner et al. (2011) reported 39% recovery of Ad2 from 20L tap water, when using a sodium polyphosphate-glycine-phosphate buffer (NaPP-G-PB) and positive pressure for elution (Table 6). Similarly, Prevost et al. (2015) reported 18-42% recovery of Ad5 from 10L source water and wastewater, using similar elution conditions. In other cases much lower recoveries (0.02 – 2.5%) have been reported (Table 6, Gibbons et al. 2010; Francy et al. 2013). Discrepancies between studies may have been due to use of different eluting buffers and methods (Table 6), sample characteristics, and/or analysis of different HAdV serotypes, since differences in the recovery efficiency of even the two AdF serotypes (Ad40 and Ad41) has been demonstrated.

In summary, use of optimised methods for EP disk filters described above, might also be validated for wastewater. Elution from EP disk filters might also be improved using elution methods such as those described by Sun et al. (2016), or by using an acidic solution for elution (Haramoto and Katayama 2013). Further investigating the effect of sample pH on adsorption (McMinn et al. 2013), NaPP-G-PB for elution (Ikner et al. 2011) and using this buffer at pH 10 (in accordance with findings of McMinn et al. 2013) may also be of value. These aspects might also be applied to EP cartridge filters, and assist in determining reasons for variability between studies.
4.3.1.4 Hollow fibre ultrafiltration.

Hollow fibre ultrafiltration (HFUF) employs inexpensive medical dialysis filters for recovery of viruses from water (Figure 4D, Hill et al. 2005). Several studies have used TFUF for concentration of wastewater, and although high levels of HAdV were detected, the recovery percentage of HAdV was not reported in these studies (Table 4). Use of HFUF (followed by PEG precipitation) was associated with 4% recovery of Ad2 from 10 L river water, with comparable recovery from wastewater, in analyses by Hewitt et al. (2013). Recovery of 1.5% was reported by Francy et al. (2013) for 10 L lake water samples (Table 6). Much higher recovery of Ad41 was recently reported by Rhodes et al. (2016), where use of HFUF resulted in Ad41 recovery of 69% and 56% for 100 L tap water and 10 L river water respectively (Table 6). Sodium polyphosphates and Tween 80 were used to minimise virus adhesion to the filter surface. Use of celite for secondary concentration reduced recovery from river water (Table 6), which might have been associated with centrifuging the primary eluate. In such cases, viral loss might be reduced by eluting the pellet after centrifuging, as described in Section 4.8 and Hewitt et al. (2011).

Nonetheless, recovery reported by Rhodes et al. (2016) was much higher compared to other studies where recovery was 1.5-4%. Reasons for this discrepancy between the studies might include the use of different elution buffers for TFUF, lower recovery of HAdV by dead end HFUF, differences in water properties or variances in the proficiencies of different laboratories. Thus, good recoveries of HAdV were obtained using HFUF and celite secondary concentration in river and tap water (Rhodes et al. 2016), suggesting higher recovery from wastewater might be possible using this method.
4.3.1.5 Comparing primary concentration methods for recovering HAdV from water.

As discussed by Cashdollar and Wymer (2013), the many variations in primary concentration methods published in the literature makes comparison between studies difficult. Further, effect of water sample properties on efficiency is a confounding factor in comparative analyses. It can also be difficult to assess performance of a primary concentration method, when results are only reported for a complete method that also involves a secondary concentration step (Table 6). For example, for methods used for analysis of smaller volume samples, in comparing HAdV recovery results using EP (1MDS) and EN (8µM) disk filters, results were not considerably different (~55-65%) for tap water and source water (Table 6; Wu et al. 2011; McMinn 2013). However, for EP disk filters this included losses from celite secondary concentration, and recovery for both methods might be improved by using various applications of recently optimised methods, and results might vary with different water types.

For smaller volume wastewater samples, the celite concentration method (as the sole method) produced higher recovery of HAdV from wastewater, compared to other reports assessed. Results with the celite method also need to be further validated with larger numbers of samples.

For all methods, a better understanding of water property and serotype dependant effects is needed.

Similarly, for larger volume samples, recovery of Ad41 was in this same range using HFUF for tap and river water (Rhodes et al. 2016), but this method also needs to be further validated using different water types. Slightly lower recoveries (22-39%) were reported using NanoCeram (cartridge or disk filters) or Filterite cartridges; recently optimised eluting buffers and secondary
concentration methods might also be assessed for these methods, and reasons for between study variability determined.

Thus, extreme caution is needed when comparing virus recovery between different reports, due to the many variations in methods and a poor understanding of effects of sample properties and between sample variability on recovery (Petterson et al. 2015). There is also requirement for larger data sets for adequate assessment of many methods and data based determination of minimum acceptable limits of recovery for QMRA (Petterson et al. 2015).

### 4.3.2 Secondary concentration methods.

Comparing secondary concentration is somewhat more practicable, when different methods are assessed within the same study and use the same water sample. Numerous studies have shown that the use of ultra-centrifugal filters (Figure 4E) is associated with high losses of HAdV, when comparing with different methods or comparing numbers in primary and secondary concentrates (Ikner et al. 2011; Wu et al. 2011; Brinkman et al. 2013). As discussed by Brinkman et al. (2013), the high concentration factor achievable using ultra-centrifugal filters, where sample volume is reduced ~37.5 fold, suggests assessing how to improve HAdV recovery may be worthwhile.

Both ultracentrifugation and PEG precipitation have often been used for secondary concentration in wastewater analyses (Table 4 and 5). Ultracentrifugation (Figure 4E) is generally associated with high viral recovery (Section 4.3.1.1), but a limitation is the need for expensive equipment. Using PEG precipitation for HAdV seeded into beef extract, resulted in ~ 40%
recovery, which was similar to that using organic flocculation (Enriquez and Gerba 1995). A disadvantage of PEG precipitation is the need for overnight stirring.

Organic flocculation has traditionally been used as secondary concentration method, where microporous filters are used for primary concentration and eluted in beef extract (Ikner et al. 2012). Use of organic flocculation resulted in 10-24% reduced recovery compared to celite secondary concentration, for HAdV eluted from EP disk filters (McMinn 2013). Increased time of exposure to low pH using organic flocculation might be associated with lower recovery compared to celite concentration. Use of celite concentration also requires less time and equipment compared to other secondary concentration methods, such as PEG precipitation and ultracentrifugation (Rhodes et al. 2011; McMinn et al. 2012).

McMinn et al. (2012) reported optimisation of celite secondary concentration procedures for Ad40 and Ad41 seeded into beef extract. Elution with PBS (pH 9) resulted in 5-fold less PCR inhibition in comparison to 0.15M sodium phosphate (pH9), although a 1/5 dilution was still required to overcome PCR inhibition. When using PBS for elution, recovery efficiencies were 81% and 124% for Ad40 and Ad41 respectively. In comparison of different celite types, smaller particle calcinated celites were most effective. Use of celite as a secondary concentration method for HAdV is increasingly reported (Table 4), and suitability of the method for wastewater analyses has been suggested (Section 4.3.1.1).

An alternative secondary concentration method, using evaporation was reported by Wu et al. 2011). The method involved use of heat (55°C) and bubbling air through the sample for 1 hour. The potential for cross contamination of samples using this method was noted, but not
observed, and might be avoided by plugging tubes with cotton wool. Using this method, Ad41 recovery of 87% was reported, a large concentration factor is achievable (in this case 20 fold) and efficiency is not affected by sample turbidity. The use of heat in the method may result in virus inactivation, so it is suited to use for molecular detection methods rather than culture methods. Evaporation might also be evaluated as a secondary concentration method for wastewater analysis. Other secondary concentration methods, such as inorganic flocculation and precipitation, are occasionally reported for HAdV analyses, and are reviewed in Ikner et al. (2012).

In summary, secondary concentration methods should be rapid and result in minimal viral loss. In this regard, celite concentration and ultracentrifugation perform favourably, and evaporation methods could be further evaluated. Further direct comparisons of secondary concentration methods may be of value for wastewater analysis.

4.4 Removal of contaminating microorganisms from virus concentrates.

Following concentration procedures, the virus concentrate is often purified, for removal of other microorganisms such as bacteria, protozoa and algae. For downstream analyses, purification removes contaminants from cell culture and also removes comparatively larger bacterial genomes that can overwhelm molecular analyses, and reduce efficiency of detection of smaller viral genomes. Use of 0.2 µM syringe filters for purification is often reported (Table 4 and 5). American Public Health Association (APHA) recommends avoiding the use of syringe filters for purification due to likely losses of virus particles (APHA 2012). The APHA (2012) instead recommends use of antibiotic treatment and/or chloroform extraction. Chloroform extraction is also frequently employed for purification in wastewater analyses (Table 4 and 5), and is useful in
cases where sample turbidity would cause syringe filter clogging. Chloroform extraction can also have the advantage of reducing PCR inhibitors (Rodriguez et al. 2012). Analyses by Enriquez and Gerba (1995) suggested little difference in recovery resulted from chloroform extraction compared to use of a low protein-binding filter pre-blocked with beef extract buffer. Pre-blocking of 0.2 µM syringe filters with newborn calf serum has also been reported (Dong et al. 2010), and presumably acts to lessen binding of the virus to the filter surface to reduce viral loss.

