Australian Water Recycling
Centre of Excellence

Project Report

National Validation Guidelines for Water Recycling: Methods for Pathogen Isolation, Culture, Detection and Enumeration

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National Validation Guidelines for Water Recycling: Methods for Pathogen Isolation, Culture, Detection and Enumeration

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About the Australian Water Recycling Centre of Excellence

The mission of the Australian Water Recycling Centre of Excellence is to enhance management and use of water recycling through industry partnerships, build capacity and capability within the recycled water industry, and promote water recycling as a socially, environmentally and economically sustainable option for future water security.

The Australian Government has provided $20 million to the Centre through its National Urban Water and Desalination Plan to support applied research and development projects which meet water recycling challenges for Australia's irrigation, urban development, food processing, heavy industry and water utility sectors. This funding has levered an additional $40 million investment from more than 80 private and public organisations, in Australia and overseas.

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Executive Summary

Issue statement and context

Water scarcity is driving increased reuse of alternative water sources, such as wastewater and storm water. The Australian Guidelines for Water Recycling (AGWR) provides a framework for the safe use of these alternative water sources. Key elements within the framework requires characterisation of the hazards in the water, such as pathogens, and the use of effective barriers to remove or control the hazards to reduce risk to end users or the environment an acceptable level. Not all utilities, particularly those in small regional locations, have the resources for detailed characterisation of hazards or measurement of the performance of treatment barriers. In the absence of such information, Phase 1 of the AGRW provides indicative values for the numbers of pathogens in sewage (Table ES1).

Table ES1 Indicative numbers of pathogens in sewage (AGWR 2006).

Organism	Numbers in sewage (per litre)
Bacteria	
Escherichia coli (indicators)	10^5–10^{10}
Pathogenic *E. coli*	Low
Enterococci (indicators)	10^6–10^7
Shigella	10^1–10^4
Campylobacter	10^2–10^5
Salmonella	10^3–10^5
Clostridium perfringens (indicator)	10^5–10^6
Viruses[a]	
Enteroviruses	10^2–10^6
Adenoviruses	10^1–10^4
Noroviruses	10^1–10^4
Rotaviruses	10^2–10^5
Somatic coliphages (indicators)	10^6–10^9
F–RNA coliphages (indicators)	10^5–10^7
Protozoa and helminths	
Cryptosporidium	0–10^4
Giardia	10^2–10^5
Helminth ova	0–10^4

a Colony-forming units for bacteria, plaque-forming units for bacteria, oocysts for *Cryptosporidium* and cysts for *Giardia*

Similarly, the AGWR also provides indicative removal values for the performance of different steps in the wastewater treatment train (Table ES2). A limitation of the values in the AGRW is that they represent ranges of pathogen numbers or treatment performance from a wide variety of locations and process designs, which may vary in terms of pathogen challenges or treatment effectiveness. Site-specific pathogen data can allow better system design to meet treatment requirements for the production of safe and fit for purpose reuse water. In addition, validation of treatment process performance will ensure that the treatment train is effective at achieving the desired level of treatment with appropriate safety factors, avoiding either under-treating the water or excessive operational or capital costs associated with over-treating the water. A difficulty with the validation of wastewater treatment processes is the lack of any national standard protocols, which are required to ensure that the validation approach is reliable and that it is consistently applied. The development of such protocols is a key element of other sub-projects within the NatVal 2 project.

A more fundamental issue that underlies process validation is the selection of appropriate representative pathogens or surrogates for use in validation studies and the availability of suitable methods for their enumeration. Within this context, a representative pathogen or indicator is used as a surrogate for a particular class of pathogens for the purposes of measuring the performance of a particular treatment process. Process indicators may be different to the reference pathogens described in the AGWR, where a reference pathogen (or pathogens in the case of viruses) has been selected to represent a class of pathogens for the purposes of hazard identification and risk characterisation for QMRA and defining health guideline values. There are known limitations with the methods used to detect some reference pathogens, including human enteric viruses (in particular adenovirus and rotavirus), *Campylobacter* and *Cryptosporidium*. Various methods are used for isolation, culture and detection of these reference pathogens, which makes comparison of data

difficult. The methods are in some cases limited in application (such as *Campylobacter*) due to highly variable results and may be improved through application of new technologies.

Table ES2. Indicative performance of different treatment processes for the removal of pathogens in wastewater (AGWR 2006, 2008).

	Indicative log reductions[a]							
Treatment	*Escherichia coli*	Bacterial pathogens (including *Campylobacter*)	Viruses (including adenoviruses, rotaviruses and enteroviruses)	Phage	*Giardia*	*Cryptosporidium*	*Clostridium perfringens*	Helminths
Primary treatment	0–0.5	0–0.5	0–0.1	N/A	0.5–1.0	0–0.5	0–0.5	0–2.0
Secondary treatment	1.0–3.0	1.0–3.0	0.5–2.0	0.5–2.5	0.5–1.5	0.5–1.0	0.5–1.0	0–2.0
Dual media filtration with coagulation	0–1.0	0–1.0	0.5–3.0	1.0–4.0	1.0–3.0	1.5–2.5	0–1.0	2.0–3.0
Membrane filtration	3.5–>6.0	3.5–>6.0	2.5–>6.0	3–>6.0	>6.0	>6.0	>6.0	>6.0
Reverse osmosis	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0	>6.0
Lagoon storage	1.0–5.0	1.0–5.0	1.0–4.0	1.0–4.0	3.0–4.0	1.0–3.5	N/A	1.5–>3.0
Chlorination	2.0–6.0	2.0–6.0	1.0–3.0	0–2.5	0.5–1.5	0–0.5	1.0–2.0	0–1.0
Ozonation	2.0–6.0	2.0–6.0	3.0–6.0	2.0–6.0	N/A	N/A	0–0.5	N/A
UV light	2.0–>4.0	2.0–>4.0	>1.0 adenovirus >3.0 enterovirus, hepatitis A	3.0–6.0	>3.0	>3.0	N/A	N/A
Wetlands — surface flow	1.5–2.5	1.0	N/A	1.5–2.0	0.5–1.5	0.5–1.0	1.5	0–2.0
Wetlands — subsurface flow	0.5–3.0	1.0–3.0	N/A	1.5–2.0	1.5–2.0	0.5–1.0	1.0–3.0	N/A

N/A = not available; UV = ultraviolet
a Reductions depend on specific features of the process, including detention times, pore size, filter depths, disinfectant

In the case of *Cryptosporidium*, well-validated methods are available for the concentration and enumeration of oocysts from surface and potable waters. However, the relatively poor quality of raw and primary treated wastewaters has been problematic for *Cryptosporidium* enumeration, often limiting the sample volume (20 mL to 250 mL) that can be processed and adversely affecting the accuracy of enumeration of pathogen loads. Direct concentration of small volumes or primary effluent is currently by centrifugation followed by oocyst purification using immunomagnetic separation (IMS). Possible alternatives include resuspension of smaller volumes of raw and primary treated sewage in large volumes of water prior to concentration, allowing dispersion of particulates and dilution of the fats and oils. Homogenization of samples is also a possible option to disaggregate particulates and expose particle-bound oocysts for more even recovery. Any improvements in the method will not compromise additional analyses such as oocyst infectivity measurement or genotyping using molecular methods.

A current key issue faced by the water industry is validation of virus removal for the production of reuse water as required by the AGWR. The use of a single standardised method (also measuring infectivity in the case of disinfection process validation) would allow direct comparison of results between schemes. The challenges related to virus enumeration include sample transport and storage, virus concentration and recovery and virus detection. Some viruses may be sensitive to storage / transport, even at 4°C. Recovery and detection can both be affected by the matrix, which can reduce recovery efficiency and also affect downstream detection by culture-based or molecular methods by interfering with binding to host cells or inhibiting the reactions used to detect the viruses. Improved recovery and detection methods will provide better process performance data and ultimately provide better data for incorporation into future revisions of the AGWR. Further work is required to improve

the recovery of viruses in raw and primary treated wastewaters and determine any factors that influence the detection of infectious viruses by cell culture. Sample stability also needs to be reviewed for samples that require transport to interstate laboratories for analysis.

Campylobacter is a key reference bacterial pathogen that is considered in the production of reuse water. The standard method for enumeration of *Campylobacter* uses a combination of membrane filtration and MPN enumeration, culture in semi selective (Preston's) enrichment broth and subculturing into enrichment agar or broth. Confirmation of *Campylobacter* species is complex, using Gram staining and biochemical markers (APHA, 9260G). There are inherent problems with this methodology due to matrix effects (especially filter blockage) and also by the use of MPN, which can have large uncertainties within the count estimate. Selective chromogenic agar offers an alternative for the enumeration of *Campylobacter*. Although still requiring membrane filtration, the detection and enumeration of *Campylobacter* species is simplified by the allowing colony counts. The applicability of chromogenic agars was originally going to be considered within this project. However, during the start-up phase of this project key technical issues were raised regarding the detection of *Campylobacter*, particularly in relation to oxidative stress and the inability of culture techniques to detect *Campylobacter* cells that are in a viable but non-culturable state. Due to these issues the proposed *Campylobacter* method development was abandoned.

Project Aims

1. The initial aim of this project was to undertake literature review of pathogens and surrogates in order to identify any appropriate pathogen-surrogate pairs that may be of use in other sub-projects within NatVal 2.2. In addition, a review was conducted of the available methods for viruses and *Cryptosporidium*.
2. Following completion of the literature review, an additional aim was to provide guidance regarding appropriate protocols for surrogates/pathogens suitable for validation activities across particular WWTP processes and source waters destined for re-use.
3. The main aim of this project was to develop improved methods for the isolation, culture, detection and enumeration of reference pathogens (eg *Cryptosporidium*, adenovirus) in wastewater matrices
4. Following completion of any method improvement, the final aim of this project was to undertake inter-laboratory trials to confirm the wider use of methods across Australian laboratories

Literature Review Outcomes

The focus of the literature review was on virus surrogates, given that viruses are the most problematic in terms of detection techniques and that there is a real need for virus surrogates for the validation of physical removal processes such as membrane filtration. The selection of a representative bacterial pathogen and surrogate was relatively straightforward and so this was not considered in great detail. *Escherichia coli* fulfilled the criteria required for both representative pathogen and indicator (cost, presence, ease of detection, behaviour) and was recommended for use in validation studies. Although *Campylobacter* is the reference pathogen used within the AGWR for QMRA and determining treatment requirements for managing hazards from bacterial pathogens in reuse water, the technical limitations for cheap and reliable detection of *Campylobacter* do not make it suitable for use in performance validation. One consideration for using *E. coli*, or more broadly for using coliforms, is the nature of the matrix and environmental conditions, bearing in mind that under favourable conditions of temperature and nutrients these faecal organisms can propagate in the environment, which could confound any validation study where there was significant time between the treatment and sample collection. There was limited literature available on surrogates for enteric protozoa. In effect there is no ideal surrogate for *Cryptosporidium*. Spores of sulphite reducing clostridia appear to be conservative indicators for *Cryptosporidium* and *Giardia* removal, but if the pathogen numbers are high enough it would be better to used *Cryptosporidium* oocysts to directly measure process performance, especially since a surrogate provides no information on inactivation of *Cryptosporidium*. Recent advances in methods now allow enumeration of total and infectious oocysts, making it possible to measure the effects of treatment processes on *Cryptosporidium* infectivity.

Of the virus pathogens, adenovirus presents benefits as an indicator due to its prevalence and the relative simplicity of the analytical method, especially for PCR-based detection. However, its relatively large size (60 – 80 nm) means that it might not be a good indicator for processes that rely on size exclusion, such as filtration. In addition, adenovirus is not suitable as a representative virus for UV disinfection on account of its high UV resistance compared with other enteric viruses. Poliovirus has appropriate properties in terms of size and the ability to measure both presence and infectivity. However, with the live vaccine no longer used it can no longer be detected in wastewater and so can no longer be used as an indicator. Enteroviruses are in the correct size range but their presence is strongly seasonal, mostly in summer and autumn. As a result, this group of viruses is at low levels or is not detected in wastewater samples during the other seasons, meaning that enteroviruses can only be used as process indicators in particular seasons. Similarly, norovirus is mostly observed in winter and such a seasonal pattern prevents further use as an indicator. In the case of norovirus, there is no readily available infectivity assay, so it is only of use for validation of processes that use physical removal. Given the absence of a more suitable representative pathogen, adenovirus would be the best option as a process indicator using indigenous human pathogenic virus.

During project workshops somatic coliphages were raised as a possible virus surrogate. The method for somatic phage detection is technically simpler than for F-RNA. The hosts for somatic phage are easier to prepare and so the assay is less likely to fail, especially in the hands of a novice user. However, some somatic phage are very large. For example, T4 is a somatic phage, the head is about 100nm, tail an extra 300 nm, so they are potentially poor surrogates for filtration validation. Additional basic research would need to be done to identify the best candidate species of somatic coliphage. Reoviruses, which are mammalian viruses, could be potential candidates as surrogates for human enteric viruses, but further research would be required to evaluate this. F-RNA phage appear to meet all the necessary requirements in terms of size, prevalence all year round and ability to measure presence and infectivity with reliable methods. A limitation of F-RNA is that their numbers can be low in some water types (eg. secondary effluent or lagoon effluent), but F-RNA such as MS2 can be readily produced by culture methods and spiked into test water for treatment performance validation trials.

The shortlist of representative pathogens and indicators for measuring wastewater treatment performance identified from the literature review is presented in Table ES3.

Table ES3. Summary of Representative Pathogens and Indicators for Wastewater.

Representative Pathogen	Indicator
Virus **By cell culture:** adenovirus reovirus enterovirus rotavirus HAV **By PCR:** norovirus adenovirus enterovirus	**Virus** **By culture:** F-specific coliphages **By PCR:** Polyoma viruses
Bacteria *E. coli*	**Bacteria** Faecal streptococci / enterococci *E. coli* Total coliforms
Protozoa *Cryptosporidium*	**Protozoa** Sulphite-reducing clostridia

Methods Review

Shortly before the commencement of this project Keegan et al., (2012) reported method improvements for virus concentration and culture. In particular, direct precipitation using PEG was reported to give better virus recovery compared with ultrafiltration followed by PEG precipitation. This same study also identified cell lines that appeared to support better virus growth and resulted in more sensitive virus detection. Based on the available information, the focus of virus method improvement was to verify the reports of Keegan et al., (2012). In terms of *Cryptosporidium*, shortly before the commencement of this project some method improvements for concentration of *Cryptosporidium* from primary effluent were made available by the AWQC NATA accredited laboratory. This method is based on dilution of the primary effluent and concentration using calcium carbonate precipitation. The recovery data suggested that this method would be ideal for analysis of primary effluents. Since the calcium carbonate method is not in widespread use, the same approach (sample dilution followed by concentration) was applied to a filtration technique for *Cryptosporidium* oocyst concentration. Another major method improvement for *Cryptosporidium* was the publication of an integrated assay to provide oocyst counts and infectivity data. The new oocyst concentration method and integrated assay were selected for evaluation within this project.

Virus Methods

The comparison of the different cell lines for supporting virus infection was largely consistent with the findings of Keegan et al., (2012). The PLC cell line supported growth of the adenovirus and enterovirus strains/species tested (Figure ES1). The BGM cell line supported enterovirus infection but was a poor host for adenoviruses, although low level of infection by adenovirus was detected. An experiment was conducted to determine if virus infectivity could be improved by adding divalent cations, which are known to improve host cell attachment for some viruses. The addition of Ca^{2+} as an infectivity supplement had no benefit and was detrimental at higher doses, causing a dose-dependent reduction in infectivity, with the impact larger for adenovirus compared with coxsackie virus (Figure ES2).

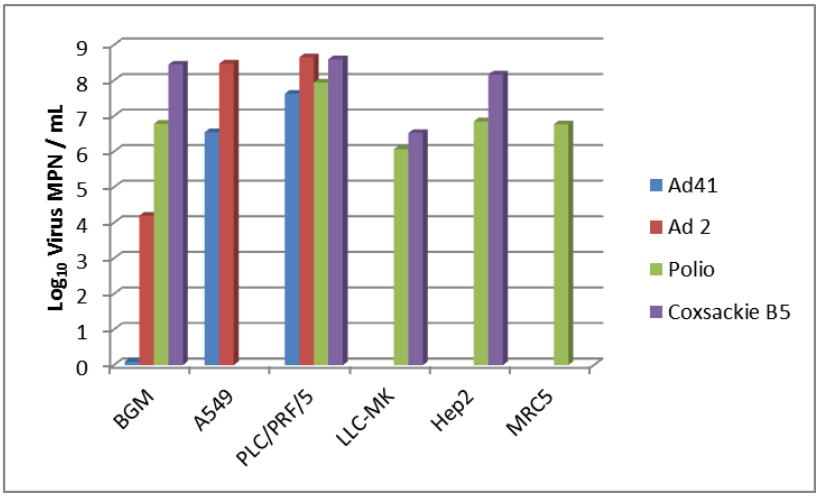


Figure ES1. Comparison of virus counts (MPN / mL) for different viruses cultured with different host cell lines.

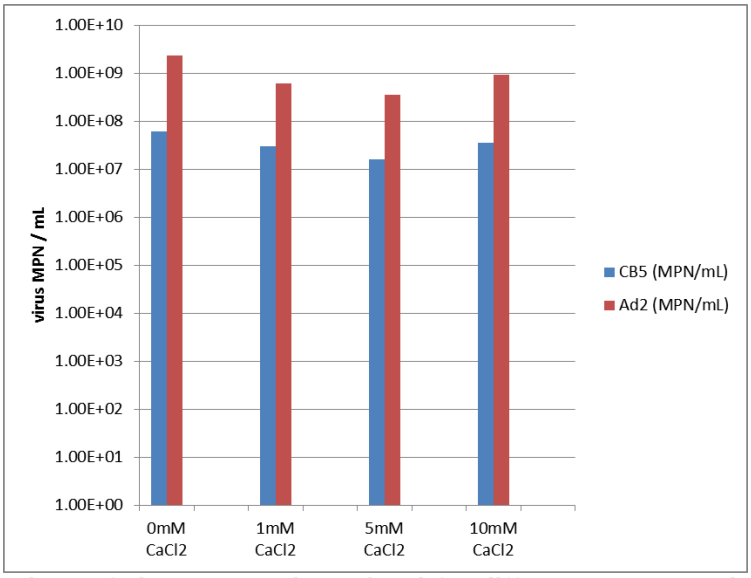


Figure ES2. Comparison of virus counts (MPN / mL) for different concentrations of calcium chloride used as a supplement for cell culture.

Virus recoveries were inconsistent for both of the viruses used and for the different concentration techniques (direct PEG or filtration + PEG). The adenovirus results in particular may have been impacted by the presence of indigenous viruses (especially adenoviruses) and appeared to be worse for infectivity compared with PCR. The detection of indigenous viruses and apparent loss of the spiked Ad 2 could indicate a specific process impact affecting the Ad 2 but not the indigenous viruses. An example of the adenovirus recovery data (detection by PCR) is given in Figure ES3.

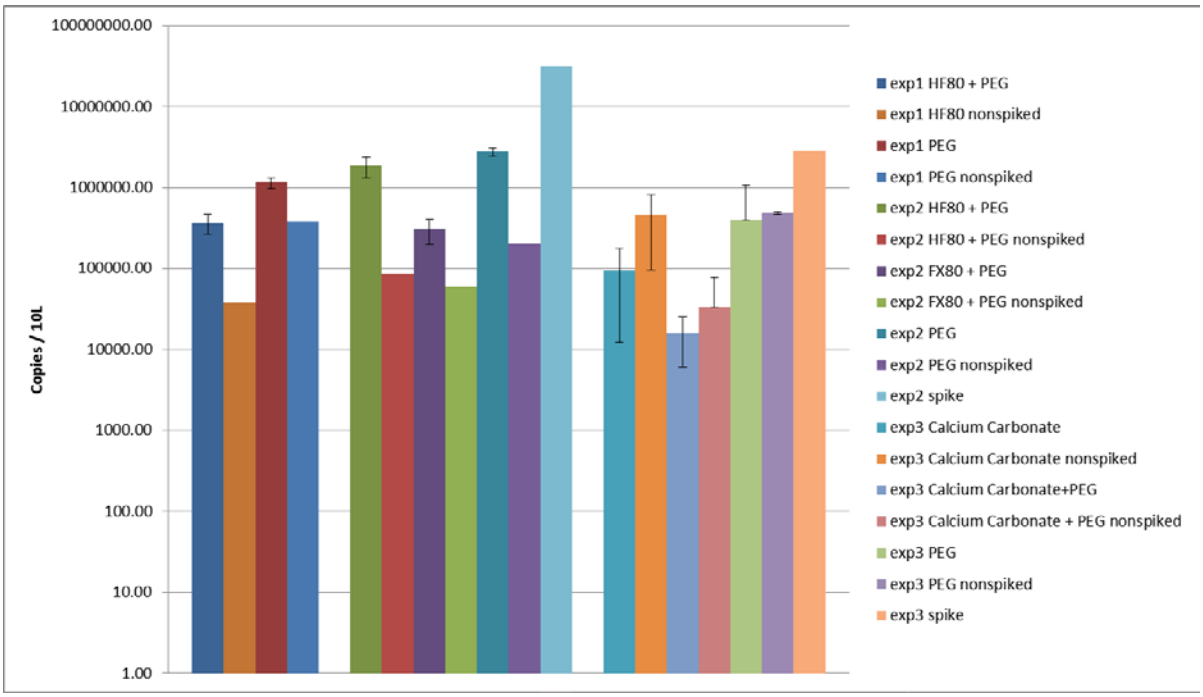


Figure ES3. Comparison of Ad 2 numbers recovered by different concentration methods in spiked and un-spiked Bolivar secondary effluent. Error bars indicate %CV.

The direct analysis of spiked samples (no virus concentration prior to cell culture or PCR) suggests that the Ad 2 were not impacted by the sample matrix and that the virus were stable in the secondary effluent for up to 168 hours (Figure ES4). This suggests that a step in the processing was responsible for the loss of the spiked virus (or loss of infectivity). A further consideration for interpreting the cell culture results is that the PLC cell line was used for all MPN analyses, this cell line supports a wide range of viruses and so detection in the un-spiked samples could be due to the presence of

adenovirus or enterovirus. To differentiate this post-cell culture PCR analysis would be required to identify the virus causing the detected infection. The PCR analysis of the un-spiked sample concentrates suggested that indigenous adenovirus were more frequently detected, with indigenous enteroviruses detected by RT-PCR in only 1 batch of samples processed.

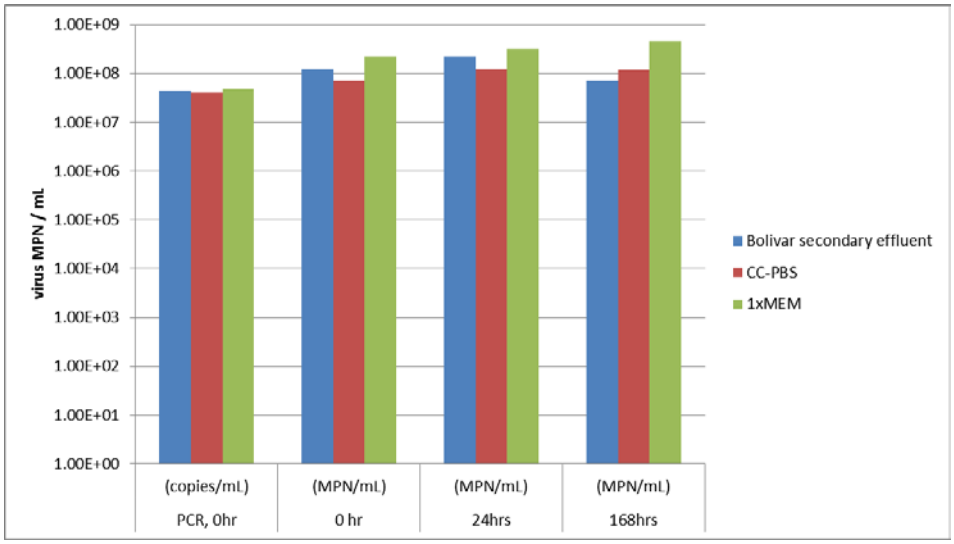


Figure ES4. Comparison of virus numbers after spiking into secondary effluent, buffer or cell culture medium and storage at 4°C.

Overall, the direct PEG method gave better recoveries for both Ad 2 and CB5, although the recovery rates were not as good as that reported by Keegan et al., (2012) for samples collected from the same locations. The recovery rates for Ad 2 in primary effluent were better (although still variable) compared with secondary effluent, which is counter to what would normally be expected (based on previous results from Keegan et al., (2012) and earlier AWQC monitoring). It is possible that factors affecting the secondary effluent quality have greatly affected some of the testing conducted in this project. Future work should focus on evaluating the concentration methods using a wider range of primary and secondary effluents. The use of a surrogate such as suitably modified nanoparticles would greatly assist with method development, allowing the use of simpler enumeration methods and also eliminating any interferences from indigenous viruses, which can complicate enumeration.

Cryptosporidium methods

The comparison of methods for processing primary effluent found similar recovery rates for all 3 methods for the first 2 rounds of testing, though in the third round both direct centrifugation and filtration of diluted primary effluent appeared to perform significantly worse. The cause for this variation is unclear, but it is possible that this could coincide with a bad batch of IMS beads (that were recalled by the manufacturer after this work was completed). For secondary effluent, the calcium carbonate flocculation method was consistently better than Envirochek filtration. Although the recoveries were lower, the oocyst counts (which incorporate recovery rate and % sample processed) were generally higher for direct centrifugation and filtration compared with flocculation. The infectivity of the oocysts recovered by the different methods appeared to vary. In the case of primary effluent, the infectivity of oocysts recovered also appeared to be higher in the oocysts recovered by direct centrifugation and filtration, although in some cases the variation between replicates was large for some of the samples because of the small number of oocysts applied to cell culture. It is possible that the calcium carbonate, which has a higher recovery rate, is better at recovering all oocysts, whereas centrifugation or filtration may be selective for healthier oocysts. If this is the case, it could account for the higher infective fraction for these samples compared with calcium carbonate. Given the relative simplicity of direct centrifugation, the method performance is adequate for oocyst concentration, but the inclusion of a recovery control is essential to identify any changes in recovery performance.

Inter-laboratory comparison

Due to time constraints, it was only possible to conduct a single round of inter-laboratory comparison for virus and *Cryptosporidium* analyses. The results for the primary effluent samples for both viruses

(Figure ES5) and *Cryptosporidium* (Figure ES6) were comparable across the different laboratories, particularly for adenovirus detection by PCR. There was greater variation between the results from the different laboratories for secondary effluent, most likely due to differences in assay detection limit, low levels of virus present and also potentially due to differences in recovery rate. The latter is less likely considering that the participating laboratories used similar filtration methods for secondary effluent. The coxsackie CB5 spiked into some of the samples did not appear to persist and there also appeared to be enterovirus within the un-spiked samples at comparable levels to the CB5 used as a spike. These factors made calculation of a recovery rate difficult and it is not clear if the obtained result was due to poor recovery of the spiked virus or due to other factors affecting the stability or culturability of the spiked CB5.

For primary effluent, the number of culturable viruses versus virus genomes detected by PCR was 2 $\log_{10}$ lower for enterovirus and up to 4 $\log_{10}$ lower for adenovirus (Figure ES5). A similar difference was observed for adenovirus but not for enterovirus in secondary effluent. The detection of indigenous adenovirus in the primary and secondary effluent samples used for the inter-laboratory comparison was similar to the results obtained during the initial method evaluation. In the earlier trials, PCR detected 10^4 – 10^5 adenovirus copies / 10 L and $<10^2$ – 10^3 culturable adenovirus / 10 L in un-spiked secondary effluent samples and 10^5 – 10^6 adenovirus copies / 10 L and 10^2 – 10^4 culturable adenovirus / 10 L in un-spiked primary effluent. Culturable enterovirus were not detected in the secondary effluent samples used for the initial method evaluation, although enterovirus were detected by RT-PCR (10^3-10^5 copies / 10 L), unlike in the case of the samples used for the inter-laboratory trial.

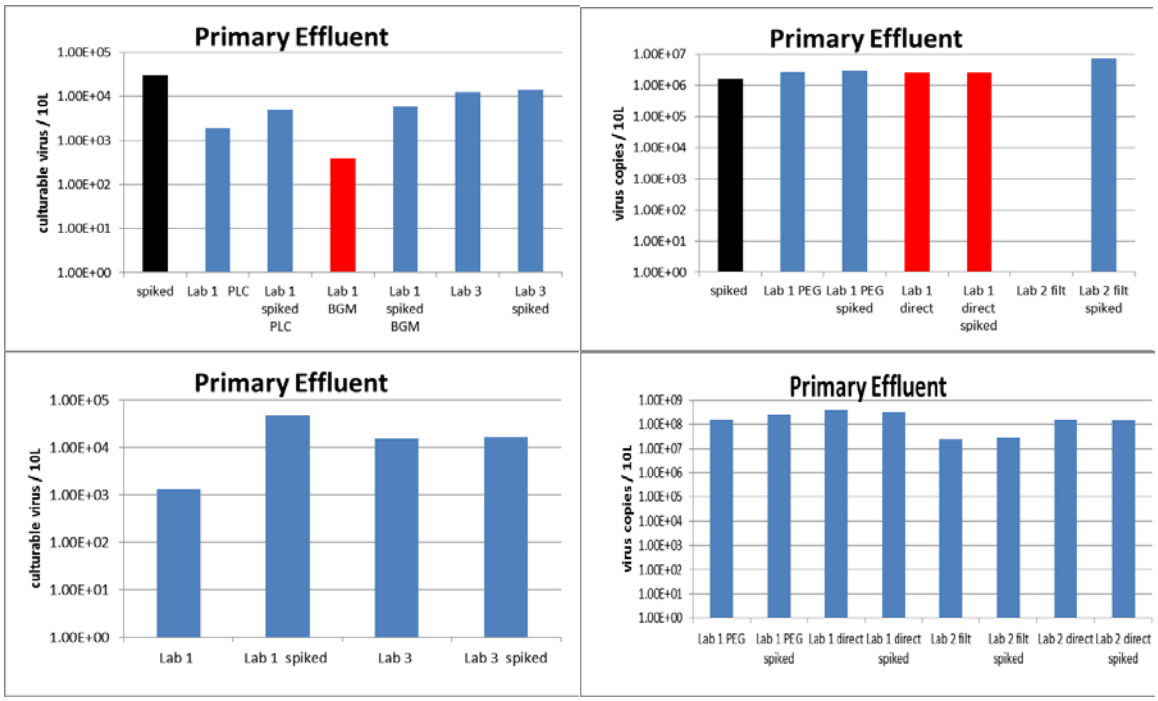


Figure ES5. Summary of virus comparison data enterovirus by culture (top left panel) and PCR (top right panel) and for adenovirus by culture (bottom left panel) and PCR (bottom right panel).

The PCR method for adenovirus appears to be suitable for directly measuring virus numbers without the need for sample concentration, provided the virus numbers are above 1 x 10^4 / 10 L. Compared with the direct detection of viruses, sample concentration appeared to result in the loss of 1 – 2 $\log_{10}$ of adenovirus. Direct RT-PCR detection of enterovirus was not successful and was only successful for primary effluent concentrates.

Based on the limited data available, direct PCR detection of adenoviruses (without any sample concentration) is recommended as a useful and cost effective option for measuring physical removal in both primary and secondary effluents. If sample concentration is required then PEG precipitation of primary effluent samples allowed detection of both enterovirus and adenovirus for cell culture and (RT-)PCR. The results for secondary effluent concentration were equivocal for the comparison of PEG versus ultrafiltration + PEG, although both methods appeared to provide better performance

than ultrafiltration + molecular weight cut-off filters. A key consideration for future method development is improvement of assay sensitivity, which at the moment can only be achieved by analysing a larger proportion of the sample concentrate, which adds to the assay cost.

While the recovery rates for *Cryptosporidium* in primary effluent were similar between the Laboratory 1 and Laboratory 3 methods, there was a large difference in recovery rate for secondary effluent, with the filtration-based method (Laboratory 3) achieving less than 5% recovery rate. No oocysts were detected in the Laboratory 1 samples but small numbers (1 – 4 oocysts) were detected in the Laboratory 3 samples. Allowing for the low recovery rate, this resulted in high apparent counts for oocysts in primary effluent (50 – 100 oocysts / 10 L), similar in magnitude to the oocyst numbers in primary effluent.

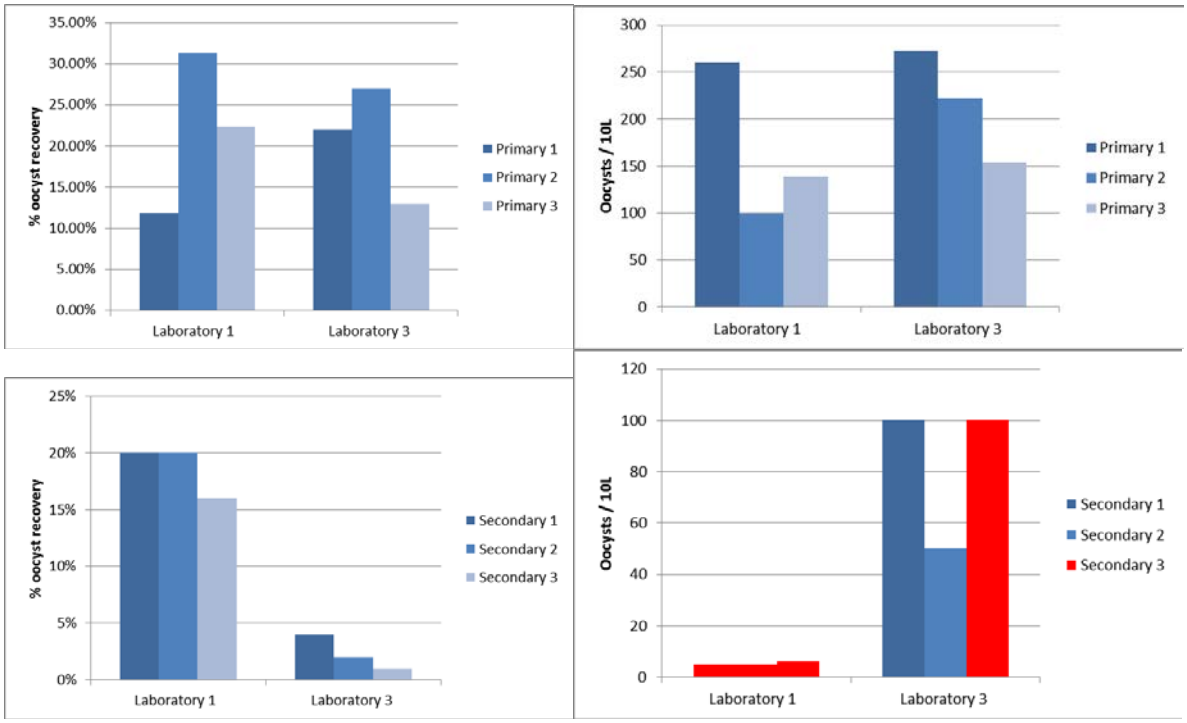


Figure ES6. Summary of *Cryptosporidium* comparison data for primary effluent recovery rates (top left panel), primary effluent oocyst counts (top right panel), secondary effluent recovery rates (bottom left panel) and secondary effluent counts (bottom right panel). The solid bars in red represent results below detection limit.

Recommendations

The literature review was used to recommend surrogates or pathogens for use in treatment validation studies in other sub-projects of NatVal 2. An obvious next step is to ground truth those selections by conducting comparative trials of the relevant pathogen-surrogate pairs. Such data may already be available from other sub-projects within the NatVal2 project and should be able to inform the design of any future surrogate validation studies.

The virus method development work that was conducted in this project suggested that some cell lines were better for detection of enterovirus and adenovirus compared with others in current use. However, not all of the cell lines behaved as anticipated with real wastewater sample concentrates. Future work should compare the performance of the different cell lines for virus detection using real wastewater samples. Many of these cell lines can host multiple virus species, so any such study would need to incorporate PCR to allow for detection of specific viruses such as enterovirus or adenovirus. A cell culture / MPN assay using PCR as the virus detection endpoint is a potential assay format for such a study to more rigorously field test the cell lines used for virus culture.

The *Cryptosporidium* method comparison suggested that the calcium carbonate method combined with IMS gave the highest recovery rates for secondary effluent, and this was confirmed in the inter-laboratory comparison. This method also performed consistently well for primary effluent samples. It

appeared that the infectivity of the oocysts recovered by the calcium carbonate method was lower than that of oocysts recovered by other methods. The reasons for this need to be investigated in future work. One possibility is that the calcium carbonate method is better at recovery of both live and dead oocysts, whereas the other methods might have poorer recoveries because they selectively recover live oocysts, resulting in a higher infectious fraction in those concentrates. Spiking trials using fresh oocysts in primary and secondary effluent would address this question.

The inter-laboratory comparison of methods yielded very promising results. Future work needs to be done using the same and different wastewater locations to determine how reproducible and robust the methods are. Prior to any further studies the baseline numbers of virus need to be determined for each matrix so that appropriate spike levels can be used to allow determination of recovery rates. In addition, virus stability needs to be determined for each matrix. Stability experiments with adenovirus suggested that Ad 2 was stable when spiked into Bolivar secondary effluent, yet these spiked virus could not be efficiently recovered using the PEG of filtration concentration methods. The possibility of matrix interference needs to be investigated – is there something in the Bolivar wastewater that is particularly challenging for virus recovery or is this a general issue for wastewater?

TABLE OF CONTENTS

Figures

Tables

Abbreviations

°C	Degrees Celsius
Ad	Adenovirus
AGWR	Australian Guidelines for Water Recycling
AWQC	Australian Water Quality Centre
AYR	All year round
BOD	Biological oxygen demand
CC	Cell culture
cDNA	Copy DNA
COD	Chemical oxygen demand
CPE	Cytopathic effect
DAPI	4',6-diamidino-2-phenyl indole
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dPCR	Digital PCR
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FITC	Fluorescein Isothiocyanate
FM	Fluorescence microscopy
g	gram
HAV	Hepatitis A virus
II	The roman numeral for 2
IMS	Immunomagnetic separation
L	Litre
LRV	Log_{10} removal value
µm	Micrometre
µL	Microlitre
mL	Millilitre
mm	Millimetre
µM	Micromolar
mM	Millimolar
MEM	Minimum essential medium
min	Minute
MPN	Most probable number
MWCO	Molecular weight cut-off filter
NaPP	Sodium polyphosphate
NEAA	Non-essential amino acids
P	Phosphorous (as in total)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque forming unit
qPCR	Quantitative PCR
qRT-PCR	Quantitative RT-PCR
rcf	Relative centrifugal force
rpm	Revolution per minute
RT-PCR	Reverse transcription PCR
RNA	Ribonucleic acid
SRC	Sulphite reducing clostridia
SS	Suspended solids
ssRNA	Single stranded RNA
TCID50	Tissue culture infectious dose 50%
TKN	Total Kjeldahl Nitrogen
U	Unit
UK	United Kingdom
USEPA	United States Environment Protection Agency
UV	Ultraviolet
VBNC	Viable but not culturable
WW	Wastewater
WWTP	Wastewater treatment plant

1 Introduction

1.1 Background

A number of issues exist with the enumeration of reference pathogens used to demonstrate treatment system performance as specified in the Australian Guidelines for Water Recycling (AGWR). The reference pathogens include human enteric viruses (in particular adenovirus and rotavirus), *Campylobacter* and *Cryptosporidium*. Various methods are used for isolation, culture and detection of reference pathogens, which makes comparison of data difficult. The methods are in some cases limited in application (such as *Campylobacter*) due to highly variable results and may be improved through application of new technologies.