When removing solids and bacteria by centrifugation, viral loss can be reduced by elution of the pellet to recover any viruses electrostatically attached to surfaces of bacteria or solids (APHA 2012). In this case, the bacteria/solids are pelleted by centrifugation and the supernatant is collected, then the pellet is resuspended in buffer and centrifuged again, and the second supernatant is combined with the first supernatant.

Pre-filtering of samples (prior to concentration) is used in some cases to reduce particulates and other organisms that may result in filter clogging. For example, Pang et al. (2012) pre-filtered water samples using paper towel and chromatography paper. Use of large pore size filters or stainless steel sieves for pre-filtering is also reported (Poma et al. 2012; USEPA-1615 2012). Rinsing/elution of the pre-filter apparatus is recommended to recover viruses that might have adsorbed to particulates or to the pre-filter (US-EPA1615 2012).

In conclusion, the potential for viral loss needs to be considered and measured in all steps of water sample processing. Most examples presented here (Tables 4-6) differ in some aspect of the method detail, which in addition to differences in water sample characteristics, makes between comparing results between reports difficult. As methods continue to be developed and
optimised, direct comparison of different methods and inter-laboratory determination of reproducibility are needed to better establish the most effective and reliable methods for recovering HAdV from wastewater, as well as other water matrices.

5. Detection of HAdV using culture methods.

Numerical evaluation of the number of viruses is critical to understanding viral recovery from concentration and purification methods, as well as accurately determining HAdV in contaminated water samples. Quantification is performed using either culture-based (see below) or molecular methods (described in Section 6).

Culture-based methods are currently the only available method for detecting viable/infective HAdV, and are thus most accepted by the water industry for QMRA (Keegan et al. 2009; Rodriguez et al. 2013b). Culture methods rely on the ability of viruses to replicate in mammalian cell lines (Figures 1 and 5), and are both costly and time consuming. Hence, culture-based estimation of HAdV concentration is typically only used in the water industry for initial validation of the efficiency of wastewater treatment (WWT) processes, rather than for routine verification monitoring (AGWR 2006). Apart from cost and time, additional limitations can include low sensitivity, particularly when sensitive methods for detecting virus infection and appropriate cell lines are not used in the analysis, as discussed below.

5.1 Most probable number (MPN) estimation and the plaque assay.

The main methods for quantification of HAdV infection of cells are outlined in Figure 5. Conventional culture methods rely on detecting cytopathic effects (CPE) produced by virus infection of cells, for estimation of MPN or TCID50 (tissue culture infective dose where 50% of
the cell cultures are infected) (Pepper et al. 2014). For MPN estimation, cells are inoculated with dilutions of the virus/sample and the lowest dilution in which cytopathic effects (CPE) occurs is recorded, and used to estimate viral titre using MPN tables or calculators (e.g. EPA Most Probable Number Calculator, USEPA-1615 2012). Similarly, TCID50 is determined using an end-point dilution assay (Pepper et al. 2014). MPN and TCID50 are often used in HAdV disinfection studies that employ laboratory strains (Thurston-Enriquez et al. 2003; Nwachuku et al. 2005; Linden et al. 2007). However, use of MPN/TCID50 in environmental samples is limited, due to the long periods (up to 28 days) required for HAdV to produce CPE, and outgrowth of HAdV by faster growing viruses such as enterovirus. It can also be difficult to visually detect CPE produced by HAdV (Figure 1), which may result in the virus concentration being under-estimated (Ogorzaly et al. 2013a; Polston et al. 2014).

Plaque assays can enable easier visualisation of virus infection of cells, in certain applications. Plaques are cleared zones in the cell monolayer (caused by lytic viral replication) that appear following staining with crystal violet (Figure 5; Pepper et al. 2014). Cromeans et al. (2008) discuss the development of a plaque assay for use in viral disinfection and concentration studies, which enabled enumeration of HAdV after 9 days. Repeated passage and selection of Ad40/41 isolates from large plaques enabled consistent results in the plaque assay, and also enabled earlier detection of CPE. As discussed by Cromeans et al. (2008), not all Ad40/Ad41 strains form plaques. As such, the reported plaque assay is more suited to use for disinfection or concentration studies rather than for quantifying HAdV in environmental water samples. Further, estimates of HAdV numbers in water samples using a plaque assay, were on average 3 to 4 logs lower than estimates using integrated culture-PCR (ICC-PCR) methods to detect virus infection of cells (Fongaro et al. 2013).
5.2 PCR based methods for detecting virus positive cultures.

Use of PCR based methods to viral infection of cells (ICC-PCR) is more rapid and sensitive compared to conventional approaches (Fongaro et al. 2013; Ogorzaly et al. 2013b; Rodriguez et al. 2013b). When the method is used to both detect and quantify viral DNA by qPCR, the test is known as ICC–qPCR. When viral mRNA (produced by replicative intermediates) is quantitatively detected the test is referred to as ICC–RT–qPCR, as the process includes reverse transcription (RT; Figure 5). The collection of a sample at T₀ (cell inoculation) ensures the increase in signal is related to virus replication using the qPCR method (Ogorzaly et al. 2013a). In the ICC–RT–qPCR method (Rodriguez et al. 2013), the signal is related to the concentration of infective viruses by measuring replicative intermediates (viral mRNA).

Detection of virus positive cell cultures by qPCR was demonstrated to be more sensitive compared with traditional PCR (Ogorzaly et al. 2013). While similar detection limits for ICC–qPCR and ICC–RT–qPCR have been reported, a much higher concentration of HAdV reported using ICC–qPCR is suggestive of a higher sensitivity for this method (Table 5). A comparison between studies is difficult, however, and parallel testing of the different methods using the same environmental samples, and employing the same cell type is needed (Polston et al. 2014). Nonetheless, initial studies suggest ICC–qPCR is a highly sensitive culture method, where HAdV numbers in the same order of magnitude as qPCR alone have been reported (Section 6).

5.3 Appropriateness of different cells lines for HAdV culture assays.

Both sensitivity and accuracy of culture-based estimates of HAdV are affected significantly by the cell line used in the analysis. This is because HAdV serotypes vary markedly in their ability to
propagate in different cell lines (Jiang et al. 2009; Ogorzaly et al. 2015). For example, the A549 cell line (lung carcinoma, Giard et al. 1973) has often been used to estimate HAdV numbers in water (Table 5). However, only non-AdF adenoviruses (such as Ad2/AdC) are detected when A549 is used in the analysis of water samples (Carducci et al. 2009; Wyn-Jones et al. 2011; Fongaro et al. 2013). AdF require several passages in cell culture to enable growth in A549 cells – thus while this cell line can be used for culture adapted AdF isolates, wild-type AdF (from environmental samples) are not propagated effectively in A549 cells (Cromeans et al. 2008; Jiang et al. 2009; Wyn-Jones et al. 2011; Ogorzaly et al. 2013a; Ogorzaly et al. 2015). Non-permissiveness of A549 for culture of wildtype AdF was confirmed in a study where the types of HAdV cultured from wastewater samples in A549 and HEK-293A cells were determined by amplicon sequencing (Ogorzaly et al. 2015; Section 9.4). Results showed that the majority of sequences from A549 cells were from AdC/Ad2, while Ad41/AdF was not detected in this cell line. Conversely, AdF species along with AdA and AdC were detected following culture in HEK-293A cells. The results of amplicon sequencing from the HEK-293A cell line, overall agreed well with diversity analysis using culture-independent analyses (Ogorzaly et al. 2015). Suitability of HEK-293A cells (and HEK-293 cells), for culture of AdF and AdC from water samples has also been demonstrated in other studies (Graham et al. 1977; Rigotto et al. 2011; Rodriguez et al. 2013; Ogorzaly et al. 2013a).

HEK-293A has improved adhesion to culture surfaces compared to the original HEK-293 line (Jiang et al. 2009). The HEK-293 line (human embryonic kidney) was transformed using fragments of Ad5 DNA (Graham et al. 1977). HEK-293 cells express E1B gene functions of Ad5. E1B is an early protein involved in viral replication, and is required for infection of the cells by Ad40/Ad41 (Mautner and Steinthorsdottir 1989; Zou et al. 2014). Polston et al. (2014) reported
further improved sensitivity of HAdV detection, using a derivative 293-CMV, which expresses the IE1 protein of cytomegalovirus.

The Hep2 cell line has also been shown to be permissive for culture of both AdF and AdC (El-Sensousy et al. 2013), but AdF was not detected in a wastewater analysis using this cell line (Amdiouni et al. 2012). Thus, the HEK-293 cell line, or its derivative 293A, is currently the most appropriate for the detection of a broad range of HAdV serotypes in water matrices, and use of the A549 cell line may underestimate HAdV numbers. It is still possible however, that not all variants of HAdV in environmental samples are propagated efficiently in cell culture, and this can result in reduced sensitivity. Currently available methods are not able to measure infective viruses that cannot be propagated in cell culture, and this presents a significant gap knowledge. The other major limitations of culture methods are that analyses are costly and they take days to obtain results.

6. Molecular methods for detecting HAdV.

In contrast to cell culture methods, molecular HAdV detection methods have the advantage of being both rapid and cost effective (Jiang 2006). Quantitative PCR (qPCR) is the predominant molecular method for HAdV enumeration (Figure 1). This method is highly sensitive, and can potentially detect variants that are not able to be cultured. Critically, qPCR would enable same-day results that would allow managers to provide more timely public health advisories (Staley et al. 2012; Symonds and Breitbart 2014).
6.1 Effect of PCR inhibitors and DNA extraction methods on the molecular detection of HAdV.

For detection of HAdV using qPCR, important aspects include effects of inhibitors, choice of DNA extraction method, primers/probes selected for the analysis, and use of methods to reduce detection of non-infective virus particles. The sensitivity of qPCR can be affected markedly by inhibitors, such as humic and fulvic acids in water (Aw et al. 2012; Schrader et al. 2012). The presence of these inhibitors in extracted DNA is dependent on the method used for extraction, as commercially available kits employ different proprietary technologies for the removal of inhibitors. For example, Sidhu et al. (2013) showed significantly higher numbers of HAdV were detected following DNA extraction using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) compared to other kits assessed. Differences in HAdV numbers detected could also be due to inherent differences in extraction efficiency of different kits. Further examples comparing the efficiency of the many available extraction methods in the literature are lacking. Importantly, for any chosen extraction method, any reduction in qPCR sensitivity due to inhibitors needs to be determined. One method used to determine levels of PCR inhibition is use of amplification controls, to test efficiency of amplification of known amounts of target or non-target DNA in spiked samples (Jiang 2006; Schrader et al. 2012; Sidhu et al. 2013). An alternative method for assessing PCR inhibition is to dilute samples, since PCR inhibitors are also diluted, such that amplification is no longer adversely affected (Brinkman et al. 2013; Schrader et al. 2012). However, the need to dilute samples can adversely affect method sensitivity (Brinkman et al. 2013). Further examples of different methods for DNA extraction and assessment of PCR inhibition in wastewater analyses are listed in Table 4.
6.2 Selection of primers for detecting HAdV by qPCR.

Numerous different primer and probe sets, designed to detect all HAdV species, have been used for quantifying HAdV in wastewaters by qPCR (Table 4 and 5). New primers continue to be reported, as further HAdV genomic sequence information becomes available (Prevost et al. 2015). As shown in Figure 6, a relatively small region of the hexon gene is usually targeted for HAdV qPCR, as this is one of the most conserved regions between different species. There is considerable overlap in many of the published primers/probes that span this genomic region. However, even small differences in primer and probe sequences can affect properties such as melting temperature, dimerization, specificity and efficiency. The presence or absence of degenerate bases might also affect qPCR results.

When using non-degenerate primers, Kuo et al. (2009) demonstrated variable efficiency in amplification of different HAdV species, which was related to the number of mismatches between primer and template. The use of degenerate and/or multiple primers-probes might enable more equivalent amplification of different HAdV species, and such an approach was used by Hernroth et al. (2002), Kuo et al. (2009) and Prevost et al. (2015).

Bofill-Mas et al. (2006) compared primers and probes described by Hernroth et al. (2002) with those of Heim et al. (2003), which are non-degenerate. They reported that the primers-probes described by Hernroth et al. (2002) produced up to thirty-times higher estimation of HAdV numbers, but an undesirable detection of animal adenovirus was also reported with these primers (Bofill-Mas et al. 2006). The non-degenerate primers described by Heim et al. (2003) were associated with the more specific detection of HAdV (Bofill-Mas et al. 2006). Further analysis using multiple sequence alignment might help determine whether more specific
detection using Heim et al. (2003) primers-probes was related to genomic location targeted or absence of degeneracy.

Along with primers-probe of Heim et al. (2003) and Hernroth et al. (2002), those reported by Jothikumar et al. (2005) have also been commonly used in wastewater analyses (Tables 4 and 5). Low levels of primers-probe degeneracy are also used in Jothikumar et al. (2005) and the newly reported ones by Prevost et al. (2015). Additionally, Prevost et al. (2015) used different forward primers for species C and A-B-D-E-F-G, targeted to smaller regions of previously published versions (Figure 6). Further direct comparison of different published primers and probes might empirically demonstrate whether any particular set provides more equivalent detection of different serotypes, as well as greater sensitivity and specificity of HAdV detection. Such an analysis might support more reliable estimation of HAdV numbers using qPCR.

6.3 Use of qPCR for quantifying HAdV in wastewater.

The typical concentration of HAdV in wastewaters determined using qPCR is in the range \(10^6 - 10^8\) gene copies (GC)/L in untreated influent and \(10^2 - 10^6\) GC/L in treated effluent (Table 4 and 5). The demonstration of log reductions in virus numbers indicates this method is highly suited for water quality monitoring (Table 4 and 5; Hewitt et al. 2013). In addition, qPCR enabled the sensitive detection of HAdV in water samples, with a limit of detection of typically \(~ \times 10\) GC/reaction (Bofill-mas et al. 2006; Dong et al. 2010). Primers and probes for the specific quantitation of AdF and AdC serotypes (Dong et al. 2010; Wolf et al. 2010) have demonstrated AdF (associated with gastroenteritis) is the most prevalent, which is in agreement with results from other studies (Haramoto et al. 2007; Ogorzaly et al. 2013a). For example, in wastewater \(1 \times 10^6\) (AdF) and \(5 \times 10^2\) (AdC) GC/L were reported (Wolf et al. 2010).
The major concern with the use of qPCR for monitoring wastewater treatment (WWT) is that both infective and non-infective viruses (that have been inactivated during treatment) can be detected (Bofill-Mas et al. 2010). For this reason, PCR-based methods have not been widely accepted for monitoring and QMRA by the water industry or regulators (Keegan et al. 2009; AGWR 2006; Rodriguez et al. 2012; Gibney et al. 2013; Mara et al. 2010). Several approaches have been used to reduce detection of non-viable viruses using PCR-based methods. One example is the use of long-range PCR, where amplification is inhibited in UV-damaged genomes (Eischeid et al. 2009). This method had varying degrees of success (Rodriguez et al. 2013a), but is not suited to qPCR, where smaller fragments are amplified. Another example is to perform a DNAse treatment of the viral samples before DNA extraction to degrade non-encapsidated DNA (Fongaro et al. 2013).

A further method proposed to reduce the detection of non-infective viruses is the use of antibodies with PCR-based methods, in immunocapture-PCR (Ogorzaly et al. 2013b). In this method, the antibody-based capture of viruses ensures only encapsidated/intact viruses are subsequently detected by PCR (Ogorzaly et al. 2013b). Experimentally, immunocapture-PCR reduced the detection of heat-inactivated HAdV inactivated by 90%. However, these viruses were inactivated by heat treatment at 95°C, which is associated with significant capsid damage; viruses inactivated at 56°C were still detected (Ogorzaly et al. 2013b). Similarly, HAdV-2 inactivated by chlorine was able to bind to hexon-specific antibodies, as well as to the cell receptor (Page et al. 2010). Thus, the high stability of HAdV hexon components may reduce the efficacy of antibody and cell–receptor-based methods in reducing the detection of non-infective particles (Ogorzaly et al. 2013b).
Similarly, efficacy of propidium monoazide (PMA) treatment (prior to nucleic acid extraction) for reducing detection of non-infectious viruses, depends on a sufficient level of capsid damage. PMA (and ethidium monoazide, EMA) are DNA intercalating dyes which can penetrate damaged capsids, and upon photoactivation, viral nucleic acids are covalently modified and not able to be subsequently amplified in PCR (Fittipaldi et al. 2012). As with immunocapture-PCR, treatment of virus particles with high heat (e.g. 95°C for 10 min) enabled effective reduction of PCR signal from non-infectious viruses following PMA treatment (Fongaro et al. 2016), while inactivation at lower temperatures was less efficient (Leifels et al. 2015). Inactivation at 65°C resulted in some reduction (2 - 3 log) of qPCR signal for Ad5 in PMA/EMA treated compared to untreated samples, but it was less than the ~6 log reduction in TCID50 determined by culture methods (Leifels et al. 2015). Similarly, ~ 3 log and 4 log reductions in qPCR signal for PMA/EMA treated samples resulted following chlorine (2 mg/L for 1 min) or UV inactivation (27-228 mJ/cm² for 30 – 120 s) respectively, while TCID50 was reduced 6 - 7 logs. Given qPCR results without PMA/EMA were not reduced at all for different the inactivation methods, there was some value in using PMA/EMA for reducing detection of inactivated HAdV. Prevost et al. (2016) reported PMA treatment resulted in Ad41 qPCR signal being reduced by <1 log for UV inactivation (50-400 mJ/cm²), and almost completely reduced (4.5 - 5.5 log) for chlorine inactivation (3, 5 and 10 min in 1mg/L chlorine). In assessment of river water and drinking water, the qPCR results were reduced by PMA treatment of virus preparations from drinking water, but not from river water (Prevost et al. 2016). Differences potentially relate to reduced detection of damaged HAdV in drinking water and detection of infectious virus in river water. However, determining effects of organic materials (such as humic acids) and sample turbidity on efficacy of PMA pre-treatment of virus preparations from river water/wastewater is an area for future attention (Fittipaldi et al.
Thus, current research suggests that in some cases PMA/EMA pre-treatment may reduce qPCR detection of HAdV inactivated by chlorine, and to a lesser extent UV. Studies that further evaluate the efficacy of PMA pre-treatment, as well as DNase treatment and antibody capture will enable the conditions in which the detection of inactivated HAdV in molecular tests is reduced, to be better defined.