1.2 Current methods and limitations for enumeration of the reference pathogens in wastewaters.

1.2.1 ***Cryptosporidium***

The poor quality of raw and primary treated wastewaters has been problematic for *Cryptosporidium* enumeration, limiting the sample volume (20 mL to 250 mL) that can be processed and adversely affecting the accuracy of enumeration of pathogen loads. Matrix effects (particle interactions, fats, oils and gross pollutants) also affect recovery rates and there is potential for differential recovery of the internal positive control (ColorSeed™) compared with natural oocysts, which may not be incorporated into the matrix in the same way (ColorSeed may be easier to recover if not enmeshed). Current method modifications at AWQC include resuspension of smaller volumes of raw and primary treated sewage in large volumes of water prior to concentration, allowing dispersion of particulates and dilution of the fats and oils. Homogenization of samples is a possible option to disaggregate particulates and expose particle-bound oocysts for more even recovery. Direct analysis of small volumes is currently by centrifugation followed by immunomagnetic separation (IMS), staining with a fluorescently labelled antibody and DAPI and visualisation by fluorescence microscopy with confirmation under DIC to visualise internal contents. Improvements in the method and consistency in approach for isolation of the oocysts will be investigated, with a preference given to improvements that do not compromise additional analyses such as oocyst infectivity measurement.

1.2.2 **Enteric viruses (rotavirus and adenovirus)**

A current key issue faced by the water industry is validation of virus removal for the production of reuse water as required by the AGWR. The use of a single standardised method (preferably also measuring infectivity) would allow direct comparison of results between schemes. For virus enumeration challenges include storage, recovery and detection. Some viruses may be sensitive to storage / transport, even at 4°C, while recovery and detection can both be affected by the matrix, which can reduce recovery efficiency and also affect downstream detection by culture-based or molecular methods. Improved recovery and detection methods will provide better process performance data and ultimately provide better data for any revisions which would better populate into the AGWR. Current molecular methods are available in the participating laboratories and incorporation of an internal positive control has been undertaken at SA Water with good initial success. Further work is required to improve the recovery of viruses in raw and primary treated wastewaters and determine any factors that impact on detection of infectious viruses by cell culture. Sample stability also needs to be reviewed for samples that require transport to interstate laboratories for analysis.

1.2.3 ***Campylobacter***

The standard method for enumeration of *Campylobacter* uses a combination of membrane filtration and MPN enumeration (3 tube multiple dilution technique using 100 mL, 10 mL or 1 mL of filtered water sample), culture in semi selective (Preston's) enrichment broth and, subculturing into enrichment agar or broth. Confirmation of *Campylobacter* species is by Gram stain, oxidase test and absence of growth in the presence of carbon dioxide (APHA, 9260G), confirmation of *Campylobacter jejuni* is by the hippurate hydrolysis test. There are inherent problems with this methodology caused by matrix effects (especially filter blockage) and also by the use of MPN, which provides questionable enumeration. Selective chromogenic agar offers an alternative for the enumeration of *Campylobacter*. Although still requiring membrane filtration, the detection and enumeration of

Campylobacter species is simplified by the allowing colony counts. A number of companies offer chromogenic agars for *Campylobacter* and Sydney Water (project partner) has trialled a limited number of these with promising results. Further application of the new chromogenic agars will be investigated in this project. It will be necessary to test the media in a range of matrices that reflect the different water qualities that require validation from primary to tertiary treated wastewaters.

1.3 Aims

1. Undertake a literature and data review to establish appropriate methods for viruses, *Campylobacter* and *Cryptosporidium* and to identify any appropriate pathogen-surrogate pairs that may be of use in other sub-projects within NatVal 2.2.
2. Provide guidance to which surrogates/pathogens coupled with appropriate protocols are suitable for validation activities across particular WWTP processes and source waters destined for re-use.
3. Develop improved methods for the isolation, culture, detection and enumeration of reference pathogens (*Cryptosporidium, Campylobacter*, adenovirus and rotavirus) in wastewater matrices
4. Undertake inter-laboratory trials to confirm the wider use of methods across Australian laboratories

2 Pathogens and surrogates literature review

2.1 Summary

The focus of this literature review is to provide the necessary background information to guide the selection of surrogates for the validation of wastewater treatment processes. A key issue is that there are many knowledge gaps regarding the behaviour of pathogens and surrogates for most treatment processes, especially at full scale. The acquisition of this information is difficult because the density of pathogens in wastewaters is generally too low to allow validation of processes, particularly for tertiary treatment, and the cost of analyses for virus and protozoan pathogens is high, limiting the scope of studies that have been conducted. Considering these factors, selection of surrogates in many cases will be based on choosing organisms with similar physical attributes to the pathogens of interest or if that is not possible will provide a conservative indicator of removal through a treatment process.

For viruses, adenovirus is currently used for testing Class A recycled water (AGWR 2006, 2008). However, this virus is not representative of the majority of human enteric viruses, being larger, relatively UV resistant (Donnellan et al. 1999, Moriyama et al. 2005) and possessing a different type of genome (DNA) compared with the other enteric viruses (RNA). Enteroviruses have been proposed as being a suitable representative virus pathogen, but the epidemiological data shows that infections are seasonal (Baek et al. 2009, Baek et al. 2011, Khetsuriani et al. 2006, Tani et al. 1995), reflected in monitoring for Australian wastewaters where enteroviruses are not always detected. Polyoma virus have been suggested as a surrogate (Bofill-Mas et al. 2006, Ling et al. 2003), being a compromise between adenovirus and enterovirus in size but possessing the same type of genome as adenovirus (dsDNA). Further study is required to demonstrate that it behaves similarly in treatment processes as enteric viruses, being dsDNA it might be more similar to adenovirus in terms of robustness and resistance to UV. Somatic phage have been proposed as a potential surrogate and satisfy criteria in terms of being the most numerous coliphage in wastewater and being cost effective to detect (Grabow 2001). However, somatic phage are generally much larger than enteric viruses (including adenovirus) and are DNA viruses. Only a single species, ΦX174, is a comparable size to enteroviruses. There is also the possibility that somatic phage can replicate in the environment, with there being a knowledge gap in terms of phage interaction with hosts in a biofilm or concentrated on a filter surface. Considering these issues, indigenous coliphage are not recommended as a surrogate, although ΦX174 could be considered as a challenge organism spiked into a test system but verification is required to demonstrate that it does not replicate under test conditions. The surrogate that best fits requirements is F-specific coliphage, F-RNA coliphage in particular. The F-RNA phage closely match enteric viruses in terms of size and genome type (ssRNA). F-specific phage cannot replicate in the environment unless the host bacteria are in log-phase growth, which is unlikely for *E. coli*. Furthermore, the assay for F-specific phage is relatively simple, F-RNA can be readily propagated for challenge testing and they have also been used in validation studies for UV disinfection and ultrafiltration (Duran et al. 2003, Harwood et al. 2003, Simpson et al. 2003, Tree et al. 2005).

Selection of a bacterial surrogate is relatively straightforward. *E. coli* is present in sufficient numbers to validate secondary treatment processes (and possibly tertiary treatment, depending on the efficiency of upstream secondary treatment), is cheap to detect and is representative of the behaviour of enteric bacterial pathogens, most of which are broadly within the coliforms (Enterobacteriaceae) (Keegan et al. 2009). *Campylobacter* are not suitable as a model pathogen because they are sensitive to oxidative stress and rapidly enter a viable non-culturable state, making detection problematic (Harvey and Leach 1998, Rollins and Colwell 1986).

There are limited data for protozoan surrogates that have been tested across a broad range of treatment processes. Spores of sulphite reducing clostridia (SRC) appear to be a good surrogate for *Giardia* through activated sludge treatment and although not tightly correlated with *Cryptosporidium* removal, may be a conservative indicator for *Cryptosporidium* (Keegan et al. 2009). In terms of size, SRC are smaller than protozoan (oo)cysts so should be a conservative surrogate for filtration processes. Particle profiling has potential as a surrogate but requires further validation (Keegan et al. 2009).

2.2 Introduction

A detailed review of the published literature has previously been conducted (WERF report 03-HHE-2, Keegan et al. (2009)) to identify pathogens and candidate indicators representative of the four groups considered to pose the greatest health risk: bacteria, viruses, helminths and protozoa. Criteria were developed to select pathogens and indicators for the validation of the performance of wastewater treatment processes. In the case of pathogens, the criteria included importance to the water industry, similarity to other pathogens, presence in sufficient numbers and persistence in the relevant waste matrix (activated sludge) where direct detection would be utilized. Where the native pathogens were not present at significant amounts or would not allow calculation of log reduction values (i.e. complete inactivation occurred) laboratory cultured microorganisms would be utilized (membrane filtration and ozone). Similar criteria were also used for the selection of indicator organisms, most importantly the indicator/surrogate organisms need to behave similarly to the target pathogen, have similar surface properties (hydrophobicity and isoelectric point) be easily cultured or detected. Chemical indicators were considered and discounted because their behaviour was unlikely to mimic that of microorganisms in the environment. This review is an update of 03-HHE-2, with the aim to provide information to support the selection of pathogens or surrogates that will be included in other sub-projects within the National Validation project.

2.3 Technologies under consideration

The three technologies under consideration for the National Validation project are activated sludge, membrane filtration and ozone. Activated sludge acts as a removal process but may also incorporate some level of inactivation for different pathogens. The selection of indicators in this case would be reliant upon detection and enumeration of viable organisms. Membrane filtration is a removal process with interaction with the membrane surface being integral to removal. A number of factors influence the removal process including nominal pore size of the membrane, size of the microorganism, surface characteristics and charge of the membrane, surface characteristics and charge of the microorganism, and the potential for interaction of microorganisms with particulates within the water matrix. Disinfection-based oxidants such as ozone act to inactivate the pathogen or indicator and thus require the use of infectivity assays or culture-based methods to determine the loss of infectivity or culturability.

2.4 Criteria for Choosing Representative Pathogens

The three major pathogen groups considered for the NatVal study were viruses, bacteria and protozoa. One or more microorganisms representing enteric pathogens that are a risk to human health have been chosen from each group to be the representative pathogen(s). The criteria used for choosing representative pathogens are given in Table 1, with each criterion ranked relative to the other criteria to assist in choosing an appropriate representative pathogen. Where criteria were unlikely to be met, they were accorded a lower ranking so as to provide a more pragmatic outcome.

Table 1. Criteria for Selection of Representative Pathogens.

Importance	Selection Criteria
1	Found in different geographical areas and represent the range of pathogens potentially present.
1	Minimal seasonal variation.
2	Pathogen is representative of other pathogens in the same group e.g. *E. coli* is considered representative of enteric bacterial pathogens.
2	Is applicable to as many matrices as possible.
2	Survives for adequate time in matrices to be useful.
3	Pathogen of human origin.
3	Analysis method can distinguish those pathogens capable of causing an infection and those which are not. Method: • is reliable • is reproducible • is robust • gives quantitative results • has confirmation step • if possible, is cost effective • is able to easily detect pathogens in wastewater
4	Ideally does not multiply in the matrix. Note that faecal coliforms, total coliforms, *E. coli* and *Salmonella* can multiply in certain wastewaters.
5	Reasonable body of knowledge available regarding the pathogen.
6	Can be handled in a Biosafety Level 2 or Class II laboratory.

2.5 Criteria for Choosing Indicators

Criteria for selection of microbial indicators are given in Table 2. Indicators were chosen for each of the pathogen groups, a representative pathogen – indicator pair(s) being set up for each group. While the density of the representative pathogen and proposed indicator may not be the same, indicators were chosen on the basis that both the representative pathogen and the indicator are removed or inactivated similarly by a treatment process. A consistent correlation between the removal of the pathogen-indicator pair will allow prediction of pathogen behaviour in response to a treatment process.

Table 2. Criteria for Selection of Microbiological Indicators.

Importance	Selection Criteria
1	Both representative pathogen and indicator are affected similarly by treatment processes e.g. similarly sensitive to a disinfection process or size/surface characteristics for removal processes, hydrophobicity and isoelectric point.
1	Minimal seasonal variation.
2	Is applicable to as many matrices as possible.
2	Represent the range of pathogens potentially present.
2	Found in different geographical areas.
3	Present in sufficiently high numbers to ensure reasonably precise quantification.
3	Analysis method: • is reliable • is reproducible • is robust • gives quantitative results • has confirmation step • if possible, is cost effective (may not currently be cost effective but could be developed to become so depending on the incentive provided) • is able to easily detect indicator in wastewater and biosolids
4	Ideally does not multiply in the matrix. Note that faecal coliforms, total coliforms, *E. coli* and *Salmonella* can multiply in wastewater.
5	Present in matrix when pathogen is present and absent when pathogen is absent.
5	Able to be easily extracted from wastewater.
6	Able to differentiate between pathogens from human and animal sources (note that some pathogens e.g. members of *Campylobacter*, *Salmonella*, *Giardia*, *Cryptosporidium* are known to infect both humans and animals).
6	Can be analysed for in a Biosafety Level 2 or Class II laboratory.

2.6 Representative Pathogens

Because of the large number of pathogens considered for the wastewater matrix, data used to assess whether or not a pathogen was representative of a pathogen group are presented in Table 3. In addition, a short information sheet on each pathogen considered for both matrices is presented in Appendix 1. The information in these sheets is not exhaustive; rather it was compiled to the point where the suitability, or otherwise, of the pathogen as a representative of that group could be established.

Table 3. Summary of Characteristics of Representative Pathogens.

	[1]Importance	Distribution	[3]Use as a surrogate	[4]Analytical method	[5]Infectivity Assay		[6]Analytical method performance		[7]Cost	[8]Ability to multiply	[9]Seasonality
Relative Importance of Criteria	1	1	2		3	3	3	3	3	4	7
Organisms						Waste Influent	Waste Effluent	Product Water			
Viruses											
Adenovirus	+++	y	y[d]	CC/PCR	y	+	++	+++	+++ / ++	n	AYR
Polyomavirus (JC, BK)[a]	?	y	?	PCR	n	+	++	+++	++	n	autumn-winter, AYR
Hep-A virus	++	y	y[e]	PCR	n	+	++	+++	++	n	AYR
Enteroviruses (coxsackie, echo)	++	y	y[f]	CC / PCR	y	+	++	+++	+++ / ++	n	summer-autumn
Calicivirus (Norovirus)	++	y	?	PCR	n	+	++	+++	++	n	winter
Reoviridae (Rotavirus, reovirus)[b]	++	y	y[g]	CC/PCR	y	+	++	+++	++	n	winter peak, AYR
Bacteria											
Clostridium	n	y	n[h]	culture	y/n	++	+++	+++	++	n	
Campylobacter	+	y	?	culture	y/n	++	+++	+++	++	?	summer
Salmonella	+	y	y?	culture	y/n	++	+++	+++	++	y	summer
E. coli	++	y	y[i]	culture	y/n	++	+++	+++	+	y?	AYR
Vibrio cholera	++	n[c]	?	culture	y/n	++	+++	+++	++ / +	y?	n/a
Aeromonas	++	y	y	culture	y/n	++	+++	+++	+	y	n/a
Enterobacter spp	n	y	?	culture	y/n	++	+++	+++	++ / +	y?	n/a
Klebsiella	+	y	y?[j]	culture	y/n	++	+++	+++	+	y?	n/a
Pseudomonas aeruginosa	+	y	y/n[k]	culture	y/n	++	+++	+++	+	y?	n/a
Mycobacterium	+	?	y?[l]	culture	y/n	?	?	?	?	y?	n/a
Protozoa											
Cryptosporidium parvum	+++	y	n[m]	FM/CC/PCR	y	+	++	+++	+++ / ++	n	variable[o]
Giardia duodenalis	++	y	y[n]	FM/PCR	n	+	++	+++	+++ / ++	n	variable

Key

y: yes n: no +: low ++: medium +++: high

?: more information required AYR: all year round FM: fluorescence microscopy CC: cell culture PCR: polymerase chain reaction

Footnotes

[1]Level of importance to water/ wastewater industry in relation to health regulation

[2]Worldwide distribution of pathogen

[3]Pathogen of interest is a good representative of its class

[4]Analytical methods suitable to detect presence of selected pathogen

[5]Analytical methods capable of detecting infectivity of selected pathogen

[6]Performance, reliability, robustness, and confirmation of selected analytical methods

[7]Cost of analytical method/s

[8]Pathogen's ability to proliferate in different wastewater matrices

[9]Pathogen's seasonal presence

[a] Pathogen is associated with renal transplants. Not thought to be a waterborne pathogen. 60-80% of humans are sero-converted.

[b] While rotaviruses are pathogens, reoviruses are not frank human pathogens and only rarely causes gastroenteritis in infants. CC detection is for reoviruses.

[c] Not expected in wastewater in developed countries. While *V. cholera* has been detected in Australian coastal waters, toxigenic strains have not been detected.

[d] But not for UV due to much higher UV resistance compared with other viruses.

[e] (Arraj et al. 2005) found similar numbers of hepatitis A virus, polioviruses and reoviruses in wastewater; also similar removal rates.

[f] Poliovirus is similar or more resistant to ozone, chloramine, chlorine and chlorine dioxide compared with calicivirus and adenovirus. Similar UV resistance to calicivirus and HAV. Similar numbers for adenovirus, reovirus, and enterovirus (pers. comm. M Angles (2005); Lodder and de Roda Husman (2005)).

[g] Reovirus numbers are within the range of norovirus. Norovirus numbers may be higher due to direct detection by PCR, which detects all viral particles, including those that can infect cell cultures and those that cannot infect cell cultures (due to culture techniques not allowing appropriate cell differentiation or expression of appropriate antigens, or due to the virus particle being defective). Reovirus is detected by cell culture.

[h] Bacterial spore numbers are generally lower than colony forming units of other bacteria; however bacterial spores are more resistant than vegetative cells.

[i] (Yanko et al. 2004) *E. coli*, *Aeromonas*, enterococci were at similar densities in final effluent. However, *E. coli* inactivate more slowly in the environment than enterococci due to solar radiation.

[j] Faecal coliform numbers are consistent with *E. coli* numbers in final effluent (Yanko et al. 2004). Inactivation by chlorine is affected by growth conditions for *K. pneumoniae* (Goel and Bouwer 2004).

[k] *Pseudomonas* showed variable results for chlorine inactivation versus indicators and *Salmonella* (Bonadonna et al. 1999). Has similar removal rate through WWTP as coliforms and faecal coliforms.

[l] Similar to *Legionella* for most disinfection - slightly more UV resistant; less chlorine dioxide resistant.

[m] Has much higher chlorine resistance than other protozoa; numbers also tend to be lower than *Giardia*.

[n] Similar to microsporidia for chlorine and ozone Ct.

[o] Australian National Notifiable Diseases Surveillance System http://www9.health.gov.au/cda/source/rpt_3.cfm

2.6.1 **Viruses**

There are more than 100 known enteric viruses that are excreted in large numbers in human faeces and are potentially transmitted by water. Those of particular significance include hepatitis A viruses, enteroviruses, noroviruses, rotaviruses, adenoviruses and reoviruses (Gerba et al. 1984). With the increase in use of recycled water, the potential exists for increased contact and potential transmission of viruses present in WWs if adequate treatment and disinfection are not applied. A range of assays are used to detect waterborne viruses including cell culture (where an adequate cell culture assay is available). Where a cell based assay is not available, molecular methods are utilised such as polymerase chain reaction (PCR) for DNA viruses and reverse-transcription polymerase chain reaction (RT-PCR) for RNA viruses. Many factors influence the recovery and enumeration of viruses from a WW sample. The methods used for concentration of viruses, such as filtration or polyethylene glycol (PEG) precipitation, will vary in the number of virus recovered based on the matrix tested. The culture methods vary depending on the cell type used for enumeration, the specificity of the virus for the chosen cell type and the culturability of the virus. For non-culturable viruses, molecular methods have been developed to allow enumeration that detects all virus particles (live and dead particles, while culture detects live, potentially infective viruses that are capable of growing in the culture system provided). This results in variability of the final results, which can result in the overestimation or underestimation of viruses in a WW sample.

The range of viruses that are of significance in WW streams are listed below and summarised in Table 4. Viruses are shed in faeces and urine in high numbers and directly contribute to the viral load in WWs. The viruses in WW are dependent on the viruses circulating within the community.

Table 4. Summary of virus characteristics.

Virus	Size nm		Genome	Isoelectric point (p*I*)
	lower	upper		
MS-2 (FRNA)	24	26	ssRNA	**3.9**
Qβ (FRNA)	24		ssRNA	**5.3**
T1 (somatic)	50	150	dsDNA	
PRD1 (DNA phage)	63		dsDNA	**4.2**
ΦX174 (DNA phage)	27		ssDNA	**6.6**
***B. fragilis* phage**	60	162	dsDNA	
HAV	27	29	ssRNA	**2.8**
Poliovirus	26	31	ssRNA	**4.5,7.0**
Enterovirus	18	27	ssRNA	**4-6.7**
Enteroviruses (animal)	15	46	ssRNA	
Polyomavirus	45	55	dsDNA	
Parvovirus	22	24	ssDNA	
Adenovirus	70	100	dsDNA	**4.5**
Rotavirus	30	37	dsRNA	**3.8, 4.0**
Norovirus	27	38	ssRNA	**5.5-6.0**
Astrovirus	27	32	ssRNA	
Echovirus	22	32	ssRNA	
Reovirus	60	80	dsRNA	

(Agosto et al. 2006, Caul and Appleton 1982, Dowd et al. 1998b, Foriers et al. 1990, Hawkins et al. 2008, Kennedy and Parks 2009, Kurogi et al. 1976, McFerran et al. 1971, Michen and Graule 2010, Oshima et al. 1995, Provost et al. 1975, Simmons et al. 1976)

2.6.1.1 Adenovirus

Adenovirus are dsDNA viruses, the only group of human enteric viruses with a DNA genome. A large range of adenoviruses are known to infect humans with the majority causing upper respiratory tract infections that are not associated with enteric symptoms. Two adenovirus serotypes are known to cause gastroenteritis (types 40 and 41), and are shed in high numbers in faecal material. Numbers of adenoviruses in WWs ranged from less than 0 to 10^2/L by culture (Sedmak et al. 2005) that tends to be lower than the enterovirus numbers detected at WW treatment plants. Standard cell culture methods are used for the enumeration of adenoviruses and PCR assays are also available. Estimations in the AGWR (2006) suggest adenoviruses are present in raw WW at 10^1-10^4 /L by culture.

A risk assessment study suggested that adenoviruses were among the most thermally resistant viruses (Gerba et al. 2002) and thus able to withstand conditions in WW treatment processes. Other studies have shown that adenoviruses survive longer in water than enterovirus and hepatitis A virus (Enriquez et al. 1995). Adenovirus serotype 2 has been used as a model for adenovirus disinfection trials with chloramines (Sirikanchana et al. 2008). Adenovirus 2 is a respiratory virus but has been detected in WW as it is shed in faeces and has a high level of resistance to chloramine disinfection while being highly susceptible to free chlorine disinfection. Adenoviruses are rated as highly important to the industry on the basis of their resistance to low pressure UV and their use by health regulators as one of the criteria in the assessment of the safety of recycled water. Adenoviruses have also been selected as a target organism in the criteria for the assessment of the safety of reuse water based on cell culture results, adenovirus numbers were reduced by 85%, while reovirus was reduced by 28% and enteroviruses by 93% across activated sludge treatment and disinfection (Long and Ashbolt 1994). Reovirus may be more difficult to remove than adenovirus and enteroviruses through secondary treatment processes (Irving and Smith 1981).

2.6.1.2 Astrovirus

Astroviruses are ssRNA viruses that have been increasingly recognised as a significant cause of diarrhoea in children, although the illness is of low severity and has a shorter duration than rotavirus. The virus causes gastroenteritis and predominantly diarrhoea, mainly in children under five years of age (although it has been reported in adults). Viral pathogen detection in water, especially in WW, is difficult and PCR is widely used to detect these viruses, although a culture based system does exist but is not frequently utilised (Abad et al. 1997). Astrovirus genomes were detected at the entrance to the sewage treatment plant, with a mean value of 4.1×10^6 astrovirus genomes per 100 mL and effluents were less strongly contaminated, with a mean value of 1.01×10^4 astrovirus genomes per 100mL (LeCann et al. 2004). Varied removal of group A and group B human astroviruses has been reported, with group A more effectively removed across WW treatment including primary sedimentation and activated sludge treatment (Morsy El-Senousy et al. 2007). RNA copies were compared to infectious units in a cell culture assay and 2-3 $\log_{10}$ higher numbers were detected in the RNA copies, although this was not consistently observed. PCR detects all viruses whether potentially infectious or inactivated and can overestimate the viral load for potentially infectious virus, while culture based methods can underestimate the viral load due to the limitations of the culture systems available. Dose response curves are usually determined with culturable viruses using culture methods to determine the titre of infectious viruses before and after disinfection. This allows the level of virus inactivation to be determined from exposure to the disinfectant.

2.6.1.3 Calicivirus

Caliciviruses are ssRNA viruses most commonly known as Noroviruses (formerly Norwalk-like viruses) and Sapoviruses (formerly Sapporo like viruses). Noroviruses are the most common cause of gastroenteritis in developed regions (Lopman et al. 2003) and are broadly distributed. The illness, although severe, is of short duration and severe complications are rare. Currently, a reliable culture system for the human caliciviruses is not available and relies on molecular methods for the detection, enumeration and speciation of noroviruses. The virus outbreaks are seasonal and tend to occur in winter in the northern hemisphere and are termed “winter vomiting disease”, while in the southern hemisphere, the opposite is true with most outbreaks occurring during summer months (Sinclair et al. 2005). Numbers of human infectious noroviruses in WW influent range from $10^{2.5}$ to 10^7 genome copies per Litre (Laverick et al. 2004, Lodder and de Roda Husman 2005), while levels in effluent range from $10^{1.6}$ to 10^5 genome copies per Litre prior to disinfection (effluent treatment consisted of primary settling, activated sludge process, and phosphorus removal) (Laverick et al. 2004, Lodder and de Roda Husman 2005). Estimates in the AGWR (2006) suggest 10^1-10^4/L. Noroviruses are listed on the USEPA Contaminant Containment List (1998).

2.6.1.4 Enterovirus

Enteroviruses are ssRNA viruses that are members of the genus *Enterovirus* within the *Picornaviridae* family. This large genus of viruses includes coxsackie A and B virus, echovirus, enterovirus types 68-71, and poliovirus. Symptoms from poliovirus infection include fever, fatigue, headaches, vomiting and muscle and joint pain, with the majority of cases being asymptomatic. Poliovirus has been eradicated in most countries due to the vaccination programmes, with wild-type poliovirus only occasionally isolated from human populations (Mulders et al. 1995). The vaccination program previously used a live attenuated virus, which meant that the vaccine strain of poliovirus could be readily detected in wastewaters and provided a useful indicator. However, in recent years there has been a change to utilise a heat inactivated /killed virus, so no replication of the virus occurs and the vaccine poliovirus

strain is no longer detected in wastewater, although rarer detection of wild poliovirus is still reported (Battistone et al. 2014). The exception for poliovirus eradication is some areas of Africa and Asia where the virus remains endemic.

Other members of the *Enterovirus* genus have been associated with a wide range of symptoms and illnesses including fever, vomiting, diarrhoea skin rashes, jaundice and conjunctivitis, however many of the infections are believed to be asymptomatic and resolve quickly and without ongoing problems. A small number of cases result in more serious complications, including meningitis, encephalitis and polio-like paralysis. Coxsackie viruses A and B cause hand, foot and mouth disease in children (often Coxsackie virus A 16) resulting in blisters on the hands and feet, in the mouth, and on the tongue and gums. Most cases have flu-like symptoms and occur most often in summer and autumn but occur year round in tropical climates. Enterovirus 71 causes similar symptoms as observed in hand, foot and mouth disease (and some severe cases progress to meningitis, encephalitis and paralysis). Enterovirus 71 does not cause gastroenteritis, although the virus is shed in faeces for periods up to 2 months after infection. Echovirus infections occur mainly in children, are highly infectious and cause acute fever in infants with early infections in infants having the potential to cause complications and fatalities.

Enteroviruses detection in WW influent may be variable, with reported concentrations of $<10^0$ (ie below detection limit) to 10^2 /L culturable (Sydney Water Corp, pers. comm.; (Arraj et al. 2008)) and up to 10^4/1L (Sedmak et al. 2005). AGWR (2006) suggest 10^2-10^6 /L. Enteroviruses are detected by cell culture base methods using plaque assays and PCR based molecular techniques are also available for enteric genome quantitation. Epidemiology data shows that community infection is highly seasonal (summer-autumn peak), although infections may occur all year round (Baek et al. 2009, Baek et al. 2011, Khetsuriani et al. 2006, Tani et al. 1995). The seasonality is likely the cause for the variability reported in wastewaters.

2.6.1.5 Hepatitis A virus

Hepatitis A virus (HAV) is a ssRNA virus that has its own genus, *Heparnavirus*, within the *Picornaviridae* family. HAV is a relatively common infection even in developed countries, but many cases are believed to pass unrecognised as they involve mild symptoms. Serious illness with significant inflammation of the liver is more common when infection occurs later in life (Sinclair et al. 2005) and the symptoms can be debilitating. In Australia, there are approximately 300-500 cases of HAV reported annually. The number of cases has been declining nationally since the late 1990's. In Australia, infection with HAV is more likely to occur in particular locations including childcare centres and preschools, residential care facilities and travellers to countries where the infection is common (Asia, Africa South Pacific, Central and South America). Infection from contaminated food or water is rare in Australia (www.HepatitisAustralia.com). HAV is listed on the USEPA contaminant containment list. HAV has been rated important to the water industry as it is used by health regulators for assessing the quality of reuse waters. The methods of detection and enumeration include PCR based assays and a culture based method using a radio-immunofocus assay or molecular methods such as RT-PCR.

2.6.1.6 Reovirus

Reoviruses are dsRNA viruses. These viruses have a broad host range and infect a number of other mammals as well as humans. Reovirus infections appear to be very common but mostly asymptomatic, and their role if any, in producing significant human disease is not clear. Reoviruses are detected through cell culture assays with discernible cytopathic effects or molecular detection using RT-PCR (Spinner and Di Giovanni 2001).

The genus *Rotavirus* is included in the *Reoviridae* family. Rotaviruses are the major cause of severe diarrhoea in young children in both developed and developing countries. Worldwide, this virus is estimated to be responsible for over 130 million episodes of gastroenteritis, 2 million hospitalisations and 440,000 deaths per year. In Australia, about 42% of cases are babies under 12 months of age and 92% of cases are children under 5 years of age. Rotavirus infections tend to be seasonal with the majority occurring in the winter months (Rao et al. 2013). The observation that rotavirus is highly prevalent among young children in both developed and developing countries, despite major differences in drinking water quality and sanitation, suggests that other modes of transmission are more important for this virus (Sinclair et al. 2005). In 2007 a routine vaccination program was introduced in Australia (and other developed countries) for children up to 6 months of age. This may potentially eliminate a broad spectrum of the disease in the community. The virus may still be

detected in sewage as the vaccine is a live virus, which may replicate in the host and be shed in faeces.

Rotaviruses were detected by RT-PCR at 10^1- 10^5 genomes /L (AGWR, 10^2-10^5 / L) in WW influent and similar levels in the effluent, with $10^{1.6}$-10^5 genomes /L indicating minimal removal of rotavirus (Lodder and de Roda Husman 2005). Reoviruses were detected at $10^{3.6}$-10^3 pfu /L using a culture-based method in wastewater influent with a reduction to 10^1-10^2 pfu /L prior to disinfection, indicating 1-2 $\log_{10}$ reduction in reoviruses from primary to secondary treatment processes. The observed reduction in reovirus may be due to removal and/or inactivation of the virus while the consistent rotavirus numbers indicates no removal of this virus and cannot establish loss of infectivity.

2.6.1.7 Which virus?

None of the human pathogenic enteric viruses possess all of the characteristics that make them ideal for the validation of treatment processes, with most of them having a seasonal prevalence. Of the viruses present in wastewater, adenoviruses and polyomaviruses are amongst the most numerous. Polyomaviruses are dsDNA viruses that infect the young, in most cases asymptomatically, and remain latent in adults with sporadic reactivation and shedding (Ling et al. 2003). Shedding is highest in autumn / winter but occurs all year round (Ling et al. 2003). While present in sufficient numbers, adenoviruses are much larger than other viruses so will not be a useful surrogate for validation of filtration-based processes. Polyomaviruses are closer in size to the other mammalian viruses so may be a suitable surrogate for filter validation, but no culture based techniques are available, so these can only be used to measure physical removal (detected by PCR) but not inactivation. Reoviruses have been reported to be present in higher numbers than enteroviruses, but they are similar in size to adenoviruses and so not any better as an surrogate for filter validation.

2.6.2 **Bacteria**

Considering that the bulk of the enteric pathogenic bacteria behave similarly to *E. coli* in response to water treatment, *E. coli* is suitable as a representative bacterial pathogen as it would allow safe handling for laboratory scale processes to be used later in the project. In addition, it is relatively inexpensive to analyse and is unlikely to regrow in domestic wastewater. Some of the opportunistic pathogens, such as *Aeromonas* and *Pseudomonas* are likely to be more problematic because they are more likely to regrow and might also have sources other than domestic influent (Banning et al. 2003, Geuenich and Muller 1984, van der Kooij 1991). *Campylobacter* have often been suggested as an indicator in wastewaters. However, current detection methods are currently problematic, with inhibition of growth observed in undiluted samples resulting in erroneous enumeration. Furthermore, *Campylobacter jejuni* have been shown to rapidly lose culturability under starvation conditions and the ability of *Campylobacter* to enter a viable but not culturable (VBNC) state may be variable among strains, with apparent VBNC cells exhibiting variable resuscitation success following passage through different hosts (Cappelier et al. 1999, Jones et al. 1991, Medema et al. 1992). Aside from starvation, another factor contributing to the failure to culture *Campylobacter* is that they are highly susceptible to oxidative stress, particularly if exposed to aerated conditions (Harvey and Leach 1998, Rollins and Colwell 1986). Considering these issues related to rapid entry into a VBNC state and resuscitation of VBNC cells, *Campylobacter* are problematic as a pathogen indicator.

2.6.3 **Protozoa**

Most studies on enteric protozoans in wastewater focus on *Cryptosporidium* and *Giardia*. There is generally a lack of information on the majority other pathogenic enteric protozoa, and also a lack of validated methods for their recovery from water or wastewater. While *Giardia* are more numerous in wastewater, *Cryptosporidium* represents a greater challenge being smaller and resistant to chlorine-based disinfection. An added advantage of *Cryptosporidium* is the availability of *in vitro* methods for infectivity assessment. Considering these, *Cryptosporidium* is the most practical pathogen to use as a representative of the enteric protozoans.

2.7 Indicators

Because of the large number of indicators considered for the wastewater matrix, data used to assess whether or not an indicator was suitable for inclusion in the study are presented in Table 6. In addition, a short information sheet on each indicator considered is presented in Appendix D. The information in these sheets is not exhaustive; rather it was compiled to the point where the suitability, or otherwise, of the indicator could be established. A summary of chosen wastewater pathogen indicators considered for this project is given in Table 5.

Table 5. Summary of Representative Pathogens and Indicators for Wastewater.

Representative Pathogen	Indicator
Virus **By cell culture:** adenovirus reovirus enterovirus rotavirus HAV **By PCR:** norovirus adenovirus enterovirus	**Virus** **By culture:** F-specific coliphages **By PCR:** Polyoma viruses
Bacteria *E. coli*	**Bacteria** Faecal streptococci / enterococci *E. coli* Total coliforms
Protozoa *Cryptosporidium*	**Protozoa** Sulphite-reducing clostridia

Table 6. Summary of Characteristics of Indicators Considered for the Wastewater Matrix.

	[a] Survival behaviour	[b] Treatment	[c] Seasonality	[d] Distribution	Analytical Method	[e] Analytical method performance			[f] Method cost	Multiplies in matrix	[g] Easy extraction
Relative Importance of Criteria	1	1	2	2		3			3	4	5
Indicator class						Waste Influent	Waste Effluent	Product water			
Virus indicator											
Enterovirus genome	?	?	summer autumn	y	PCR	+	++	+++	mod	n	[16]+
Reovirus	?	[17]y	winter peak, AYR	y	CC / PCR	+	++	+++	mod / high	n	+/++
Polyoma virus	?	?	autumn-winter, AYR	y	PCR	?	?	?	mod	n	[18]+++
PRD1 phage	?	?	AYR	?	culture	?	?	?	low / mod	?	+++
Somatic coliphage	?	[19]y/n	AYR	y	culture	+	++	+++	low	[11]n	+++
F-RNA phage	?	y/n	AYR	y	culture	+	++	+++	low	n	+++
B. fragilis phage	?	[12]y	AYR	y	culture	+	++	+++	low	[13]maybe	+++
Bacterial indicator											
Total coliforms	?	y	AYR	y	culture	++	++	+++	low	[14]y?	+++
Faecal coliforms	?	[15]y	AYR	y	culture	++	++	+++	low	y?	+++
E. coli	y	y	AYR	y	culture	++	++	+++	low	y?	+++
enterococci / faecal streptococci	?	[4]y	AYR	y	culture	++	++	+++	low	[5]?	+++
SRC/ *C. perfringens* spores	[6]y/n	[7]n	AYR	y	culture	++	++	+++	low / mod	n	+++
aerobic spores	?	[8]n	AYR	y	culture	++	++	+++	low	y	+++
particle profiling	na	[9]y	AYR	y	particle counter	[10]?	[10]?	[3]+++	[2]mod	na	na
Protozoan indicator											
C. perfringens spores	?	[1]y / n	AYR	y	culture	++	++	+++	low / mod	n	+++

Key

y: yes n: no na: not applicable +: low ++: medium +++: high

?: more information required AYR: all year round CC: cell culture PCR: polymerase chain reaction

[a] Survival behaviour similar to that of representative pathogen.

[b] Treatment effect: treatment has similar effect on indicator and representative pathogen.

[c] Seasonality: present in all seasons

[d] Wide distribution: wide geographical distribution.

[e] Method performance: is reliable, reproducible, robust, quantitative, has confirmation step, cost effective, easy.

[f] Method cost: cost of analytical method

[g] Easy extraction: is easily extracted from matrix.

[k] Source tracking: allows differentiation of pathogen sources.

[1] (Galofre et al. 2004) suggest anaerobic spores as a potential surrogate.

[2] Although purchase of a particle counter can be expensive, analysis is relatively inexpensive.

[3] Assume particle counter will perform adequately in product water.

[4] (Rose et al. 2004) show enterococci to have similar overall removal rate as faecal coliforms.

[5] (Gauthier and Archibald 2001) suggest enterococci present in pulp and paper mill waste in the absence of sewage.

[6] Spores are more persistent than vegetative cells.

[7] Rose (2004) *C. perfringens* results not consistent with those of faecal coliforms.

[8] (Galofre et al. 2004) suggest spores grew on GAC filter and that removal rate for entire WWTP was lower than for anaerobic spores.