A knowledge gap also exists, regarding the persistence of non-infective viral particles in water. For example, how long do inactivated viruses persist in water environments, and thus continue to be detected by DNA, antibodies or cell receptor-based methods? Would addressing these questions affect the log reduction in HAdV as measured by qPCR? Answers to these questions would allay the concerns of regulators in the water industry for the implementation of molecular methods for HAdV detection. However, given the underestimation of culture methods (described in Section 5) and the inability of culture methods to provide results timely to water recycling, the benefits of qPCR implementation presumably outweigh a possibility of over-estimation, as discussed in Section 6.5.

6.4 Comparison of culture- and qPCR-based estimates of HAdV concentration.

How much does qPCR over-estimate numbers of HAdV in environmental water samples? Numbers of HAdV detected in water using qPCR are typically 1 to 2 logs higher than estimates using culture-based methods (Table 5). However, these comparative analyses used the A549 cell line, which does not support growth of the most prevalent serotype in water, Ad41. Therefore, analyses using the A549 cell line have likely under-estimated HAdV numbers, and the difference with qPCR methods will therefore be less than indicated in these studies (Wyn-Jones et al 2011; Ogorzaly et al. 2013a; Ogorzaly et al. 2015). To establish differences between culture and
molecular tests more accurately, studies are needed to compare enumeration results obtained by qPCR and culture in 293A cells (which supports the growth of AdF, as well as AdC and AdA). Importantly, sensitive methods to detect virus infection of cultures, such as ICC-qPCR are needed in these studies. It would also be beneficial to include analyses of methods for removing free viral DNA and reducing detection of non-infective virus particles (DNase treatment, antibody capture and PMA/EMA pre-treatment) in these analyses. Such studies may enable better establishment of the relationship between virus concentration estimated using culture and qPCR methods and reduce the discrepancy between results obtained using the different methods.

6.5 Use of qPCR results for water quality monitoring.

Of importance for monitoring viral disinfection during wastewater treatment processes, is that qPCR can measure log reductions of HAdV (Table 4 and 5; Hewitt et al. 2013). The recent introduction of regulations permitting the use of HAdV qPCR for validating WWT processes in Victoria, Australia (VIC-Guidelines 2013) is indicative that molecular methods are becoming more accepted for monitoring the virological quality of water. The time and cost effectiveness of qPCR may also promote more routine verification monitoring of human viruses in wastewater treatment processes in the future, which is increasingly recognised as a priority by regulators. Early notification of water users is imperative to limit exposure to recycled water or recreational water contaminated with human viruses. Our analysis supports that public health will be better protected by use of rapid qPCR methods, even if there is some degree of over-estimation of risk (Ashbolt et al. 2010; Kumar et al. 2012; Staley et al. 2012; Symonds and Breitbart 2014).
7. Implications of method accuracy and limitations for QMRA.

Culture based analyses reporting infectious units have conventionally been relied upon to inform QMRA studies, and used to determine suitable purposes for recycled water. However, as discussed, culture methods may not measure HAdV numbers adequately, due to their inherently lower sensitivity and/or an inappropriate choice of cell line and/or where sensitive PCR methods are not used to detect virus infection of cells (Section 5). Underestimating HAdV numbers when modelling wastewater treatment efficiency, and subsequently in QMRA, means risks for a given application may be underestimated. This was demonstrated for different exposure scenarios for bio-solids, where approximately 10-fold differences in risk estimates were reported depending on the cell line used for analysis of echovirus (Kumar et al. 2012). This study also used a “cell-line based infectivity parameter” in QMRA, to account for the difference in infectious viruses (culture methods) and gene copies (molecular methods), to enable risk assessment based on qPCR data. Similarly, others assumed 50% of norovirus GC were infectious to enable risk assessment based on qPCR data (Schoen and Ashbolt 2010).

The use of HAdV qPCR results for QMRA for various water-related applications were also reported by Ahmed et al. (2009) and Kundu et al. (2013), and involved conversion of GC to infectious units. A better understanding of the relationship between culture methods (such as ICC-qPCR) and molecular estimates of HAdV numbers is expected to support the increased use of qPCR data for routine monitoring of wastewater treatment, and enable more precise corrective factors to be incorporated into QMRA.

Viral enumeration data also needs to be corrected in QMRA to account for viral loss during concentration procedures (Petterson et al. 2015). Adequately correcting for high levels of
between sample variability in viral recovery presents a significant challenge (Petterson et al. 2015), and would be assisted by improved and consistent HAdV concentration methods and identification of suitable surrogates. Currently, in cases where recovery is extremely low (e.g. < 0.1%) the need to correct viral enumeration data by several orders of magnitude is a significant source of uncertainty in QMRA, and thus minimum acceptable limits of recovery need to be determined (Section 4.3.5; Petterson et al. 2015).

QMRA may also become more refined by greater attention to assessing different genotypes present (measuring HAdV associated with respiratory illness or gastroenteritis) as appropriate to different water re-use applications (Section 2). Additionally, the limited availability of dose-response models is a constraint in QMRA. The dose-response model for inhalation of the Ad4 respiratory pathogen has been used to assess risk of drinking water and swimming in recreational water contaminated with HAdV (Crabtree et al. 1997). Ad4 is highly infective (Mena and Gerba 2009; Ward et al. 1984), but it is not commonly found in environmental waters (Table 1). It is not known whether the infectivity of A4 is comparable to that of serotypes that are commonly found in environmental waters. The use of the dose response-model for rotavirus and occurrence data for HAdV in QMRA is recommended in the Australian Guidelines for Water Recycling (AGWR 2006). Thus, the reference pathogen is an amalgam of rotavirus and adenovirus (as stated), and risk is assessed for rotavirus using HAdV enumeration data.

Considerable research effort is being focussed on how to correct for sources of error and uncertainty related to viral enumeration data and limited dose-response models and within the QMRA modelling process (Petterson et al 2015; Ashbolt 2015).
8. Recent technological developments and future directions.

8.1 A molecular beacon assay for early detection of virus infection of cell cultures.

Potential for dramatically reducing time for culture results was shown using a molecular beacon assay to detect viral replication in inoculated cells within 3 hours (Dunams et al. 2012). The molecular beacon (MB) enabled specific detection of HAdV E1A gene products. Detection of E1A expression is the earliest known indicator for confirming virus infectivity, and can be detected before viral genomic DNA replication (Sirikanchana et al. 2008; Page et al. 2010). A MB is an oligonucleotide, that forms a stem and loop structure in the unbound state, such that the fluorescent label and quencher (at either end of the MB) are in contact. Upon binding of the MB to target nucleic acid, the fluorescent label and quencher are separated, and a fluorescent signal is produced and detected. Dunams et al. (2012) added a TAT-peptide for intracellular delivery of the MB and chemical modification of specific bases for improved nuclease resistance. Detection limits for HAdV were 1 PFU/mL after 7 hours, 10 PFU/mL after 4.5 hours and 100 PFU/mL after 2 hours using fluorescent microscopy. The assay was further able to multiplexed, to enable the simultaneous detection of echovirus. The intensity of the fluorescent signal was highly correlated to the number of plaques, showing potential for use as a quantitative assay. Disadvantages of the method include the use of chamber slides (which limits analysed sample volume), time and facilities required for fluorescent microscopy.

8.2 Fluorescence-activated cell-sorting (FACS) assay for rapid detection of infectious HAdV.

FACS is another innovative approach for detecting early stages of viral replication in inoculated cells. FACS is a type of flow cytometry that can be used to quantify fluorescent signals from individual cells in a heterogeneous population (Li et al. 2010). Li et al. (2010) used FACS to
quantify recombinant Ad5, which express green fluorescent protein in inoculated cells. Following incubation for 1, 2 and 3 days method sensitivity was 100, 10 and 1 PFU respectively.

In a modified version of the method, fluorescently labelled antibodies were used to detect replication of HAdV from wastewater in cell cultures (Li et al. 2010). Using antibodies targeted to the hexon protein, sensitivity of 1-10 PFU after 3 days incubation was demonstrated. Numbers of HAdV reported were 10 - 160 PFU/100 mL in primary effluent and 10 – 160 PFU/100 mL in secondary effluent. The reporting of a range of PFU is necessitated by use of different standard curves for different serotypes. Direct comparison with other culture methods is needed to better interpret relative sensitivity of the FACS method. The time required for sample preparation (cell collection, washing, successive incubations with antibodies and fixation), as well as high cost of equipment and need for trained personnel are limitations to the routine use of the FACS method.