[9] (Chavez et al. 2004) examined flocculation for the removal of bacteria and Helminth ova and correlated this with removal of particles. Removal of particles <8µm diameter could be used to predict removal of *Salmonella* and faecal coliforms. Correlation was linear but slopes and intercepts slightly differed for faecal coliforms and *Salmonella*.

[10] Samples may require dilution to allow analysis if turbidity is too high.

[11] Conditions are unlikely to be present to support replication. Conditions required include a specific host and log phase growth of the host, the latter requiring the presence of nutrients and a suitable temperature.

[12] (Gantzer et al. 1998) showed significant correlation between numbers of *B. fragilis* phage ($>10^2$/L) and presence of infectious enterovirus. Isolation rate and concentration of *B. fragilis* phage was lower than somatic phage and matched more closely the infectious virus isolation rate.

[13] Depends on the ability of the host to multiply which is thought only possible in extreme circumstances.

[14] No reference found for wastewater. Gauthier and Archibald (2001) suggest faecal coliforms and *E. coli* can grow in pulp and paper mill effluent.

[15] (Jimenez-Cisneros et al. 2001), (Koivunen et al. 2003) found faecal and total coliform removal to be the same as *Salmonella*.

[16] RNA viruses are technically slightly more difficult to isolate for molecular work.

[17] Assumes reovirus can be used as a model for rotavirus.

[18] Polyomavirus should be relatively easy to handle since its genome is dsDNA.

[19] (Gantzer et al. 1998) found a significant correlation between numbers of somatic coliphage and the presence of infectious enterovirus. No infectious enterovirus were present when somatic coliphage numbers were $<10^4$. Only 22% of samples with $>10^4$ somatic coliphage had infectious enterovirus. Rose et al. (2004) and (Harwood et al. 2005)) found no correlation between coliphage and enteric viruses. Viruses appeared to be more sensitive to filtration and disinfection than somatic coliphage.

2.7.1 **Virus Indicator Summary**

A recent EU-funded program has shown the utility of *B. fragilis* B124 to identify human-specific phage in high numbers from a range of U.K. sewage treatment works (Ebdon et al. 2007). However, an earlier study has suggested that densities may be too low for reliable detection (Chung and Sobsey 1993). Phage numbers aside, *B. fragilis* phage was rejected as a virus indicator because the analysis procedure is complicated, requiring anaerobic conditions, the phage has a dsDNA genome unlike the majority of human enteric viruses and the phage are much larger than human enteric viruses (Hawkins et al. 2008).

Enteric virus genomes are also proposed a viral indicator, along with reoviruses and polyomaviruses. Polyomavirus is a dsDNA virus reported to be widely present at concentrations higher than adenovirus in sewage (Bofill-Mas et al. 2006). Analysis is currently by PCR. Neither reovirus nor polyomavirus are considered to be a risk to human health making both attractive as indicators. As outlined above, enteric virus genomes are proposed as indicators.

The behaviour of viruses, both indicators and pathogens, was variable depending on the treatment regime, with large differences in removal efficiencies observed. Polyomavirus was the most promising virus indicator, behaving similar to adenovirus and being a very conservative indicator of removal for rotavirus (Keegan et al. 2009). Virus genomes appear to be the most reliable method of enumerating virus in removal processes such as ASP and membranes although the process detects both live and inactivated virus and is therefore conservative.

Studies investigating the removal of F-RNA bacteriophage and adenovirus genomes demonstrated no correlation (R^2= 0.0726) between the indicators for removal across activated sludge most likely due to the variability in virus numbers that occur in wastewaters and the higher numbers of indicators that are observed (Keegan et al., 2009).

Demonstrating the link between virus and indicator removal is difficult. Reports have demonstrated that plaque counts and PCR based detection differs by approximately 4 $\log_{10}$ for MS-2 (Sherchan et al. 2014). For adenovirus is has been suggested that 0.1% of the virus that is detected by PCR is infectious out of 10^5 genomes (He and Jiang 2005). Plaque-forming efficiencies vary widely in different virus species and serotypes with enumeration also affected by the cell line utilized.

Regel et al. (2012) reported a 2-2.5 log removal of MS-2 bacteriophage across UF membrane filtration after challenge testing. No comparison was made with pathogens due to the absence of a suitable numbers of viruses for detection in the filtrate.

2.7.1.1 Enteric virus genomes

Molecular detection of water borne viruses has been utilised in the past for those viruses where a suitable culture method was not available, such as noroviruses and rotaviruses. Quantitative PCR for DNA based viruses (adenovirus) or RT-PCR for RNA based viruses (*Picornaviruses*, reoviruses) is now widely available for both culturable and non culturable viruses as an alternative to the cell culture detection methods, and these have been investigated by a number of researchers. The method involves recovery of the viral particles from a water sample, extraction of the nucleic acid (DNA or RNA) and quantification of the virus present using an internal standard with PCR and specific primers to allow detection of the viruses of interest. The methods are reliant on the selection of specific primers for the virus(es) of interest, adequate optimisation of the assay and determination of non-specific amplification of other closely related viruses. The sensitivity of individual assays also requires determination to ensure low levels of viruses are detectable. The limitation of the method is that both infectious and non-infectious virus particles will be detected, possibly providing an overestimate of the health risk. In contrast, culture based enumeration using cytopathic effect (or plaque formation) can fail to detect a large number of viruses as suitable culture systems do not exist and as a result can potentially underestimate the number of infectious virus particles present (Lee and Jeong 2004). No correlation has been found between the presence of adenovirus or enterovirus genomes with infectious virus (Choi and Jiang 2005, Gantzer et al. 1998).

Viral genomes (either enterovirus or adenovirus based) are becoming more widely accepted as an indicator for the removal of viruses though treatment processes and is suitable for most processes. The exception for viral genomes is the assessment of disinfection efficacy, where the virus may be damaged and unable to replicate within a suitable host, but is still capable of being detected through PCR. This includes treatments such as UV, chlorine, chloramines and ozone. When assessing

disinfection kinetics, the detection of false positives i.e. detection of inactivated virus would be encountered unless degradation of the nucleic acid was achieved during the disinfection process. Culture-based methods are better suited to determining inactivation due to a disinfectant.

Enteric virus genomes tend to be present at significantly higher numbers when compared to culturable viruses. Numbers for astrovirus have been reported to be between 2 and 4 $\log_{10}$ higher for virus genomes compared with virus enumerated by culture. For adenoviruses, numbers detected were between 10^3 to 10^6 PFU/L by PCR (Keegan et al. 2009), while cultured adenovirus numbers are usually between 10^0-10^2 /L (Sedmak et al. 2005).

2.7.1.2 Somatic bacteriophage

Somatic phage are a collection of diverse and predominantly dsDNA viruses (except for ΦX174 which is ssRNA), including different genera and families of bacteriophage. The definition of somatic phage is somewhat loose, generally being used to describe any phage that initiates infection by binding to a host's cell wall. As a result, there is a large variation amongst different somatic phage species for size, structure and cell surface targets used for infecting the host cell. There is conflicting information regarding the ability of somatic phage to replicate in the environment. Somatic and F-RNA phage do not appear to replicate in the tropical aquatic environment (Hernández-Delgado and Toranzos 1995) and infection studies have shown that *E. coli* isolated from wastewater are poorer hosts for somatic phage compared with the lab strain of *E. coli* (WG5) used for routine detection of somatic phage (Muniesa and Jofre 2004). It has also been suggested that the densities of somatic phage and host bacteria in wastewater are below the threshold required for a productive infection, such that there should not be any significant replication of somatic phage in wastewater (Jofre 2009). However, much of the case against the replication of somatic phage in wastewater is based on assumptions regarding the host bacteria present and theoretical considerations of multiplicity of infection based on laboratory infectivity experiments of planktonic bacteria. The interaction of somatic phage with hosts within biofilm (or concentrated at a filter surface) has not been considered, but it is possible that under such circumstances infection will be more likely if host bacteria are concentrated. It is known that the host cells for somatic phage continuously express phage receptor sites and it has been reported that somatic phage are capable of attaching to dead cells (Grabow 2001). Anecdotally, it is also known that the host bacteria for somatic phage do not need to be in log phase growth for infection to be possible. This makes somatic phage technically simple to detect because there are no specific host growth requirements and an overnight broth culture is sufficient for use in a plaque assay (unlike F-RNA which require actively growing cells expressing the F pilus for infection). A broad range of coliforms can be hosts to somatic phage (not just *E. coli*) and some of these coliforms can grow or remain metabolically active to support somatic phage growth (Grabow 2001) and there have been reports of increases of somatic phage numbers in certain aquatic environments (Grabow 1990, Grabow et al. 1998).

Somatic phage have been studied in wastewater treatment process and are always present at much higher numbers (500-fold) than observed for mammalian viruses and are also about 5-fold more numerous than F-RNA (Grabow 2001). Studies have generated conflicting results, reporting either a significant correlation between the presence/absence of infectious enterovirus and the number of somatic coliphage in samples after secondary treatment (Gantzer et al. 1998) or no correlation (in particular for filtration and disinfection) between coliphage and enteric virus removal (Carducci et al. 1995, Harwood et al. 2005). Concentrations in WW influent has been reported at 10^6 PFU/L (AGWR, 2006, 10^6-10^9 PFU/L), while concentrations in effluent decreased to 10^5 PFU/L (Lodder and de Roda Husman 2005). The use of somatic phage as an enteric virus surrogate requires caution. Consideration needs to be given to whether the validation conditions might support host/phage replication and what the primary mechanism of pathogen removal will be. In terms of size, only the *Microviridae* (ΦX174) are comparable with human enteric viruses. Being DNA-based, somatic phage may be more robust than the RNA-based enteric viruses in terms of survival in the environment. The method of Grabow et al. (1998) is recommended for somatic phage.

2.7.1.3 F-specific bacteriophage

F-specific bacteriophage have been utilised in wastewater treatment process analysis as a surrogate for waterborne viruses with variable success. There are two types of F-specific phage. F-DNA phage are large (>100nm) with a filamentous structure. F-RNA phage are small (24-26nm) round viruses with a shape more similar to many of the enteric viruses. The presence of these two types can vary depending on host species and environment, with similar numbers reported in environmental waters,

but 3-fold more F-DNA phage in samples from a wastewater treatment plant (Cole et al. 2003). The survival of F-DNA and F-RNA in spiked lake water microcosms was similar at 4°C but varied for different species for both groups at 20°C (Long and Sobsey 2004). The most robust F-specific phage was the F-DNA phage M13, while the most robust F-RNA was MS-2 (Long and Sobsey 2004). With the exception of M13, the other F-DNA behaved similarly to group IV F-RNA, which were inactivated rapidly at 20°C (Long and Sobsey 2004).

One of the most commonly used F-RNA in validation studies is MS-2, which has been suggested as a conservative indicator because it is more chlorine and UV resistant than *E. coli* and poliovirus (Tree et al. 2005). However, specific studies of treatment processes found no correlation between enteric viruses and F-RNA or other traditional indicators (Harwood et al. 2005, Simpson et al. 2003), with enteric viruses more greatly affected by filtration and disinfection compared with F-RNA (Harwood et al. 2005). F-RNA have similar sensitivity to poliovirus when disinfected with chlorine (Duran et al. 2003).

F-specific phage, including both F-RNA and F-DNA phage, satisfy many of the criteria to be a useful indicator. They are unlikely to replicate in the environment, requiring a host cell in log phase growth (which requires a temperature >30°Cand specific nutrient requirements) for expression of the pilus to which the phage attach (Grabow 2001, Woody and Cliver 1997). In addition, F-RNA are a similar size to human enteric viruses and possess ssRNA genomes, comparable with many of the human enteric viruses. F-RNA phage have been well studied, particularly as a biodosimeter for UV and as a challenge organism for validation of ultrafilters. Another advantage of F-RNA phage is lower analysis costs compared with culture or PCR-based detection of enteric viruses (Stewart-Pullaro et al. 2006). Concentrations of F-RNA phage in waste influent were $10^{3.5}$- 10^{6} PFU/mL (AGWR, 2006; 10^{5}-10^{7} PFU / L) and $10^{0.5}$-10^{2} PFU / mL in waste effluent (Lucena *et al*., 2004). The recommended method is the double agar layer technique specified in the Standard Methods for the Examination of Water and Wastewater (APHA 2012). This method has worked reliably at AWQC, provided care is taken in the preparation of the host bacteria. The default method analyses a total volume of 10 mL (10 standard agar plates, 1 mL of sample per plate), if that detection limit is not required, costs can be reduced by analysing 1 mL only. Monitoring of F-specific phage may be sufficient for validation purposes, especially if phage such as MS-2 are being spiked into the wastewater and so will be present at levels that are orders of magnitude higher than the indigenous phage. If a specific count for F-RNA is required then samples need to be analysed with and without RNase present in the medium, with the number of F-RNA being the differential count between total F-specific phage (no RNase in medium) and F-DNA phage (RNase in medium).

2.7.1.4 Numbers of viruses in wastewaters targeted for recycling

Virus numbers in wastewaters vary from plant to plant with the numbers influenced by (i) the inputs to sewage (whether domestic, industrial wastes or a mixture of both), (ii) the incidence of disease within the community (most enteric viruses are seasonal), (iii) the treatment processes used at the treatment plant and (iv) the end point use of the water (this determines the level of treatment applied to the wastewater). In the literature, this variation is also influenced by the concentration, recovery and detection methods that are used for viruses. Few studies have investigated the removal of viruses across the full treatment train with the inclusion of a large range of pathogens and indicators in the assessment. The numbers of viruses in primary wastewaters varies considerably and ranges have reported in the AGWR, with enteroviruses present at 10^{2}-10^{6} / L, adenovirus at 10^{1}-10^{4} / L, norovirus at 10^{1}-10^{4} / L, rotavirus at 10^{2}-10^{5} / L, somatic coliphage at 10^{6}-10^{9} / L and F-RNA coliphage at 10^{5}-10^{7} / L.

Estimated $\log_{10}$ removals of viruses and bacteriophage for individual processes are provided in Table 6. The virus $\log_{10}$ removals are process dependant and reductions are dependent on specific features of the process, including detention times, pore size, filter depths and disinfectant type. When under consideration by a state Department of Health, a system or process will be attributed the default minimum $\log_{10}$ credit listed in Table 7 unless it has been proven otherwise that greater removal of inactivation is achievable. The default values are accumulated across the treatment train processes for a total $\log_{10}$ removal achievable by the system with a maximum of 4 $\log_{10}$ credit attributed to any single process. The level of removal required is dependent on the quality required in the product water whether it be Class A, Class B or other.

Table 7. Indicative log_{10} removals of enteric viruses and indicator organisms.

	Indicative log_{10} removals[a]		
Treatment	**Enteric Viruses**	**Phage**	**E. coli**
Primary treatment	0-0.1	N/A	0-0.5
Secondary treatment	0.5-2.0	0.5-2.5	1.0-3.0
Dual media filtration with coagulation	0.5-3.0	1.0-4.0	0-1.0
Membrane filtration	2.5->6.0	3.0->6.0	3.5->6.0
Reverse osmosis	>6.0	>6.0	>6.0
Lagoon storage	1.0-4.0	1.0-4.0	1.0-5.0
Chlorination	1.0-3.0	0-2.5	2.0-6.0
Ozonation	3.0-6.0	2.0-6.0	2.0-6.0
UV light	>1.0 adenovirus >3.0 enterovirus, hepatitis A virus	3.0-6.0	2.0->4.0

[a] Reductions depend on specific features of the process, including detention times, pore size, filter depths, disinfectant. Adapted from AGWR (2006).

2.7.2 **Bacterial Indicators**

Enterococci/faecal streptococci have potential to be used for source tracking and do not appear to multiply in municipal wastewater. Some reports question whether these are a good indicators for pathogenic enteric bacteria and more information is required. Based on the limited information from available studies, total coliforms appear to be worthwhile along with *E. coli* as a surrogate/indicator for other enteric bacteria. Few of the microbiological indicators evaluated proved to be good predictors of pathogen removal for full-scale treatment for wastewater. Coliforms generally correlated with *E. coli* removal, but in most wastewaters *E. coli* is the dominant coliform so there is little advantage to test for both (Keegan et al. 2009).

Particle profiling using a Coulter Counter Multisizer II was used to determine the particle profile of water samples (Chavez et al. 2004). A good correlation was found between the removal of particles <8 microns and the removal of faecal coliforms and *Salmonella* spp (R^2 = 0.8217 and 0.7148 respectively). Using a LISST-100 Particle Size Analyser Type C (Sequoia, Redmond, WA, USA), Keegan et al., (2009) reported no correlation between pathogen removal and the removal of particles of any particular size category, but the data suggested a direct correlation between the volume of particles removed and the numbers of pathogens removed across ASP.

2.7.3 **Protozoan Indicators**

In the absence of a better indicator, sulphite-reducing clostridia (SRC) are often used as an indicator for protozoa. There are conflicting reports on their suitability as an indicator as well as some variation in methodologies. A study investigating pathogen removal across activated sludge treatment for different sites showed that SRCs and particle profiling generally provided a conservative measure of removal comparable with *Cryptosporidium* (within the same order of magnitude), while *Giardia* cysts were better removed at most plants (Keegan et al. 2009). However, more detailed comparison of removal at a single site demonstrated a lack of correlation for *Cryptosporidium* and SRC, although the correlation between SRC or particle removal with *Giardia* removal was good (Keegan et al. 2009). The removal of SRC reported by Keegan et al (2009) was higher than that reported by Rose et al. (2004), although the two studies reported similar removals for *Cryptosporidium* and *Giardia*. A strong correlation has been reported between particle volume (2 – 14 micron size class) and *Cryptosporidium* and *Giardia* numbers (Keegan et al. 2009).

Fluorescent microspheres are spherical micro particles having similar size and density characteristics to microorganisms. They are available in a size range of 0.08–3,300 µm and are generally made from latex or polystyrene. Fluorescent microspheres have successfully been used in the laboratory as a surrogate for *Cryptosporidium parvum* oocysts in assessing disinfection employing ozone followed by chlorine (Baeza and Ducoste 2004). They have also been used to successfully measure UV fluence distribution to complement biodosimetry validation of a pilot scale UV reactor (Bohrerova et al. 2005). They can provide a conservative estimate of *C. parvum* oocyst removal in hydrophilic negatively charged filter media (e.g. sand) but are insufficiently conservative for hydrophobic media such as GAC (Dai and Hozalski 2003). Although not present naturally in wastewater, they may be added to treatment processes making them a possible model indicator. The cost of employing them in full-scale processes, however, detracts from their use.

3 Virus method review

3.1 Summary

This section provides a review of methods used for virus concentration and detection and considered factors affecting these processes. The nature of the sample matrix can have both positive and negative impacts on virus recovery. Water with high turbidity and organic content may be challenging to concentrate, especially for methods such as filtration. However, poorer water quality may overcome losses of viruses due to adsorption to surfaces, with organics and other material providing a carrier effect (Pang et al. 2012). Another key factor identified was nucleic acid extraction, which needs to balance yield and quality since both of these factors influence assay sensitivity and the accuracy of quantitation. Cell line selection is critical for optimal detection of infectious viruses and previous work has reported that the inoculation method for virus cell culture influences detection rates (Keegan et al. 2012).

3.2 Concentration

Most virus recovery methods identified in this literature review had a focus on surface water or tap water. One report described concentration of viruses from wastewater using a molecular weight cut-off filter, followed by PCR-based detection and enumeration for adenovirus (Sidhu et al. 2013). The sample volumes were relatively small, using Ultracel 50K molecular weight cut-off filters to concentrate 10 mL of sample down to 750 µL. Different DNA extraction kits were evaluated, with the Qiagen blood extraction kit providing the best overall performance, extracting a reasonable amount of high quality DNA compared with the other kits evaluated. The combination of yield and quality was important, resulting in the best detection of adenovirus by PCR. Kits with high yield but low quality DNA gave similar or better results compared with kits with low yield and better quality.

The virus concentration method in use at AWQC, published in SWF 62M-2114, was originally transferred from Sydney Water as part of a project funded by the Cooperative Research Centre for Water Quality and Treatment. In brief, the method concentrates volumes of water 10 – 50L, depending on turbidity, using Hemoflow F filters, which are polysulfone ultra-filters normally used for blood dialysis. The method uses modified end-caps (designed by Sydney Water) for the filters, which increase the inlet and outlet diameters and improve filter performance. Elution of the viruses (and particulates trapped in the filter capillaries) uses back flushing with deionised water / carbonate buffer. The eluate is further concentrated by precipitation using polyethylene glycol (PEG). An aliquot of the final concentrate is filtered using a 0.2µm syringe filter and the filtrate (containing virus) is then analysed for infective viruses using mammalian cell culture or total nucleic acid is extracted and viruses are detected using polymerase chain reaction (PCR) or reverse transcription (RT)-PCR. For quantitation of RNA viruses by quantitative RT-PCR, RNA standards are produced using specially modified primers that include a T7 promoter in the primer sequence, resulting in the production of amplicons that can be used in a T7 *in vitro* transcription system to produce RNA copies of the amplicon fragment (equivalent to virus RNA for that gene region).

Concentration methods were evaluated for virus recovery in project SWF 62M-2114. A 10 L sample of secondary treated effluent (post ASP) was spiked with Coxsackie virus B5. The sample was split, with 9.25 L processed by the AWQC/Sydney Water method (ultra-filtration followed by PEG precipitation) and the remaining 750 mL directly concentrated by PEG precipitation. Direct PEG precipitation demonstrated a 10-fold improvement in recovery compared with filtration. Interestingly, most probable number (MPN) counts were generally 2-fold higher than plaque counts. The reason for this is not clear, possibly it is due to the better performance of inoculation using a centrifugation within the MPN method. Taking the volume analysed into consideration, the two methods provided comparable detection limits, with direct PEG being faster and cheaper (saving on the materials and labour costs for the filtration step).

A recent review (Ikner et al. 2012) summarised methods for virus concentration and recovery from water. For filtration methods, membrane charge was identified as a critical factor. Depending on charge interactions, adsorption can be a major mechanism of virus retention, in addition to any physical capture due to enmeshment in the filter matrix. The isoelectric point for most viruses is less than 5 (Table 8), which means that viruses will be negatively charged in wastewaters, which are usually between pH 7 and 8. Electronegative membranes are cost effective but require the use of

cations to allow binding of viruses to the membrane (as in the above example using the CAE negatively charged filters and Mg^{2+}). Electropositive membranes are more expensive but allow direct filtration of samples without adjustment. Recovery of viruses requires pH adjustment to enhance desorption from the membranes. An advantage of this approach is that filters with larger nominal pore sizes (e.g. microfilters) can be used for sample concentration, which can be useful for processing samples with higher turbidity. However, the impact of any pH changes or addition of cations on downstream virus analysis needs to be considered. Ultrafilters, which have nominal pores sizes in the same range as viruses, primarily rely on physical capture based on size, though adsorption can still occur. For this reason, the elution technique is still critical to ensure release of any virus particles that have adsorbed to the membrane surface. Recovery of different viruses using the same elution buffer has been shown to be variable, which is likely to be a reflection of differences in virus size, charge and composition. Another potential disadvantage of charged membranes is that they can co-purify undesirable compounds, such as humic substances, which have similar isoelectric points (Table 9).

Table 8. Isoelectric points of Waterborne Viruses (from Keegan et al. (2012)).

Virus	Virus Type	Isoelectric point (p*I*)
Reovirus 3	Mammalian	3.8, 4.0
Vaccinia	Mammalian	3.9, 4.8
Coxsackie A21	Mammalian	4.8, 6.1
Poliovirus type 1 (Brunhilde)	Mammalian	4.5, 7.0
Poliovirus type 1 (Mahoney)	Mammalian	8.2
Echovirus 1 (V248)	Mammalian	5.0
Echovirus 1 (V212)	Mammalian	6.4
Tobacco mosaic virus	Plant	3.8, 4.1
MS2	Bacterial phage	3.9
T2	Bacterial phage	4.2
T4	Bacterial phage	4.2
Qβ	Bacterial phage	4.1, 5.3
Fr	Bacterial phage	9.0

Note: More than one p*I* value shown for the same virus strain reflects results of different p*I* measurement methods.

Table 9. Isoelectric points of common solids (from Keegan et al. (2012)).

Solid	Solid type	Isoelectric point (Ip)
Quartz	Mineral	2.0-3.5
Kaolinite	Mineral	2.0-4.6
Humic matter	Organic	<3.0
Cellulose nitrate	Filter material	1.5-2.0

An important consideration for selection of an elution buffer is the nature of any downstream applications. Some compounds can result in virus inactivation, cell cytotoxicity or PCR inhibition. Glycine is effective for virus elution but the high pH used can cause virus inactivation. Beef extract has also been successfully used for elution of viruses from charged membranes and ultrafilters but there is some concern on the contribution of organics from this on PCR inhibition. Sulphuric acid has been used for virus elution from electronegative membranes. Most recently, sodium polyphosphate has been used successfully for virus recovery by elution / backwashing of ultrafilters and in combination with glycine for elution from an electropositive membrane. Another factor that influences virus recovery is the sample volume concentrated, with recoveries generally decreasing as the volume concentrated increases. The reason for this was not considered by Ikner et al., (2012), but it is possible that large volumes / long filter run times can lead to viruses becoming embedded in the filter matrix or that other interactions with components in the water, such as particles, may lead to aggregation and failure to isolate the viruses.

Secondary concentration methods identified in the review by Ikner et al., (2012) included aluminium hydroxide precipitation, adsorption-elution and organic flocculation (PEG was not covered but it is in use at the Sydney Water and AWQC laboratories and is described above). Organic flocculation has

been reported to have variable recovery, thought to be caused by differences in surface properties for the different viruses. Molecular weight cut-off filters have also been used for secondary concentration. An advantage of molecular weight cut-off filters is that sample processing is relatively rapid and only requires a centrifuge with the appropriate filter tube adaptors.

A method has been described for simultaneous concentration of viruses and protozoa from tap or river water (Haramoto et al. 2012). This method uses an electronegative HA mixed cellulose ester membrane (0.45µm pore size, 47 mm diameter). Water samples have cations added ($MgCl_2$ was identified as the most effective) to aid binding of target particles to the membrane, presumably by overcoming any charge repulsion between negatively charged microorganisms and the negatively charged membrane. Samples were eluted in a 15 mL volume, *Cryptosporidium* / *Giardia* samples were resuspended into 10mL PBS. The eluted sample was centrifuged to concentrate the protozoan (oo)cysts, with the resulting supernatant concentrated using molecular weight filters to isolate viruses. Quantitative RT-PCR was used for poliovirus and Qβ phage (FRNA). Recoveries appeared to be good, although volumes tested for recovery were relatively small (500 – 1000 mL). The method was applied to wastewater (50mL raw and 800-1000mL secondary treated) but details of water quality was not provided, making it difficult to anticipate relative method performance for different wastewater matrices, which are generally more challenging to concentrate compared with tap water and surface waters. The same type of filter has been used to concentrate pepper mild mottle virus from drinking water sources (Haramoto et al. 2013).

Granular activated carbon (GAC) has potential for virus concentration, with a report of GAC (1 g / 500mL) being used to concentrate MS2 phage from seawater (Cormier et al. 2014). Best recovery was observed at a pH of 6, with salinity having minimal effect. The incubation temperature was a large factor affecting recovery, with highest virus numbers binding at 37°C. Elution required trypsin and EDTA, potentially limiting the utility of this approach for concentrating viruses for cell culture analysis. The viruses needed to be eluted prior to downstream analysis, with RNA extraction directly from the GAC not successful using a QIAamp viral RNA mini kit.

Concentration and detection of enteric viruses in water (deionised, tap, river) has been assessed using a positively charged NanoCeram 90mm disc filter (Pang et al. 2012). Variable recovery was observed, depending on the water quality and virus. Norovirus recovery decreased as water quality decreased (42 - 18%). In contrast, echovirus had higher recovery in raw water compared with pure water (78-47%). Echovirus recovery was impacted by the numbers of virus in the sample, with poorer recovery for lower numbers of virus, suggesting loss could be due to adsorption to surfaces. This might explain the improved recovery of echovirus in dirtier water, with organic matter potentially acting as a carrier / reducing losses due to binding to surfaces. Coxsackie and adenovirus recoveries were not impacted by virus numbers, although adenovirus generally had poorer recovery in pure water (20-50%) compared with Coxsackie virus (32-69%).

3.3 PCR-based Detection

Molecular assays are well established for the detection of human enteric viruses. The assays in use at the AWQC are listed in Table 10. In some cases PCR-based methods are the only methods available for virus detection because cell-based infectivity assays are not available, eg. noroviruses require fully differentiated enterocytes for successful infection, such a culture system is not amenable for a routine diagnostic assay. The key requirements for PCR-based detection of viruses are a robust nucleic extraction technique that will provide sufficient yield / quality while removing PCR inhibitors and appropriate positive control material / assay validation to determine method performance for a given matrix. In the case of quantitative PCR or RT-PCR assays two key considerations are the use of appropriate reference material for the preparation of standards and the incorporation of appropriate controls to detect / correct for any PCR inhibition, which can impact the accuracy of quantitation. Digital PCR (dPCR) is an emerging technique that allows absolute quantitation (without the need for a DNA standard) and is claimed to be resilient in the presence of PCR inhibitors (Yang et al. 2014). Provided an accurate DNA standard is used, conventional qPCR will provide comparable accuracy to dPCR, but at lower cost for both equipment and materials/labour (Yang et al. 2014). A strength of dPCR is in the preparation of reference material for qPCR.

A multiplex PCR assay is available for the simultaneous detection of adenovirus, enterovirus and HAV (Formiga-Cruz et al. 2005). Evaluation of the performance of the assay demonstrated that detection of adenovirus was not impacted by the presence of other viruses. However, detection of enterovirus or

HAV was reduced in the presence of other viruses if there was a 2 or more log-fold difference in virus numbers, with the virus at lower concentration not being detected. This PCR method was used to analyse raw sewage samples (42 mL), which were concentrated by ultracentrifugation. Viruses were eluted from the pellet using a glycine buffer, followed by additional concentration by ultracentrifugation. This method will not be useful for processing larger sample volumes and requires access to an ultracentrifuge, which are not standard equipment in most water testing laboratories but should be available in any university. Nucleic acid extraction was via a published method using silica powder and guanidine thiocyanate, which is the same basic principle by which most commercial kits work. The multiplex assay had a 5 – 10 fold decrease in sensitivity for sewage extracts, likely due to either the presence of interfering substances not removed by the extraction method used or poorer performance of the extraction method for sewage concentrates (or a combination of both). In terms of extraction, Jebri et al. (2014) compared extraction methods for detection of enteroviruses and phage in sludge. The matrix is not relevant to this project but it is noteworthy that RNA extraction was via the QIAamp viral RNA kit.

Table 10. Primers currently in use at AWQC.

Virus	Primer Name	Type	Sequence	Ref
Poliovirus	ENT1	Forward	5'-GGCCCCTGAATGCGGCTAAT-3'	1
	ENT2	Reverse	5'-CACCGGATGGCCAATCCAA-3'	1
	ENTERO	Taqman	5'-FAM-CGGACACCCAAAGTAGTCGGTTCCG-BHQ1-3'	1
Reovirus	ReoL3f	Forward	5'-CAGTCGACACATTTGTGGTC-3'	2
	ReoL3r	Reverse	5'-GCGTACTGACGTGGATCATA-3'	2
Group II Norovirus	GIIf	Forward	5'-CARGARBCNATGTTYAGRTGGATGAG-3'	3
	GIIr	Reverse	5'-TCGACGCCATCTTCATTCACA-3'	3
	NORO	Taqman	5'-FAM-GAGGGCGATCGCAATCT-BHQ1-3'	4
Adenovirus	AQ1	Forward	5'-GCCACGGTGGGGTTTCTAAACTT-3'	5
	AQ2	Reverse	5'-GCCCCAGTGGTCTTACATGCACATC-3'	5
	Adeno	Taqman	5'-FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1-3'	5
Rotavirus	MRD145	Forward	5'-GCTGGCGTGTCTATGGATTCA-3'	6
	MRD155	Reverse	5'-CAAAACGGGAGTGGGGAGC-3'	6

1 Donaldson et al. (2002)
2 Spinner (2001)
3 Trujillo et al. (2006)
4 Radcliff pers comm.
5 Heim et al. (2003)
6 Fout et al. (2003)

Another multiplex assay for HAV and norovirus has been described for shellfish and water samples (Fuentes et al. 2014), using Probit analysis to determine the limit of detection (LOD) of the assay. Zeta plus filters were used to concentrate river water samples, these filters are positively charged cellulose membranes that are relying on virus adsorption since the smallest pore size of these filters (based on the manufacturer's information) is nominally 0.5 µm. To improve detection of norovirus in water Gregory et al., (2011) developed a synthetic norovirus standard and a competitive internal positive control. The inclusion of the control did not appear to impact sensitivity, with a reported KOD of 5 copies / reaction and the limit of quantification (LOQ) being 50 copies / reaction (Gregory et al. 2011).

3.4 Cell culture

Numerous cell lines are used for the detection of infectious viruses (Table 11). The cells are generally from the type of organ that would be infected in the host animal and all of the cell lines in use either are cancer cell lines or are transformed cell lines. This means that the cell lines can be serially cultured and do not die after certain number of cell divisions (as would be the case with cells directly isolated from an organ, known as a primary cell line). In the case of the cell lines used for the detection of human enteric viruses, these cells form monolayers that adhere to the surface of the culture plate. Mammalian cell culture requires a dedicated clean laboratory and adherence to strict protocols to minimise contamination of the cultures by bacteria or fungi, which can rapidly outgrow the mammalian cells and will kill them or otherwise impair the assay. Virus infectivity is typically measured by observing the host cells for any evidence of death or morphological change, known as cytopathic effect. The type of cytopathic effect can be diagnostic for infection by particular viruses. Confirmation of infection by a particular virus can be done using PCR-based assays.

Table 11. Summary of cell lines used for virus culture.

Virus	Cell Line	Reference
Echovirus	BGM	Pang et al. 2012
Poliovirus	BGM [1]	Keegan et al. 2012
	PLC/PRF/5 [2]	Keegan et al. 2012
Coxsackie	MA104	Pang et al. 2012
	BGM [1]	Formiga-Cruz et al. 2005, Keegan et al. 2012
	PLC/PRF/5 [2]	Keegan et al. 2012
Adenovirus 41	MA104	Pang et al 2012
	LLK	Sidhu et al 2013
	A549 [1]	Formiga-Cruz et al. 2005
	PLC/PRF/5 [2]	Keegan et al. 2012
Adenovirus 2	A549 [1]	Keegan et al. 2012
	PLC/PRF/5	Keegan et al. 2012
Hepatitis A	Vero	Formiga-Cruz et al. 2005
	FRhK-4	Fuentes et al. 2014

[1] Based on AWQC research, this is the recommended cell line for inactivation or removal studies when large cell numbers are required due to the faster growth of this cell line
[2] Based on AWQC research, this cell line provides the best recovery and so is recommended for analysis of wastewater samples to detect infectious virus

Aside from detection / enumeration of infectious viruses, cell culture is also used to propagate viruses for use as controls or use in experiments such as disinfection or filtration trials. It is important to note that not all viruses produced in cell culture are infectious. Virus quantitation by transmission electron microscopy has been compared with enumeration of infectious viruses determined using the $TCID_{50}$ (infectious dose required to infect 50% of wells inoculated) for canine adenovirus and feline calicivirus (Malenovska 2013). This report demonstrated that production of virus through culture resulted in a stable ratio of infectious virions to total virus particles, with an approximate 2 log difference. This means that the measurement method will affect the number of viruses enumerated, with infectivity providing a lower number of viruses compared with direct enumeration by microscopy of PCR, even for freshly cultured viruses. In the study of Malenovska et al., (2013), virus particles remained intact but lost infectivity after prolonged exposure to 37°C in culture medium. This is an important consideration for the production of viruses in culture, with a specific window for harvesting infectious virus particles to provide the maximum yield of infectious viruses.

Cell lines were evaluated for infection by particular viruses in project SWF 62M-2114. The yields of Coxsackie virus and poliovirus in BGM and PLC/PRF/5 cells were comparable. However, the yields of adenovirus 40 and 41 were 1 to 2 orders of magnitude better using PLC/PRF/5 cells compared with BGM or A549. For detection of adenovirus 2, which is a respiratory adenovirus, detection in PLC/PRF/5 was slightly better than in A549. However, A549 is a faster growing cell line and more amenable for high volume screening, so is recommended for the detection of adenovirus 2. This study also evaluated modifications to the inoculation technique to improve virus recoveries. Reduction of the inoculum volume improved recovery from 1% to approximately 30%. Centrifugation of the plates after inoculation also increased recovery, from 1% to approximately 50%. The change in inoculum volume presumably had an effect on cell surface area to volume ratio, which may have increased contact between viruses in the medium and cells on the plate surface. The reason for the improvement using centrifugation was not clear. The speed used would be insufficient to affect the viruses unless the viruses were aggregated or attached to particles in the medium. The particle size in the virus preparation should be less than 0.2 µm, which is the pore size of the filter used to prepare the viruses recovered from cell culture or from sample concentrates. The report used the centrifugation technique for MPN counts but not for plaque assays, expressing concern of reduced counts due to virus aggregation. However, this was not investigated to determine if it was a valid limitation.

The cell line used for virus detection has been shown to be critical ((Keegan et al. 2012)). Testing of secondary treated effluent demonstrated consistent detection of adenovirus using PLC/PRF/5, while only 1/4 samples were positive using A549 cells and none were detected using BGM. The cell line evaluation demonstrated a 10-fold difference between PLC/PRF/5 and A549, but the performance of A549 was poorer for real samples (all samples should still have been positive for the A549 considering that the results for PLC/PRF/5 were 30 MPN/L or higher). Infectious enteroviruses were not detected in secondary treated effluents that were positive for infectious adenovirus. Screening of secondary treated effluents from a wider range of locations using PCR assays supported the absence of enteroviruses in the concentrates, with adenoviruses detected in the majority of samples (Table 12). Norovirus and rotavirus were also detected in approximately half of the samples. Detection of reovirus appeared to be highly variable, with high numbers (10^8) or no virus detected in samples from the same location.

Table 12. Virus genomes detected in secondary treated effluent from 5 Australian WWTPs adapted from (Keegan et al. 2012).

Sample Location	Adenovirus genomes/L	Enterovirus genomes/L	Reovirus genomes/L	Norovirus genomes/L	Rotavirus genomes/L	F-RNA phage/L
A	1.59 x 105	0	3.7 x 108	2.7 x 105	1.63 x104	5.0 x 105
A	9.3 x 106	0	0	2.0 x 103	>6.0 x 103	3.7x104
B	2.8 x 105	0	1.16 x 108	2.3 x 104	1.05 x 104	5.0 x 103
C	8.1 x 105	ND	ND	ND	ND	ND
D	1.7 x 106	ND	ND	ND	ND	ND
E	ND[1]	ND	ND	4.0 x 103	ND	2.1 x 101

[1] ND – not detected

4 Evaluation of methods for virus and *Cryptosporidium* concentration

4.1 Introduction

4.1.1 **Culture methods**

The literature review identified areas for improvement in sensitivity/recovery: using different cell lines; using smaller inoculation volumes or centrifugation of the sample onto the cell culture plate; testing the influence of metal cations on virus infection rate. The verification of these findings was the focus of the virus methods research.