8.3 Digital PCR.

Digital PCR (dPCR) is a recently developed technology enabling absolute quantification, unlike qPCR where sample concentration is determined relative to a standard/standard curve, using rate-based measurements (Baker 2012; White et al. 2015). Compared to qPCR, dPCR can enable improved precision and has a greater resistance to inhibitors (Hall Sedlak and Jerome 2014). Digital PCR is an extension of end-point PCR and fluorescent probe-based detection, based on the premise of limiting dilution (White et al. 2015). Thus, dPCR uses the same chemistries and primers/probes as qPCR, but additionally the sample is compartmentalised or partitioned. Dependant on the platform, sample are partitioned into capillaries, microfluidic chambers or droplets (White et al. 2015. At the reaction end-point, the proportion of partitions with
fluorescence (+/-) enables absolute quantification by applying Poisson’s distribution. The use of positive (1) and negative (0) data explains the term “digital” PCR (Hall-Sedlak and Jerome 2014; Gutiérrez-Aguirre et al. 2015). An internal amplification control is used to ensure reproducibility (Sedlak et al. 2014).

Digital PCR has been used to detect AdF in a small number of clinical samples (Sedlak et al. 2014). In promising examples, dPCR has been applied to virus particle detection in different water matrices. Detection of enterovirus and pepper mild mottle virus in wastewater using dPCR, demonstrated resilience to PCR inhibitors and suitability of the method for quantifying these two viruses in water (Rački et al. 2014a; Rački et al. 2014b).

A further advantage of dPCR, is that compartmentalisation can reduce certain PCR biases, such as the preferential amplification of more abundant templates (Gutiérrez-Aguirre et al. 2015). Thus, dPCR can enable better amplification of rare sequences, which would be valuable for diversity analysis using amplicon sequencing and viral metagenomics (Hall-Sedlak and Jerome 2014; Gutiérrez-Aguirre et al. 2015). In its current format, dPCR has comparable sensitivity to qPCR, but carrying out the dPCR can take up to five times longer (Hall-Sedlak and Jerome 2014). The major advantages of dPCR, of improved precision and reproducibility, will likely drive the development of more high-throughput, robust and validated methods that will increasingly be used for quantifying waterborne viruses such as HAdV.

8.4 Next generation sequencing (NGS).

Next generation methods for massively parallel sequencing have enabled unprecedented developments in the field of microbial ecology (Radford et al. 2012). NGS can be performed in
different ways, including amplicon sequencing (single gene/PCR product survey) or by metagenome shotgun sequencing (sequencing all of the nucleic acids in a sample) (Zhou et al. 2011; Thomas et al. 2012). Amplicon sequencing is a cost effective approach to targeted diversity assessment (~ US $80 for 20 000 reads). Further, data analysis can be relatively fast, as much fewer reads are produced and requirement for assembly is minimal.

Kuo et al. (2015) sequenced 128 bp HAdV amplicons from raw sewage in Taiwan using an Illumina HiSeq2500. The majority of sequences were Ad41 (99.71%), while AdD and AdC were detected at lower frequency (0.25% and 0.03% respectively). Interestingly, there was a high level of sequence diversity within these species groups, where 2748, 314 and 33 operational taxonomic units (OTUs) were detected for AdF, AdD and AdC respectively. Sequences were grouped into OTUs based on 97% similarity. Absence of AdA and AdB was confirmed using species-specific assays (Kuo et al. 2015).

Ogorzaly et al. (2015) also reported dominance of Ad41 in amplicon sequencing analyses of HAdV in wastewater, but higher proportions of Ad2, Ad12, Ad31 and Ad40 were detected, dependant on location of the treatment plant in Luxembourg (Table 1). Amplicons were sequenced using an Illumina MiSeq (300bp paired-end reads), which provides longer sequence reads that can assist in serotype classification. Amplicon sequencing has not only been shown to be an exceptional means for assessing diversity of HAdV species in wastewater, Ogorzaly et al. (2015) also demonstrated it is a valuable tool for assessing different aspects of method efficiencies. For example, the use of amplicon sequencing better established that different cells lines are permissive to the culture of different HAdV species (Section 5.3). Other methods that might also be evaluated using HAdV amplicon sequencing in wastewater analyses include different concentration methods and PCR methods (e.g. PMA pre-treatment, antibody capture
and primer comparison). For instance, are different HAdV species recovered using different concentration methods? In the future, increased availability of benchtop machines and devices, such as MiniSeq (Illumina) and MinION (Oxford Nanopore) are likely to see uses of NGS expanded even more in different aspects of HAdV research in wastewater/water, including routine application for obtaining enumeration data for different serotypes.


All current viral testing methods have limitations. According to the detailed literature analysis undertaken in this review, we highlight the following for estimating HAdV numbers in wastewater:

1. Celite concentration has shown to be effective for recovering HAdV from small volume wastewater samples. Good recovery of HAdV using EN and 1MDS disk filters and HFUF in particular, have been recently reported for tap water and source water, and these methods might also be evaluated for wastewater. Further evaluation and direct comparison is needed to better establish the most efficient and reproducible methods for recovering HAdV from different volumes of wastewater.

2. Estimating HAdV recovery is important using any concentration method. Spiking a control sample with the target virus is suggested to be most accurate for determining recovery.

3. For culture estimates, use of the HEK-293 cell line (and its derivatives) are more appropriate for estimating total HAdV in wastewater samples, compared to the A549 cell line. Using PCR methods for detecting HAdV infection of cells provides improved sensitivity, which can allow more accurate enumeration.

4. For qPCR estimates, method used for nucleic acid extraction can affect estimates of HAdV numbers, where one study showed the commonly used Qiagen Blood and Tissue kit was
more effective compared to other methods assessed. PCR inhibition should be assessed for any extraction method. The qPCR primers described by Heim et al. (2003) and Jothikumar et al. (2005) have been shown to be suitable for assessing HAdV in wastewater. Further direct comparison of published primers, as well as extraction methods for wastewater analysis may be of value.

Studies directly comparing molecular and cell culture methods are required to ascertain the comparability of molecular methods for determining log reduction values and validation of viral disinfection during WWT processes. Comparisons should use (1) cell culture methods based on lines permissive for all HAdV types and enumerate using ICC-qPCR and ICC-RT-PCR, and (2) molecular methods that assess benefits of PMA/EMA, DNase treatment and immuno-capture for reducing detection of non-infective HAdV in molecular analyses.

Improved viral testing methods can enable public health to be better protected, through data based management of hazards and risks. This applies to both the management of recycled water schemes and to the routine monitoring of recreational waters, where same day results would enable more immediate action to be taken to protect against public health risks. In addition, the current use of culture methods may unacceptably underestimate HAdV exposure risks, depending on the potential re-use and exposure scenarios and monitoring protocols in place. Reliable and timely notification of HAdV contamination is imperative for the protection of public health and the qPCR method performs favourably in this regard. Applied carefully qPCR methods are not likely to greatly over-estimate numbers, and thus should be more widely adopted for routine monitoring of HAdV and in QMRA.
Population growth, water scarcity and stress issues are anticipated to continue to drive greater demands on water resources, such that re-use of water will be increasingly relied upon (WHO 2006; Mara et al. 2010). Thus, robust and dependable tools for monitoring HAdV, as a reliable indicator of human virus contamination of water, will advance the management of water quality for protecting public health.

Acknowledgements

We thank Tim Beales for careful reading of the manuscript. This research was funded by the University of the Sunshine Coast (Australia), the Smart Water Research Centre (Griffith University, Australia) and the Queensland Government, Department of Science, Information Technology, Innovation and the Arts (DSITIA) Science Fund (Australia).
Table 1. Human adenovirus serotypes detected in water matrices in various countries.

<table>
<thead>
<tr>
<th>Human Adenovirus detected</th>
<th>Context</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 and D (17, 32 or 49)</td>
<td>Wastewater</td>
<td>Morocco</td>
<td>Amdiouni et al. 2012</td>
</tr>
<tr>
<td>3, 22, 27, 31, 46, 51</td>
<td>Wastewater</td>
<td>Canada</td>
<td>Vidovic et al. 2011</td>
</tr>
<tr>
<td>2, 3, 12, 40, 41</td>
<td>Wastewater</td>
<td>USA</td>
<td>Fong et al. 2010</td>
</tr>
<tr>
<td>3, 10, 41</td>
<td>Wastewater</td>
<td>Greece</td>
<td>Kokkinos et al. 2010</td>
</tr>
<tr>
<td>1, 2, 12, 31, 41</td>
<td>Wastewater</td>
<td>USA</td>
<td>Kuo et al. 2010</td>
</tr>
<tr>
<td>2, 31, 45</td>
<td>Wastewater</td>
<td>Italy</td>
<td>Carducci et al. 2009</td>
</tr>
<tr>
<td>11, 12, 31, 34, 35, 40, 41</td>
<td>Wastewater</td>
<td>Spain</td>
<td>Bofill-Mas et al. 2006</td>
</tr>
<tr>
<td>1, 2, 3, 6, 12, 31, 40, 41</td>
<td>Wastewater, river water</td>
<td>Luxembourg</td>
<td>Ogorzaly et al. 2015</td>
</tr>
<tr>
<td>1, 2, 5, 6, 31, 41</td>
<td>Wastewater, river water</td>
<td>Luxembourg</td>
<td>Ogorzaly et al. 2013a</td>
</tr>
<tr>
<td>2, 5, 15, 40, 41</td>
<td>Wastewater, river water</td>
<td>USA</td>
<td>Jiang et al. 2006</td>
</tr>
<tr>
<td>1, 2, 3, 5, 6, 7, 15, 40, 41</td>
<td>Wastewater, surface</td>
<td>Various</td>
<td>Mena and Gerba 2009</td>
</tr>
<tr>
<td>1, 2, 3, 12, 31, 40, 41</td>
<td>Marine, fresh water</td>
<td>Europe</td>
<td>Wyn-Jones et al. 2011</td>
</tr>
<tr>
<td>3, 4, 7, 14</td>
<td>Swimming pools</td>
<td>Various</td>
<td>Mena and Gerba 2009</td>
</tr>
</tbody>
</table>
Table 2. Diseases associated with human adenoviruses, which have been detected in water.