4.1.2 **Recovery methods**

The current method used to concentrate large volumes uses HF80 ultra filters (normally used for dialysis), which are no longer readily available. Similar filters used in the literature are also not available (eg the filters used in work published in recent years by the Centres for Disease Control in the USA). A new filter has been sourced (FX80), which has the same filter material but different filter housing fittings. Trials were conducted to compare the old and new filters as part of the research investigating virus recovery rates.

Earlier research at AWQC suggested that direct PEG precipitation provided good recoveries and equivalent detection limit to filtration/PEG of larger volumes (Keegan et al. 2012). This work was repeated to compare recovery rates in wastewater effluent. PCR assays were initially used for virus enumeration, followed by cell culture using the most appropriate cell lines identified in the culture methods research.

Previous *Cryptosporidium* method development at AWQC has shown that dilution of primary effluent in a larger volume (e.g. 500 mL into 10 L) followed by primary concentration and purification using immunomagnetic separation (IMS) provides better recovery rates than direct centrifugation/IMS of effluent. Experiments were conducted to verify method performance using Bolivar primary effluent. This work was originally planned to be done using HF80 filters, but given the stock availability issue, this was done using calcium carbonate flocculation (the standard AWQC NATA accredited method) and Envirochek filters (one of the filters validated for use in US EPA methods 1622 and 1623 for concentration of *Cryptosporidium* in water samples).

Virus stability in samples has previously been identified as an issue for virus analysis. Once the concentration method has been selected, sample stability will be assessed. This area of research will be of primary relevance in cases when validation locations requires significant transport (eg overnight shipping) to testing laboratories and sample holding times may exceed 24 hours before processing. Time limitations did not allow an extensive investigation of this subject.

Ideally data will be gathered from this research to demonstrate recovery rates. The original proposal mentioned development of an internal recovery control for viruses. However, this is not feasible within the project timeframe. It is possible that artificial nanoparticles could be used as a future recovery control, considering recent work using these as surrogates for groundwater studies (Farkas et al. 2015, Pang et al. 2014).

4.1.3 **PCR methods**

During the scoping of this work it was originally proposed to review / evaluate PCR assays used for virus detection. However, the literature review did not identify this as a major need, with many virus PCR assays well established in the literature and in use by the partner laboratories participating in this project. The virus PCR assays in use at AWQC and CSIRO are being routinely used for research projects and do not appear to require further assay development. Furthermore, a standard operating procedure for using an adenovirus TaqMan assay was recently published as part of Water RA project 1035, addressing some of the concerns regarding the virus PCR assays that were the original reason for the inclusion of this module in SP5.

The existing PCRs will be used in the method evaluation and inter-laboratory trials as one of the analyses, so PCR will be part of the project but not as method development.

4.1.4 **Inter-laboratory method comparison**

A series of inter-laboratory trials was originally planned to allow method comparison, identify opportunities for development of methods and to provide guidance on method suitability for different wastewater matrices. Since the start of the project, an additional laboratory has agreed to participate in the method comparison. However, due to time constraints this component of the project has been limited to a single round of testing using primary and secondary wastewater from a single location. The scope of the trial was to compare the performance of the methods in use at CSIRO, AWQC and Sydney Water laboratories for the concentration and detection of human enteric viruses (adenovirus and enterovirus) and *Cryptosporidium*.

4.2 Materials and Methods

4.2.1 **Source water**

Primary settled wastewater and secondary treated wastewater was collected from the Bolivar wastewater water treatment plant (South Australia). Typical water quality data for primary and secondary effluent from this site are presented in Table 13.

Table 13. July 2015 average wastewater quality data for Bolivar primary and secondary effluent.

Analyte	Primary effluent	Secondary effluent
BOD (mg/L)	213	9.8
COD (mg/L)	485.5	99.5
Total P (mg/L)	8.3	1.5
Ammonia (mg/L)	41.7	0.2
TKN (mg/L)	70.7	2.5
SS (mg/L)	128	10.7
pH	7.3	7.3

4.2.2 **Virus concentration**

4.2.2.1 Filtration

Samples (10 L) were concentrated using Hemoflow® hollow-fibre ultra-filtration dialysis filters (HF80S, Fresenius Medical Care) with modified end caps to improve filter performance (developed by Sydney Water). The sample was pumped through the filter using a peristaltic pump at a flow rate of approximately 0.4 L/min. The filter was back-flushed with carbonate buffer (500 mL, pH 9.6) and all liquid dispelled with nitrogen gas. Due to the limited availability of the HF80 filters in Australia, an alternative filter (FX80, Fresenius Medical Care) was trialled. These filters have a fixed case and small diameter inlet / outlet that could not be modified. The tubing from a dialysis pack (that fits the proprietary connectors on the filter) was adapted to fit a small peristaltic pump, and the flow rate was adjusted manually to between 0.2 – 0.4 L/min.

4.2.2.2 PEG precipitation

The filter eluate or primary effluent (up to 0.5 L) was concentrated by adding polyethylene glycol 6000 (8% w/v), tween 80 (1% w/v) and calcium chloride (0.5% w/w). After stirring overnight at 4°C, the material was centrifuged (11,344 *x* rcf 60 min in a Sorvall Evolution with a SLC-6000 rotor) and the pellet resuspend in minimal essential medium (Life Technologies, 40 mL). The suspension was sonicated in an ultrasonic bath (NEY Ultrasonik 57X) for 1 min and place in a flask shaker for 15 min. Centrifugation (6,196 *x* rcf 30 mins, Sorvall Evolution with a SS34 rotor) pelleted the suspended solids and the concentrated supernatant was stored at -80°C until nucleic acid extraction. **Note:** during method documentation for this report a training error was identified, the speeds used in experiments contained in this report were 7,250 rpm (converted to rcf above), these speeds should have been rcf as per Keegan et al (2012).

4.2.2.3 Laboratory 2 concentration methods

Primary Effluent: For adenovirus, 10 mL of primary effluent was concentrated using an Amicon ultra centrifuge filter (30 K). The sample was centrifuged at 4,750 rpm for 30 mins to obtain a final volume of 180-200 µL. Triplicate samples were used for each primary effluent sample [non-spiked (n = 2) and spiked with CB (n = 2)]. For enterovirus, primary wastewater samples were concentrated and purified with Amicon® Ultra centrifugal filters (Ultracel-50K) (Millipore, Billerica, MA) in triplicate. Briefly, 20 mL sample was centrifuged at 4,500 rpm for 5 minutes at 4 °C and the resulting supernatant was added to an Amicon® column and centrifuged at 1,000 rcf for 10 min to obtain a final volume of 1,000 µL, which was then mixed with the pellet from the initial centrifugation step and frozen at -80 °C prior to batch processing.

Secondary Effluent: A 10 L secondary effluent sample was concentrated using hollow-fibre ultrafiltration using a Hemoflow FX 80 dialysis filters (Fresenius Medical Care, Lexington, MA, USA). The sample was amended with a solution of sodium polyphosphate (NaPP; Sigma #305553) to achieve a final concentration in the water samples of 0.01% w/v. Each secondary effluent sample was pumped with a peristaltic pump in a closed loop with high-performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole-Parmer Instrument Co.). Tubing was sterilized by soaking in 10% NaClO for 10 min, washed with sterile distilled water, and autoclaved at 121°C for 15 min. The sample was concentrated to approximately 150 to 200 mL, depending on the turbidity. A 500-mL elution solution consisting of 0.01% Tween 80, 0.01% NaPP, and 0.001% Antifoam A was recirculated through the ultrafilter for 5min, according to Hill et al. (2007), and then allowed to concentrate to 150 mL-200 mL. This elution solution was added to the concentrated sample to achieve a final volume of approximately 320-500 mL. A new filter cartridge was used for each sample. Secondary concentration of viruses in the concentrate was performed by using 50 mL from each sample, centrifuging at 4,750 rpm for 10min to obtain a pellet. The pellet was retained and the supernatant was passed through the JumboSep filtration device (100 K MWCO, Pall, Australia). The final JumboSep concentrate (2-3 mL) was combined with the pellet. From the combined sample, 200 µL was used to extract DNA using Blood and Tissue Kit (Qiagen). A total of 4 DNA samples was extracted from 4 secondary effluent samples.

4.2.2.4 Laboratory 3 concentration methods

Samples were concentrated as described in 4.2.2.1 and 4.2.2.2, except that up to 1 L of primary effluent was used for concentration (rather than 0.5 L).

4.2.3 **Human enteric virus culture**

4.2.3.1 Cell lines

The cell lines used for this project are listed in Table 14. Cells were cultured in complete Eagle's minimum essential medium (MEM; Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), 2 mM L-glutamine and 10 mM non-essential amino acid (NEAA; Gibco).

Table 14. List of cell lines used in this project.

Cell line	Cell type	Origin
A549	Human lung adenocarcinoma	ATCC (CCL-185)
BGM	Buffalo Green Monkey Kidney	Commonwealth Serum Laboratories
HEp- 2	Human larynx epidermoid	ATCC (CCL-23)
LLC-MK2	Rhesus Monkey Kidney	ATCC (CCL-7)
MRC	Human foetal lung fibroblast	ATCC (CCL-171)
PLC/PRF/5	Human liver	ATCC (CRL-8024)

4.2.3.2 Virus culture

Virus stocks were prepared by infecting cells in culture. Confluent cell monolayers in 175 cm^3 flasks were rinsed with Phosphate Buffered Saline (PBS) and infected with respective virus at a concentration of approximately 1 multiplicity of infection, diluted in 3 mL media without foetal bovine serum (FBS). The flasks were incubated at 37°C in a CO_2 incubator for 90 minutes with rocking every 10 minutes, after which the inoculum was replaced with 15 mL of complete media. Infected flasks were incubated at 37°C in a CO_2 incubator until >90% cell monolayer destruction, due to cytopathic effect (CPE), was observed. One to three freeze-thaw steps were performed to release virus particles from host cells. The supernatant was centrifuged at 4°C and 10,000 rcf for 10 minutes to remove cell debris. Further purification was accomplished by filtering supernatant through a 0.2 µm Acrodisc syringe (Pall Life Sciences, USA). All viral stocks were titrated using the plaque assay method as described below and stored in 1 mL lots at -80°C.

Coxsackie B5 (CB5) (ATCC VR-185) was cultured in buffalo green monkey kidney (BGM) cells; adenovirus 2 (Ad 2) was originally obtained from the National Institute of Allergy and Infectious Diseases (NIAID) and cultured in human lung adenocarcinoma epithelial (A549) cells. All viral stocks were titrated using a previously described plaque assay method (Kahler et al. 2011). Overnight confluent cell monolayers were washed and infected with 100 or 200 µL of serially diluted (10^{-1} – 10^{-6}) supernatant or sample as described above. Following infection, inoculum was removed and cells were washed and overlaid with 2 or 4 mL of a 1:1 mix of 2% SeaPlaque Agarose (Lonza Rockland, Inc, USA) and 2X MEM plus 10% FBS. Agarose was allowed to set, and plates were inverted and incubated at 37°C for 3 or 10 days for CB5 or Ad 2 respectively. After appropriate incubation time, cells were fixed with 1% formalin for 30 minutes. The overlaid agar was removed and cells were stained with 0.2% crystal violet and rinsed with distilled water to visualise the plaques. Levels of infectious virus were reported as PFU per mL.

4.2.3.3 Enumeration of viruses by plaque assay

Sample concentrates were assayed for infectious viruses using a plaque assay in 12 or 6 well tissue culture plates. Overnight confluent cell monolayers were washed and infected with 100 or 200 µL of serially diluted (10^{-1} – 10^{-6}) sample concentrate. Following infection, inoculum was removed and cells were washed and overlaid with 2 or 4 mL of a 1:1 mix of 2% SeaPlaque Agarose (Lonza Rockland, Inc, USA) and 2 x MEM plus 10% FBS (for adenovirus an additional 25 mM $MgCl_2$ was added to the medium (Williams 1970)). After the agarose had set, plates were inverted and incubated at 37°C for 3 or 10 days for enterovirus or adenovirus respectively. After the appropriate incubation time, cells were fixed with 1% formalin for 30 minutes. The overlaid agar was removed and cells were stained with 0.2% crystal violet and rinsed with distilled water to visualise the plaques. Levels of infectious virus were reported as PFU per mL. The reproducibility of the plaque assay results was determined in triplicate.

4.2.3.4 Enumeration of viruses by most probable number

The most probable number (MPN) for enteroviruses and adenoviruses was calculated using the MPN General Purpose program by Hurley and Roscoe (Hurley and Roscoe 1983). Four replicate wells were inoculated for each dilution and the number of wells displaying cytopathic effect were scored as positive/negative and entered into the MPN program for determination of virus MPN / L.

4.2.3.5 Laboratory 3 method for virus enumeration

Quantitative estimation of culturable adenovirus and enterovirus from primary and secondary effluents was performed by the Cell Culture Most Probable Number (MPN) method. Aliquots of the supernatant from filtration PEG (secondary effluent) or direct PEG (primary effluent) was divided and used to inoculate cultured cells derived from monkey kidney cells (LLCMK2), human epithelial cell lines (Hep-2) and human foetal lung fibroblast cell line (MRC5).The inoculated cells were incubated at 37°C and examined regularly under an inverted microscope for up to a month for signs of virus specific Cytopathic effect (CPE). Most Probable Number Infectious Units (MPNIU) of adenovirus and enterovirus for the sample was calculated from the number of CPE positive tubes as per MPN calculations (APHA Standard Methods 22nd edition).

4.2.4 **PCR detection of viruses**

4.2.4.1 Nucleic acid extraction

Laboratory 1: Viral nucleic acid was extracted directly from 200 μL of sample or sample concentrate using the QIAamp DNA extraction kit (Qiagen, Germany) for DNA viruses and the QIAamp Viral RNA extraction kit (Qiagen, Germany) for RNA viruses, both according to the manufacturer's instructions.
Laboratory 2: Viral DNA was extracted directly from 200 μL of sample or sample concentrate using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. Fpr RNA extraction, 280 μL of sample was extracted using the QIAamp Viral RNA extraction kit (Qiagen, Germany).

4.2.4.2 Enterovirus reverse transcription qPCR

Laboratory 1: One step RT-PCR, originally described by Donaldson et al (2002), was performed to detect and quantify enterovirus (coxsackie CB5). Sample extract (5 µL) was added to a mastermix (25 µL final volume) containing 0.2 mM dNTP mix (Applied Biosystems Pty Ltd., Scoresby, VIC, Australia), 3 mM MgCl2, 1 x PCR buffer (Applied Biosystems), 2.5 units of Amplitaq Gold™ DNA Polymerase, 0.6 µM ENT1 (5'-GGCCCCTGAATGCGGCTAAT-3') and ENT2 (5'-CACCGGATGGCCAATCCAA-3') primers, 5 units MuLV Reverse Transcriptase (Applied Biosystems), 20 units of RNAse Inhibitor (Applied Biosystems), and 0.4 µM of Entero TaqMan probe (5'-FAM-CGGACACCCAAAGTAGTCGGTTCCG-BHQ1-3'). Reverse transcription was performed at 43°C for 30 minutes, followed by heat treatment at 95°C for 10 minutes, followed by 45 cycles of; 95°C for 10 seconds, followed by 55°C for 15 seconds and 72°C for 15 seconds. Reactions were performed on either a RotorGene 6000HRM (Qiagen) or a Light Cycler 96 (Roche). Signal was acquired on the Green (FAM) channel. Positive control material utilised was CB5 stock virus, batch date 22.7.2013. DNA standards for quantitation were produced by purifying the CB5 RT-PCR amplicon, determining the concentration of the purified DNA and then converting this first to molarity using the molecular weight of the amplicon (size = 192 bp) and then to copy number using Avogadro's number.

Laboratory 2: The oligonucleotide primer and probe sequences used for enteroviruses detection are previously published (Katayama et al. 2002, Shieh et al. 1995). In brief, forward primer was CCTCCGGCCCCTGAATG, reverse primer ACCGGATGGCCAATCCAA, and probe internal oligoprobe was (FAM)CCGACTACTTTGGGTGTCCGTGTTTC (TAMRA). One step qRT-PCR assays were performed in 20 µL reaction mixture using iScript RT-PCR probe mix (Bio-Rad, Hercules, CA, USA). Quantitative RT-PCR reactions were performed on Bio-Rad CFX96 (Bio-Rad, Hercules, CA, USA). In order to improve detection of the enterovirus two step RT-PCR was also performed in addition to single step RT-PCR. In the RT step 5 µL of the extracted RNA with random primers (Invitrogen). Two step RT-PCR was performed with SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) for the generation of cDNA. In the second step 2 µL of the cDNA was used to set up qPCR assays (20 µL reactions) using Sso Advanced™ Universal Probe Supermix (Bio-Rad Laboratories). All PCR/RT-PCR assays were performed in 20 µL reactions. The reaction mixtures contained 10 µL of respective RT/PCR mixture, 250 nM of each forward and reverse primers and 250 nM of probe, RNase inhibitor (40 U / µL) (Roche Diagnostics). The temperature cycle used for RT-PCR reaction was as follows: 30 min of reverse transcriptase at 50°C to create cDNA copies of RNA, then 45 cycles of 95°C for 30s, 55°C for 20s, and 68°C for 20s.

4.2.4.3 Adenovirus qPCR

Laboratory 1: Adenoviruses were detected using a Taqman PCR originally described by Heim et al (2003). The 25 µL reaction included 1 x FastStart Essential Probe Master (Roche), 0.5 µM each of forward primer AQ1 (5'-GCCACGGTGGGGTTTCTAAACTT-3') and reverse primer AQ2 (5'-GCCCCAGTGGTCTTACATGCACATC-3'), 0.3 µM Adeno Taqman probe (5'-FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1-3') and 5 µL DNA. Taq DNA Polymerase activation was performed at 95°C for 6 minutes, followed by 60 cycles of 94°C for 5 seconds, 59°C for 20 seconds, and 72°C for 10 seconds. Amplifications were performed on either a RotorGene 6000HRM (Qiagen) or a Light Cycler 96 (Roche). Signal was acquired on the Green (FAM) channel. Positive control material was extracted from adenovirus type 41 (ATCC VR-930). For quantitative PCR known numbers of plasmid containing the AQ1/AQ2 fragment were used for the PCR standards.

Laboratory 2: Standards for qPCR of HAdVs were prepared from plasmid DNA, ranging from 3×10^5 to 3×10^1 (Ahmed et al., 2015). Primers and probe used for the HAdV qPCR were: F:GCC ACG GTG GGG TTT CTA AAC TT, R: GCC CCA GTG GTC TTA CAT GCA, P:FAM-TGC ACC AGA CCC GGG

CTC AGG AGG TAC TCC GA BHQ1 (Heim et al. 2003). qPCR was carried out by using SsoAdvanced™ Universal Probe Supermix (Bio-Rad Laboratories). The qPCR mixtures contained 10 µl of Supermix, 250 nM concentrations of each primer, and 3 µl of template nucleic acid. For each PCR run, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included. The thermal cycling conditions were: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 20 s at 95°C (Ahmed et al. 2015).

4.2.5 *Cryptosporidium* concentration

4.2.5.1 Filtration using Envirochek filters

Envirochek capsule filters (Pall Gelman) were used to concentrate 10 L secondary effluent samples following the manufacturer's instructions. Briefly, samples were filtered at a flow rate of 2 L / s, connected to a pressure regulator to limit the pressure to ≤30 psi and a flow restrictor to limit the flow rate to 2 L / s. A variation of the method of Pezzana et al. (2000) was used for filter elution, which itself is based on the manufacturer's instructions. Elution buffer (10 mM Tris pH 7.4, 2mM EDTA, 0.1% (w/v) Laureth 12, 0.015% (v/v) Antifoam A made up in deionised water) was added to the filter to approx. 10 mm above the pleated filter module (holding the filter vertically). The filter was allowed to stand vertically for 15 min, inverted and left to stand vertically for further 15 min and then decanted into a centrifuge tube. Elution buffer was again added to the filter and the filter was vortexed 3 times for 30 s (upright, inverted then upright) and the buffer decanted into the same centrifuge tube. Elution buffer was added to the filter (as above) and the filter was shaken on a wrist action shaker at 600 rpm for 5 min, then turned 90° and shaken for a further 5 min at the same speed. This eluate was also added to the same centrifuge tube as previous. Centrifugation of the eluate was as per 4.2.3.3.