<table>
<thead>
<tr>
<th>HAdV serotype</th>
<th>Disease</th>
<th>Affected groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>3, 7,</td>
<td>Fatal disseminated infection</td>
<td>Neonates</td>
</tr>
<tr>
<td>1, 2, 5, 7, 14</td>
<td>Pharyngitis, Coryza</td>
<td>Infants, young children</td>
</tr>
<tr>
<td>3, 7, 14</td>
<td>Pharyngoconjuctival fever</td>
<td>Children</td>
</tr>
<tr>
<td>1, 2, 3, 4, 6, 7, 14</td>
<td>Upper respiratory disease, pneumonia</td>
<td>Children</td>
</tr>
<tr>
<td>2, 3, 5, 31, 40, 41</td>
<td>Gastroenteritis</td>
<td>Children</td>
</tr>
<tr>
<td>1, 2, 4, 5</td>
<td>Intussusception</td>
<td>Children</td>
</tr>
<tr>
<td>3, 5, 6, 7, 12</td>
<td>Meningoencephalitis</td>
<td>Children, immunocompromised, transplant recipients</td>
</tr>
<tr>
<td>34, 35</td>
<td>Haemorrhagic cystitis</td>
<td>Children (boys), immunocompromised, transplant recipients</td>
</tr>
<tr>
<td>3, 4, 7, 14</td>
<td>Acute respiratory disease</td>
<td>Military recruits, adults</td>
</tr>
<tr>
<td>10, 15, 22-29, 34, 37</td>
<td>Epidemic keratoconjunctivitis</td>
<td>All ages</td>
</tr>
<tr>
<td>5, 31, 34, 35, 45, 46</td>
<td>Pneumonia (disseminated)</td>
<td>Immunocompromised</td>
</tr>
<tr>
<td>14, 34, 35</td>
<td>Urinary tract infections</td>
<td>Immunocompromised</td>
</tr>
<tr>
<td>7, 12</td>
<td>CNS disease, encephalitis</td>
<td>Immunocompromised</td>
</tr>
</tbody>
</table>

a. Diseases listed are associated with HAdV detected in clinical scenario. The ability of many of these serotypes to be transmitted in water is poorly understood. Adapted from Wold and Ison (2013), Mena and Gerba (2009) and Roche et al. (2002).

b. Human adenovirus serotypes detected in water belong to the following species: Species A (12, 31); Species B (3, 7, 14, 34, 35); Species C (1, 2, 5, 6); D (10, 15, 22, 27, 51); Species E (4) and Species F (40, 41).
Table 3. Example sample volumes for virus analysis in different applications.

<table>
<thead>
<tr>
<th>Water Type</th>
<th>Volume (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw wastewater</td>
<td>( \leq 1 )</td>
</tr>
<tr>
<td>Treated wastewater (not re-used, research purposes)</td>
<td>1-10</td>
</tr>
<tr>
<td>Recycled water (non-potable, no direct or indirect contact)</td>
<td>10</td>
</tr>
<tr>
<td>Recycled water (Class A/direct or indirect contact/indirect potable)</td>
<td>50-120</td>
</tr>
<tr>
<td>Recycled water (direct potable)</td>
<td>500-1000</td>
</tr>
<tr>
<td>Drinking water</td>
<td>1500</td>
</tr>
</tbody>
</table>

Table 4. Analysis methods and numbers of human adenovirus detected in wastewater using qPCR.

<table>
<thead>
<tr>
<th>Treatment Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration Method&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Recovery&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>Vol&lt;sup&gt;d&lt;/sup&gt; (L)</th>
<th>DNA Extraction Kit</th>
<th>PCR inhibition test</th>
<th>Concentration of HAdV</th>
<th>Log removal</th>
<th>Primers used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Influent (GC/L)</td>
<td>Effluent (GC/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>Celite</td>
<td>55</td>
<td>1</td>
<td>QIAamp DNA Blood Mini</td>
<td>Dilution</td>
<td>5 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>n/a</td>
<td>n/a</td>
<td>Jothikumar et al. 2005</td>
</tr>
<tr>
<td>Untreated</td>
<td>Celite + UC</td>
<td>14</td>
<td>1</td>
<td>QiaGen DNeasy Blood and Tissue</td>
<td>Spike with phage pP7</td>
<td>n/a</td>
<td>n/a</td>
<td>Heim et al. 2003</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>UC</td>
<td>NR</td>
<td>0.01</td>
<td>QiaGen DNeasy Blood and Tissue</td>
<td>Spike with phage pP7</td>
<td>5.4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>n/a</td>
<td>Heim et al. 2003</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>PEG</td>
<td>NR</td>
<td>0.1</td>
<td>High Pure Viral Nucleic Acid</td>
<td>NR</td>
<td>1.9 x 10&lt;sup&gt;6&lt;/sup&gt;–4.6 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>n/a</td>
<td>Heim et al. 2003</td>
<td></td>
</tr>
<tr>
<td>MBR, AS</td>
<td>GW, PEG</td>
<td>0.09–5.1</td>
<td>4</td>
<td>QIAamp DNA Blood Mini</td>
<td>Spike with Hepatitis G</td>
<td>2 x 10&lt;sup&gt;2&lt;/sup&gt;–2 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1 x 10&lt;sup&gt;2&lt;/sup&gt;–2 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2</td>
<td>Cromeans et al. 2005</td>
</tr>
<tr>
<td>S, MBR</td>
<td>AE, SF</td>
<td>NR</td>
<td>20, 400</td>
<td>QIAamp Viral RNA Mini Kit</td>
<td>NR</td>
<td>1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;–10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.1–3.6</td>
<td>Jothikumar et al. 2005</td>
</tr>
<tr>
<td>S, AS, C</td>
<td>AE, UC</td>
<td>0.2–7.0</td>
<td>0.1, 2</td>
<td>QiaGen DNeasy Blood and Tissue</td>
<td>NR</td>
<td>1.1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.3 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2</td>
<td>Jothikumar et al. 2005</td>
</tr>
<tr>
<td>S, AS</td>
<td>U</td>
<td>34</td>
<td>.04</td>
<td>High Pure Viral Nucleic Acid</td>
<td>Spike target, Dilution</td>
<td>A) 4 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>8 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4</td>
<td>A) Hernroth et al. 2002</td>
</tr>
<tr>
<td></td>
<td>AE, U</td>
<td>2–25</td>
<td>NR</td>
<td>QIAamp Viral RNA Mini Kit</td>
<td>NR</td>
<td>B) 2 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>n/a</td>
<td>Heim et al. 2003</td>
<td></td>
</tr>
<tr>
<td>AS, C</td>
<td>TFUF, UC, CE</td>
<td>NR</td>
<td>1, 10</td>
<td>QIAamp DNA Stool Mini Kit</td>
<td>Uracil N-glycosylase</td>
<td>6 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.1</td>
<td>Hernroth et al. 2002</td>
</tr>
<tr>
<td>AS, C</td>
<td>TFUF, UC, CE</td>
<td>NR</td>
<td>1, 10</td>
<td>QIAamp DNA Stool Mini Kit</td>
<td>Uracil N-glycosylase</td>
<td>6 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.1</td>
<td>Hernroth et al. 2002</td>
</tr>
</tbody>
</table>

a. MBR = membrane bioreactor, AS = activated sludge, S = sedimentation, C = chlorination.
b. Celite = celite concentration method, UC = Ultrafilter centrifugation, PEG = PEG precipitation, GW = glass wool, AE = adsorption–elution, SF = syringe filtered, U = ultracentrifugation, TFUF = tangential flow ultrafiltration, CE = chloroform extraction.
c. NR = not reported; n/a not applicable.
   QiaGen Blood and Tissue Kit resulted in higher numbers of HAdV compared to Macherey-Nagel Nucleospin, Epicentre MasterPure complete DNA and RNA purification kit, QIAamp DNA Stool Mini kit, and Mo-Bio PowerSoil DNA isolation kit.
d. Volume analysed, where influent and effluent volumes were different, a comma separates the...
Table 5. A comparison of human adenovirus concentration in wastewater using culture-based and qPCR methods.

<table>
<thead>
<tr>
<th>Treatment Type</th>
<th>Concentration Method</th>
<th>Vol (L)</th>
<th>Methods</th>
<th>HAdV concentration</th>
<th>qPCR Primers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety of types</td>
<td>AE, OF</td>
<td>20, 410</td>
<td>qPCR</td>
<td>7 x 10^5 GC/L</td>
<td>Jothikumar et al</td>
<td>Simmons and</td>
</tr>
<tr>
<td>(MBR, AS, OD, UV and C)</td>
<td>Cell culture (A549)</td>
<td></td>
<td></td>
<td>4 x 10^3 GC/L</td>
<td></td>
<td>Xagoraraki 2011</td>
</tr>
<tr>
<td>Large WWTP</td>
<td>PEG, CE</td>
<td>1</td>
<td>qPCR</td>
<td>1 x 10^6 GC/L</td>
<td>Heim et al. 2003</td>
<td>Hewitt et al. 2011</td>
</tr>
<tr>
<td>(e.g., MBB, AS, TB, WSP)</td>
<td>Cell culture (A549)</td>
<td></td>
<td></td>
<td>3 x 10^4 GC/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small WWTP</td>
<td>PEG, CE</td>
<td>1</td>
<td>qPCR</td>
<td>1.6 x 10^5 GC/L</td>
<td>Heim et al. 2003</td>
<td>Hewitt et al. 2011</td>
</tr>
<tr>
<td>(WSP)</td>
<td>Cell culture (A549)</td>
<td></td>
<td></td>
<td>0–1 x 10^3 IU/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>OF, PEG, CE</td>
<td>1</td>
<td>qPCR</td>
<td>760–1.5x10^6 GC/L</td>
<td>Jothikumar et al</td>
<td>Rodriguez et al. 2013b</td>
</tr>
<tr>
<td></td>
<td>ICC-q-RT-PCR (293)</td>
<td></td>
<td></td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not specified</td>
<td>U, SF</td>
<td>0.2, 0.4</td>
<td>ICC-qPCR (293a)</td>
<td>3.15 x 10^8 IU/L</td>
<td>Hernroth et al. 2002</td>
<td>Ogorzaly et al. 2013a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.5 x 10^7 – 2.2 x 10^8 IU/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>U, CE</td>
<td>0.04</td>
<td>qPCR</td>
<td>3.52 x 10^6 GC/L</td>
<td>Hernroth et al. 2002</td>
<td>Calgua et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.22 x 10^3 FFU/L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- MBR = membrane bioreactor, AS = activated sludge, OD = oxidation ditch, UV = ultraviolet, C = chlorination, MBB = moving bed biofilm bioreactor, TB = trickling bed, WSP = waste stabilisation pond.
- AE = adsorption–elution, OF = organic flocculation, PEG = PEG precipitation, CE = chloroform extraction, U = ultracentrifugation, SF = syringe filtered. Recovery efficiency was not reported, with the exception of Hewitt et al. (2011) who reported 10% recovery.
- Cell type used in the culture assay is indicated in parentheses.
- IFA = immunofluorescence assay, FFU = measuring focus forming units.
Table 6. Optimal concentration method for HAdV using different filter types, elution buffers and elution methods in a range of water types.

<table>
<thead>
<tr>
<th>Filter*</th>
<th>Spiked virus</th>
<th>Water Typeb</th>
<th>Sample (L)</th>
<th>Virus recoveryc (%)</th>
<th>Sample Amendment/ pH adjusted</th>
<th>Elution buffer* (volume)</th>
<th>Elution method</th>
<th>Z° Conc*</th>
<th>Final vol. (mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA 90mm, 0.45 µM sewage</td>
<td>R</td>
<td>1</td>
<td>C: 32</td>
<td>pH 3.5</td>
<td>Not eluted</td>
<td>Direct extraction</td>
<td>...</td>
<td>...</td>
<td>Ahmed et al. 2015</td>
<td></td>
</tr>
<tr>
<td>SCWP 47mm, 8 µM Ad41</td>
<td>So</td>
<td>1</td>
<td>P: 55 ± 19 C: &gt; 40</td>
<td>pH 3.5 and 10 mM MgCl₂</td>
<td>1.5% BE/ 0.75% glycerol, pH 9 (4 mL)</td>
<td>Vortex 2min</td>
<td>Eva</td>
<td>0.1</td>
<td>Wu et al. 2011</td>
<td></td>
</tr>
<tr>
<td>HA 47mm, 4 µM Ad7</td>
<td>T, S</td>
<td>1</td>
<td>P: 79 ± 5</td>
<td>25 mM MgCl₂</td>
<td>1mM NaOH, pH 10.8 (5 mL)</td>
<td>Stirring 30min</td>
<td>...</td>
<td>5</td>
<td>Sun et al. 2016</td>
<td></td>
</tr>
<tr>
<td>Filterite cartridge Ad40</td>
<td>WWs</td>
<td>65</td>
<td>C: 23 ± 2</td>
<td>pH 3.5 and 0.5 mM AlCl₃</td>
<td>1.5% BE + 0.05M glycine, pH 9.5 (900 mL)</td>
<td>+ve pressure</td>
<td>OF</td>
<td>20</td>
<td>Enriquez and Gerba 1995</td>
<td></td>
</tr>
<tr>
<td>1MDS cartridge T</td>
<td>113</td>
<td>C: 37 ± 5</td>
<td>pH 7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1MDS cartridge T</td>
<td>113</td>
<td>C: 27 ± 7</td>
<td>pH 7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NanoCeram cartridge Ad41</td>
<td>S, So, T</td>
<td>40</td>
<td>P: 1.4-2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 Gibbons et al. 2010</td>
<td></td>
</tr>
<tr>
<td>NanoCeram cartridge Ad2</td>
<td>T</td>
<td>20</td>
<td>P: 39 ± 13 C: 14 ± 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NanoCeram 47mm Ad41</td>
<td>T</td>
<td>0.1</td>
<td>C: 20 ± 9</td>
<td>pH 8</td>
<td>1.5% BE/0.05 glycine, pH 10 (100 mL)</td>
<td>Filtration</td>
<td>Cel</td>
<td>5</td>
<td>McMinn 2013</td>
<td></td>
</tr>
<tr>
<td>NanoCeram 47mm Ad40</td>
<td>T</td>
<td>0.1</td>
<td>C: 39 ± 2</td>
<td>pH 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1MDS 47mm Ad41</td>
<td>T</td>
<td>0.1</td>
<td>C: 64 ± 4</td>
<td>pH 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1MDS 47mm Ad40</td>
<td>T</td>
<td>0.1</td>
<td>C: 52 ± 22</td>
<td>pH 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NanoCeram 90mm Ad41 Dl, R, T</td>
<td>10</td>
<td>C: 19-21</td>
<td>prefilter (R)</td>
<td>1.5% BE, pH 9.75 (1.8L)</td>
<td>+ve pressure 2-30min</td>
<td>FeCl₃</td>
<td>30</td>
<td>Pang et al. 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NanoCeram cartridge Ad41</td>
<td>L</td>
<td>10</td>
<td>C: 0.02</td>
<td>pH 6.5-7</td>
<td>1.5% BE/0.05 M glycine, pH 9 (1 L)</td>
<td>Not reported</td>
<td>Cel, PEG</td>
<td>2.1</td>
<td>Francy et al. 2013</td>
<td></td>
</tr>
<tr>
<td>ViroCap NanoCeram</td>
<td>L</td>
<td>10</td>
<td>C: 0.04</td>
<td>pH 6.5-7</td>
<td>OptimaRE® solution (0.5L)</td>
<td>Not reported</td>
<td>PEG</td>
<td>2.8</td>
<td>2013</td>
<td></td>
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<tr>
<td>Asahi KR 25S (TFUF)</td>
<td>L</td>
<td>10</td>
<td>C: 1.4</td>
<td>pH 6.5-7</td>
<td>0.01% T80 (1 L)</td>
<td>Recirculation</td>
<td>PEG</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>Asahi KR 25S (DE)</td>
<td>L</td>
<td>10</td>
<td>C: 0.6</td>
<td>pH 6.5-7</td>
<td>0.5% T80/0.01% NaPP/AF (0.5L)</td>
<td>Backflush</td>
<td>PEG</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asahi Kasei Reed 25S (TFUF) Ad41</td>
<td>T</td>
<td>100</td>
<td>P: 69 ± 12 C: 68 ± 14 R: 56 ± 8</td>
<td>0.01%</td>
<td>0.01% T80/0.01% NaPP, AF (300-350 mL final volume)</td>
<td>Recirculation</td>
<td>Cel</td>
<td>30</td>
<td>Rhodes et al. 2016</td>
<td></td>
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<tr>
<td>T</td>
<td>100</td>
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<td>R</td>
<td>10</td>
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</table>

- Disk filters are indicated by 47mm and 90 mm, followed by pore size; DE = dead end.
- R = river water; So = source water; T = tap water; S = seawater; WWs = secondary wastewater; DI = deionised water.
- P = recovery from primary concentration method; C = recovery using complete method (primary and secondary); values rounded to nearest integer.
- BE = recovery from primary concentration method; PB = phosphate buffer; T80 = Tween 80; NaPP = sodium polyphosphate; AF = 0.001% antifoam.
- Secondary concentration: Eva = evaporation; OF = organic flocculation; UC = Ultrafilter centrifugation; Cel = celite concentration; FeCl₃ = FeCl₃ flocculation; PEG = PEG precipitation.
References


of Applied Microbiology, 113, 1014-1026.


Genomics. Methods in Microbiology, 42, 555-567.


Figure 1. An overview of the concentration and detection process for waterborne viruses. The first step in the process is concentration of viruses such that they can be detected using conventional methods. During primary and secondary concentration viruses are extracted from large volume water samples into smaller volumes. In this example, primary concentration reduces the sample from 10 L to 100 mL. Secondary concentration is used to reduce the sample volume further (to 10 mL), so that more of the sample can be analysed. Following concentration viruses can then be detected using culture-based or nucleic acid-based methods. Culture based methods rely on the ability of viruses to proliferate in mammalian cell lines. Cell lines are inoculated with the virus concentrate, and viruses are detected by observing the cytopathic effects they cause in the cells or by detecting the viral nucleic acids in the cell cultures (also see Figure 5). Alternatively, viral nucleic acids can be detected directly (without culture in cells) in nucleic acid based detection such as qPCR. This involves extraction of DNA (in the case of HAdV) from the virus concentrate, and measuring virus numbers as gene copies (GC) by qPCR. During qPCR, viral nucleic acid is amplified and higher numbers/GC of viruses produce a detectable signal earlier (red line) compared to lower numbers of viruses (blue line) as shown in Figure 1c. A standard curve, produced from known numbers of GC, is used to determine the number of virus GC in a sample. Figures 1A and 1B (cell culture figures only) from Brown et al. (1992) are used with permission, Copyright © 1992 American Society for Microbiology.
Figure 2. Human adenovirus: structure and morphology. Human adenoviruses (Mastadenovirus genera, Adenoviridae family) are icosahedral, 90–100 nm, non-enveloped, linear double-stranded DNA viruses. The major proteins of the icosahedral capsid include the hexon, fibre and penton base proteins (Vellinga et al. 2005). For fibres, there is a high level of intraspecies sequence divergence (Darr et al. 2009). Conserved regions in the gene encoding the hexon proteins are targeted in molecular analyses designed to detect all HAdV. Figure adapted from Feldmann (2001).
10 000 virus particles ($N_i$)

Treatment
process

3 log$_{10}$ reduction
= 99.9% or 1000 fold reduction

10 virus particles ($N_o$)

$\text{LRV} = \log_{10} (N_i) - \log_{10} (N_o)$

Figure 3. Log reduction value (LRV) for a wastewater treatment process. In this example, a 3-log$_{10}$ reduction is a 99.9% or 1000 fold reduction in virus numbers. A 1-log reduction is a 90% or 10 fold reduction in virus numbers and so on (VIC-Guidelines 2013). $N_i$ = number of viruses at the inlet of a treatment; $N_o$ = number of viruses at the outlet of a treatment.
A) Adsorption–elution using electronegative or electropositive filters. Water is passed through a disk or cartridge filter, viruses are adsorbed to the filter surface, and then eluted in a smaller volume of alkaline beef extract or buffer (APHA 2012). Viruses adsorption is via electrostatic and hydrophobic interactions. Most viruses have a negative charge in ambient waters, and bind to positively charged filters. For adsorption to negatively charged filters, sample pH is lowered below the viral isoelectric point, so that the viral charge becomes positive; or multivalent cations are used for adsorption via salt bridges. **Applications:** Cartridge filters are used for primary concentration of large volume water samples (10s to 1000s L). Disk filters are used for smaller volumes, up to ~10L. **Disadvantages:** Poor elution of enteric HAdV can result in low recovery (current research is focussing on improving elution); clogging of the filter from suspended materials, and virus inactivation when using electronegative filters and low pH (Gerba 1984; Li et al. 1998; Gibbons et al. 2009; Fong et al. 2010).

B) Organic flocculation (OF) and celite concentration. During OF, the pH of a beef extract solution is lowered and flocs of proteins and virus are formed. Flocs are collected by centrifugation, and viruses are eluted in a smaller volume of alkaline buffer. Celite also acts as a binding agent, similarly to flocs. Sample pH is lowered to effect virus binding, celite is collected using centrifugation or filtration, and are viruses are eluted in a resuspended in a smaller volume (Katzenelson et al. 1976; McMinn et al. 2012). **Applications:** Typically used for secondary concentration, and in some cases used for primary concentration. Larger volume samples can be processed when flocs are recovered using filtration. **Disadvantages:** Virus inactivation from the pH range used (pH 4–9), and proteins can interfere with downstream analysis (Rhodes et al. 2011).

C) PEG Precipitation. Viruses are precipitated from solution by the addition of PEG (an inert, synthetic polymer) and salts, while stirring at 4°C (4h to overnight). The viruses are then pelleted using centrifugation. The viruses are driven from solution so that the system can return to a more favourable thermodynamic dynamic state and/or because of saturation of the solution (Atha and Ingham 1981; Lee and Lee 1981; Lewis and Metcalf 1998). **Applications:** Often used as a secondary or tertiary concentration method, or as the primary concentration method for small volume samples (~1 L) of high turbidity such as wastewater. **Disadvantages:** More hydrophobic or negatively charged proteins may be precipitated more. Lengthy incubation time.

D) Hollow fibre ultrafiltration. Viruses are retained in large surface area dialysis filters (with hollow fibres) and in the filtration system, while water is extracted from the sample. A peristaltic pump is used to recirculate the sample/retentate until the desired level of concentration is achieved. Viruses bound to the filter surface are released using Tween 80 and sodium polyphosphate surfactants. The method is adaptable to field collection, and is less affected by turbidity (Hill et al. 2005; Smith and Hill 2007). **Application:** Primary concentration from 10 L to large volume samples. **Disadvantages:** Entrapment of viruses on the filter surface and inhibition of molecular analysis from the use of sodium polyphosphate.

E) (i) Ultra centrifugal filter centrifugation and (ii) Ultracentrifugation. Using ultra centrifugal filters, water is extracted from the sample to concentrate the viruses. In ultracentrifugation, very high speeds (180,000 to 210,000 xg) are used to pellet or fractionate viruses in a sucrose gradient (Prata et al. 2011; Sidhu et al. 2013). **Application:** Typically secondary or tertiary concentration and in some cases primary concentration of small volume, turbid samples. **Disadvantages:** Only small volumes can be processed (~15 – 70 mL); entrapment of viruses in ultracentrifugal filter resulting in poor recovery; expensive equipment is required for ultracentrifugation.

Figure 4. Overview of the common methods used for the concentration of viruses from water. Figure 1A from Fout et al. (2015) is used with permission Copyright © 2015 JoVE; Figure 1B is adapted from Birdle et al. (2014); Figure 1D is adapted from Hill et al. 2005; Figure 1Ei is used with permission Copyright © 2002 Nature Publishing Group.
Inoculate cells with virus (or sample) dilutions

**MPN/Endpoint**
- 14-28 day incubation, passaging cells as needed
- Determine MPN/endpoint by observing CPE.

**Plaque Assay**
- 7 day incubation, following overlay of cells with agar
- Remove agar, stain cells with crystal violet and count plaques..

**ICC-RT-qPCR**
- 3-5 day incubation, followed by mRNA extraction from cells
- Reverse transcription followed by qPCR, LOD 10/reaction

**ICC-qPCR**
- 2 day incubation, followed by DNA extraction from cells
- Quantitative PCR, LOD 10 MPN/flask

**Figure 5. Overview of different culture-based detection methods for viruses.** Conventional culture methods (plaque assay and MPN/endpoint detection based on cytopathic effects), are much more lengthy compared with newer methods that use PCR to detect virus-positive cultures (ICC-RT-qPCR and ICC-qPCR). ICC-qPCR has demonstrated slightly higher sensitivity (Ogorzaly et al. 2013a) compared to other molecular methods used to detect virus infection of cells.
Figure 6. Multiple sequence alignment of the partial hexon gene for representative HAdV species and published PCR primers and probes. Numbering is relative to genome position in Ad41 AB330122 (Tak isolate). Dots indicate the sequence is the same as the anchor sequence (Ad41 Tak). Primer name denotes reference, F = forward primer, P = probe, R = reverse, and any further designation. Primers references: Allard et al. 2001; Heim et al. 2003; Hernroth et al. 2002; Jothikumar et al. 2005; Prevost et al. 2015. Representative HAdV sequences were downloaded from GenBank.
Highlights

- Concentration and detection methods for human adenovirus (HAdV) are evaluated.
- Methods chosen affect the accuracy of enumerating HAdV in water.
- HAdV culture analyses using non-permissive lines are underestimating numbers.
- HAdV qPCR performs favourably for water quality monitoring and risk assessment.
- Quantitative PCR provides capacity for early notification of water contamination.