4.2.5.2 Calcium carbonate flocculation

A NATA accredited commercial in confidence method was used by laboratory 1 to concentrate samples using calcium carbonate flocculation. This protocol was originally described by Vesey et al. (1993). Briefly, 10 L of water sample (or 0.5 L primary effluent + 9.5 L reagent water) was decanted into a container (volume determined by weight), followed by addition of ColorSeed (to allow determination of oocyst recoveries), then addition of 100 mL of 1 M $CaCl_2$ with thorough mixing and addition of 100 mL of 1M $NaHCO_3$ with thorough mixing. The pH was increased to 10 using NaOH. Following dosing, the water sample was allowed to stand overnight to allow for the sedimentation of the flocc containing any oocysts present. The supernatant was removed by aspiration and the pellet then dissolved in 200 mL of 10% sulphamic acid. Particulates (including any oocysts) were concentrated by benchtop centrifugation (4.2.3.3), with the resulting pellet being washed in phosphate buffered saline to remove residual acid. Once the pH was near neutral the oocysts were further purified using immunomagnetic separation (IMS).

4.2.5.3 Centrifugation

Primary effluent, or filter eluate or redissolved flocc were centrifuged at 2200 rcf for 20 min with no braking to obtain a pellet. The supernatant was aspirated, leaving sufficient volume not to disturb the pellet. The pellet was resuspended in the remaining supernatant, transferred to one or more 50 mL tubes and centrifuged at 2200 rcf for 10 min with braking. The supernatant was aspirated and the pellet weighed prior to resuspension for IMS.

4.2.5.4 Dynal Immunomagnetic separation

Concentrates containing oocysts were further purified using a *Cryptosporidium* IMS kit (Dynal) following the manufacturer's instructions. The sample pellet (0.5 mL packed pellet volume) was resuspended up to a volume of 10 mL using phosphate buffered saline. One mL each of 10x SL-buffer-A and 10x SL-buffer-B were added to a flat-sided Dynal tube, followed by addition of the 10 mL sample concentrate and 100 µL of well mixed *Cryptosporidium* Dynabeads. The tube was mixed on a rotator at 18 rpm for 1 hr at room temperature. Following mixing, beads were captured using a magnetic particle concentrator as per the manufacturer's instruction. The captured beads were either processed as per 4.2.4 or dissociated from the beads (using 50 µL of 0.1 N HCl for 10 min, with the supernatant without beads transferred to a fresh tube containing 5 µL of 1.0 N NaOH) and enumerated by fluorescence microscopy.

4.2.5.5 Laboratory 2 concentration methods

Due to a technical failure the Laboratory 2 *Cryptosporidium* samples were not included in this study.

4.2.5.6 Laboratory 3 concentration & enumeration methods

Secondary effluent samples (10 L) were filtered through polycarbonate membrane (293mm, 2.0µm pore, Osmonics, USA) placed in a flatbed membrane filtration apparatus attached to a peristaltic pump (Watson Marlow 620S). The residue from the membrane was scraped using a small rubber squeegee and eluted to a 50 mL centrifuge tube with elution buffer (0.05% Tween® 80 (v/v), 2mM (TSPP) made up in DI water). The tubes were centrifuged at 1730 x rcf for 10 minutes at 4°C. The pellet was resuspended in 50 mL suspension buffer (5%w/v BSA**;** 0.05% w/v sodium azide; 0.05% Tween® 80; 2mM TSPP) then centrifuged at 1730 x rcf for 10 minutes at 4°C and aspirated to 5 mL.

Primary effluent samples were concentrated by centrifugation. Briefly, the sample was centrifuged at 5000 x rcf for 30 minutes at 4°C. The supernatant was aspirated and the pellet mixed with 50 mL elution buffer. The sample was further centrifuged at 1730 x rcf for 10 minutes at 4°C. The pellet was resuspended in 50 mL suspension buffer, then centrifuged at 1730 x rcf for 10 minutes at 4°C and aspirated to 5 mL.

Cryptosporidium from sample concentrates were purified by IMS as described above. Oocysts were stained by Fluorescein Isothiocyanate (FITC) conjugated anti-*Cryptosporidium* antibody (Easystain™ (BTF, Australia) and 4'6-diamidino-2-phenyl indole (DAPI). Stained oocysts were identified and enumerated using a fluorescent microscope (Leica Microsystems).

4.2.6 ***Cryptosporidium*** **integrated assay (enumeration + infectivity)**

A cell culture assay was used to measure oocyst infectivity. This method was a simplified version of the assay described by King et al. (2015). In brief, oocyst counts were determined by fluorescence microscopy prior to temperature exposure experiments. Set numbers of oocysts were processed by the infectivity assay. Infectious oocysts were detected using the focus detection method, which uses a specific fluorescent antibody to detect infection of host cells by *Cryptosporidium*. The % infectious oocysts was determined using the number of infectious oocysts detected by cell culture and the total number of oocysts applied to cell culture. A limitation of this method is the number of oocysts that can be applied to a single cell culture well and the number of infectious oocysts that can be counted in a single cell culture well. The total number of oocysts applied across multiple cell culture wells was used to allow calculation of maximum log removal values (LRVs) for large numbers of oocysts.

4.2.7 ***Cryptosporidium*** **enumeration by microscopy**

For direct enumeration by microscopy, oocysts were air-dried onto Dynal Spot-On slides, fixed with 50 µL 100% methanol (for as long as required for the methanol to evaporate) and then stained with EasyStain (BTF) following the manufacturer's instructions. In the case of the integrated assay, oocysts recovered from cell culture plates, the oocyst suspension was stained using 30 µL of EasyStain for 1 h in the dark at room temperature. Following incubation, the sample was filtered through a 13-mm black polycarbonate membrane with a nominal pore size of 0.8 µm(Rowe Scientific) on a vacuum manifold fitted with Swinnex filter holders using a vacuum pressure of 200 mbar. The membranes were mounted on glass slides with 7 µL of mounting medium (BTF). Slides were scanned by fluorescence microscopy, with + green fluorescent + red fluorescent objects in the correct size range scored as ColorSeed oocysts and + green fluorescent - red fluorescent objects in the *Cryptosporidium* size range scored as presumptive oocysts.

4.2.8 **Inter-laboratory method comparison**

Samples used for the inter-laboratory comparison were collected from the Bolivar WWTP on 10/8/2015.

The following samples were collected for each participating laboratory:

Virus testing:
2 x 0.5L Primary effluent un-spiked
2 x 0.5L Primary effluent spiked with CB5 (at AWQC prior to dispatch)
2 x 10L Secondary effluent un-spiked
2 x 10L Secondary effluent spiked with CB5 (at AWQC prior to dispatch)

Analyses: Adenovirus & CB5 by (RT)PCR and / or cell culture

Methods:
Laboratory 1: HF80 UF Filtration + PEG, PEG, direct DNA extraction (no concentration), cell culture, (RT)PCR
Laboratory 2: UF Filtration + MWCO filtration, MWCO filtration, direct DNA extraction (no concentration), (RT)PCR
Laboratory 3: HF80 UF Filtration + PEG, PEG, cell culture MPN

Cryptosporidium testing:
3 x 0.5L Primary effluent un-spiked
3 x 10L Secondary effluent un-spiked

Spiked with ColorSeed (by participating lab) when the sample is processed

Methods:
Laboratory 1– Calcium Carbonate precipitation for both primary/secondary + IMS, integrated infectivity assay for counts and infectivity analysis
Laboratory 2: Due to a technical failure these *Cryptosporidium* samples were not included in this study
Laboratory 3: Flatbed membrane filtration + IMS, fluorescence microscopy for oocyst counts

4.3 Results for method evaluation

4.3.1 **Virus culture**

Experiments were conducted in triplicate to evaluate different virus host cell lines, with 3 technical replicates used in each experiment. One of the replicate experiments for Ad 41 had a cell count approx. 2 log higher than the other experiments so it was not used for calculation of average cell counts. The experiment had the same trend as that reported by Keegan et al., (2012), with PLC cells providing a higher virus count compared with A549. The PLC/PRF/5 cell line gave the highest overall virus counts for adenovirus (Ad 2) and enterovirus (CB5), suggesting it is the best all round cell line in terms of maximising virus detection (Table 15). The A549 cell line, which is a faster growing cell line compared with PLC cells, gave comparable virus counts for Ad 2. Similarly, the BGM cell line was only slightly poorer than the PLC cells in terms of enterovirus counts. From a logistical viewpoint, faster-growing cell lines are preferable to use for frequent or large-scale virus testing because the cells are easier / faster to prepare for use in virus testing. The cells for fast / slow growing cells can be prepared (passaged) in the same time frame (3-4 days), but the seeding density required for slower growing cells is much larger. This means that more plates for analysis can be prepared from a plate of fast growing cells compared with slow growing cells, making it easier to prepare the numbers of cells / plates required for experiments. For this reason, A549 was used for Ad 2 plaque assays and BGM was used routinely for CB5 plaque assays because these cell lines are faster growing and easier to maintain (taking approximately half the resources required to produce the same number of PLC cells). However, for detection of all adenoviruses and enteroviruses, the PLC cell line appears to be the best choice and this cell line was used for MPN assays. Another consideration was the finding that PLC and A549 had different performance for wastewater sample concentrates, so it is recommended that cell line performance be determined for any given matrix.

Table 15. Virus counts (MPN / mL) for Ad 41, Ad 2, poliovirus (vaccine strain) and Coxsackie B5 obtained using different cell lines. Percent CV is shown in parentheses.

Virus/Cell line	BGM	A549	PLC/PRF/5	LLC-MK	Hep2	MRC
Ad 41	1.30E+00 (141%)	3.60E+06 (98%)	4.30E+07 (62%)	ND	ND	ND
Ad 2	1.60E+04 (83%)	3.00E+08 (41%)	4.50E+08 (31%)	ND	ND	ND
Polio	6.10E+06 (81%)	ND	8.80E+07 (68%)	1.20E+06 (94%)	7.10E+06 (76%)	5.90E+06 (148%)
Coxsackie B5	2.80E+08 (105%)	ND	3.50E+08 (81%)	3.30E+06 (90%)	1.50E+08 (138%)	Fail

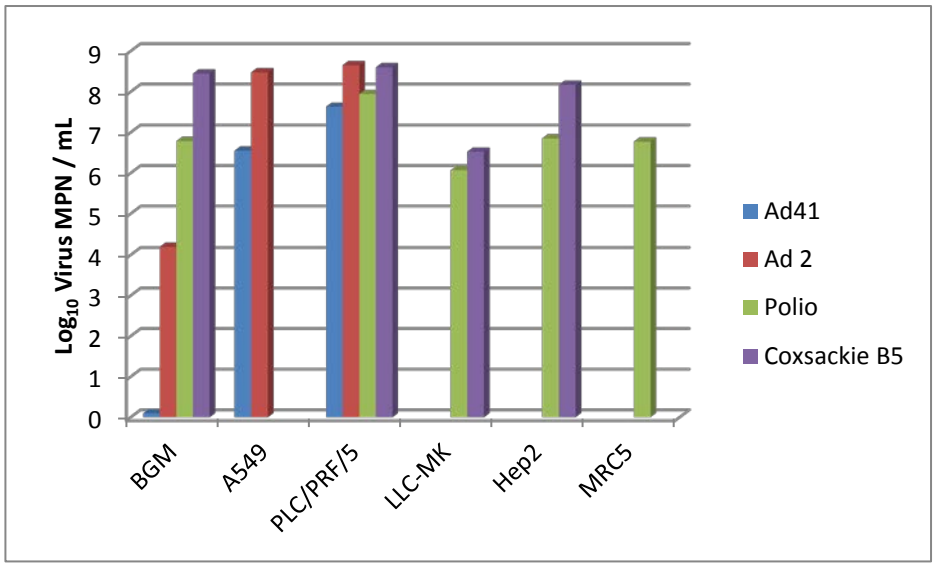


Figure 1. Comparison of virus counts (MPN / mL) for different viruses cultured with different host cell lines.

The impact of $CaCl_2$ on virus infectivity was assessed for Ad 2 and CB5. The rationale for assessing this was taken from the reported importance of divalent cations for the infectivity of some bacteriophage. The addition of low concentrations of calcium did not improve virus counts and appeared to cause a small reduction in the number of viruses detected (Table 16). Interestingly, the virus counts appeared to have a greater reduction for 5mM $CaCl_2$ compared with either 1 mM or 10 mM $CaCl_2$. This occurred for both the CB5 and Ad 2 cultures. There appears to be no benefit from adding $CaCl_2$, although this has not been trialled on viruses in wastewater / wastewater concentrates, which could potentially be affected by chelating agents or other contaminants.

While the tested calcium concentrations did not have any benefit, it is possible that a higher dose might yield some improvement and it is also possible that the presence of calcium might have a different effect for viruses in wastewater sample concentrates. Sample concentrates from virus recovery experiments will be further tested using addition of calcium chloride to determine if that improves virus detection in these samples

Further examination of published methods found that one of the variations on the adenovirus plaque assay used $MgCl_2$ as an additive. Experiments are in progress to test if this has any benefit.

Table 16. Virus counts in the presence of different concentrations of calcium chloride.

	CB5 (MPN/mL) % control	Ad 2 (MPN/mL) % control
0mM $CaCl_2$	6.10E+07 100%	2.30E+09 100%
1mM $CaCl_2$	3.00E+07 49%	6.10E+08 27%
5mM $CaCl_2$	1.60E+07 26%	3.60E+08 16%
10mM $CaCl_2$	3.60E+07 59%	9.30E+08 40%

Centrifugation was identified through the literature review as an option to improve virus infection rates. The mechanism for this is not fully understood because the low centrifugation speed that needs to be used to prevent damaging the host cells is insufficient to pellet virus particles. However, it is possible

that centrifugation would improve contact between host cells and any viruses associated with particulates, which could promote infection from particle-associated viruses. Due to concerns related to virus contamination of the AWQC cell culture clean room, the clean room plate centrifuge was unavailable for evaluating this approach. Given the project timelines (and budget), sourcing a new centrifuge was deemed not to be feasible. Alternative benchtop centrifuges at AWQC were not in an appropriate containment area and would also need the purchase of specific plate holder adaptors, so this option for improving infection efficiency was not pursued.

4.3.2 **Virus recovery methods**

Experiments were conducted to assess the recovery of Ad 2 and CB5 spiked into Bolivar secondary treated effluent. Concentration was either by ultrafiltration + PEG or by PEG alone. Detection of viruses was by PCR and by cell culture in the case of Ad 2 and by cell culture for CB5 (the PCR detection for CB5 is still in process because this assay needed to be re-established in the AWQC lab).

The first experiment dosed 11L of spiked secondary effluent with Ad 2. 1 L of this sample was directly concentrated using PEG precipitation, the remaining 10L was concentrated by ultrafiltration (HF80 filter) and the eluted filtrate was concentrated by PEG precipitation. A non-spiked sample was also processed in parallel to test for the presence of viruses already in the water. The results for PCR and cell culture are presented in Table 17 and Table 18. Due to a laboratory error, the virus dilution used for the spike could not be enumerated, so recovery rates could not be determined for this experiment. Taking into account the assay sensitivity, sample concentration factor and sample volume analysed, the assay detection limits (minimum number of virus particles / 10L sample required for a positive result) were calculated.

Table 17. Ad 2 recovery from spiked secondary effluent measured using qPCR. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive PCR result.

	Ultrafiltration (HF80) + PEG (copies/10L)	PEG (copies/10L)
A (Ad 2 spiked)	3.03E+05 (4.40E+03)	1.03E+06 (5.50E+04)
B (Ad 2 spiked)	3.10E+05 (3.80E+03)	1.35E+06 (3.30E+04)
C (Ad 2 spiked)	4.76E+05 (2.70E+03)	1.05E+06 (3.40E+04)
D (non-spiked)	3.80E+04 (4.50E+03)	3.81E+05 (2.90E+04)

Table 18. Ad 2 recovery from spiked secondary effluent measured using cell culture. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive MPN result.

	Ultrafiltration (HF80) + PEG (MPN/10L)	PEG (MPN/10L)
A (Ad 2 spiked)	1.01E+03 (1.10E+03)	0 (1.38E+04)
B (Ad 2 spiked)	0 (9.50E+02)	0 (8.25E+03)
C (Ad 2 spiked)	6.21E+02 (6.75E+02)	0 (8.50E+03)
D (non-spiked)	1.04E+03 (1.13E+03)	0 (7.25E+03)
Estimated Spiking stock 1.10E+05 MPN / 10L		

In general culture allows for a better detection limit than PCR due to the larger volume of sample concentrate analysed. Direct PEG analysis was 10-fold less sensitive due to the smaller sample volume used for virus concentration, but it allowed for the detection of more viruses by PCR, suggesting a higher virus recovery rate. Indigenous adenoviruses were present in the wastewater and these appeared to have a higher recovery efficiency in the direct PEG method compared to the spiked Ad 2. Significantly, no culturable Ad 2 were detected by the infectivity method. It appears that indigenous adenovirus were detected near the detection limit of the cell culture assay for the filtration + PEG samples, with similar virus numbers in the spiked and non-spiked samples. No infectious viruses were detected by the direct PEG method, possibly because the number of infectious viruses was below the assay detection limit.

A second spiking experiment using Ad 2 was conducted to compare direct PEG with filtration + PEG using either the old HF80 or new FX80 filters. The experimental protocol was the same as the previous experiment except a fresh batch of Bolivar secondary effluent was collected for this round of testing. The results are presented in Table 19 and Table 20. Assuming that the spike stock had similar virus numbers compared with that used in the previous experiment (being the same dilution from a virus stock concentrate), the direct PEG performance was similar to the previous round, with comparable numbers of virus copies detected for spiked and indigenous adenovirus. The PEG method had a recovery between 8 – almost 10%, which is much lower than that reported previously. Based on virus numbers, the performance of the FX80 filters in this round was comparable to the performance of the HF80 filters in the previous round and approximately 10-fold poorer recovery compared with direct PEG. In this round, the HF80 filters had recovery rates comparable with direct PEG. The reason for the improved result is not known. Of particular interest, the number of indigenous adenovirus copies recovered after filtration was similar for both the HF80 and FX80, which were both similar to the previous round for indigenous adenovirus recovered by the HF80 filter. This suggests that there may be some difference between Ad 2 and other serotypes of adenovirus in terms of filtration recovery efficiency and also behaviour / survival in wastewater. The culture results did not detect any infectious indigenous viruses in the non-spiked sample this round, and there was very low or no recovery of infectious Ad 2 (possibly the detections were background from indigenous adenoviruses).

Table 19. Ad 2 recovery from spiked secondary effluent measured using qPCR. % value indicates the percentage recovery calculated using the spiking stock enumerated by qPCR and correcting for the presence virus in the non-spiked water. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive PCR result.

	HF80 + PEG (copies/10L)	FX80 + PEG (copies/10L)	PEG (copies/10L)
A (Ad 2 spiked)	2.23E+06; 6.9% (1.80E+03)	2.31E+05; 0.6% (1.80E+03)	2.55E+06; 7.5% (3.00E+04)
B (Ad 2 spiked)	1.47E+06; 4.4% (1.80E+03)	3.77E+05; 1.0% (1.80E+03)	3.01E+06; 9.0% (3.00E+04)
C (non-spiked)	8.53E+04 (1.80E+03)	6.08E+04 (1.80E+03)	1.99E+05 (3.00E+04)
Spiking stock 3.12E+08 virus genomes / 10L			

Table 20. Ad 2 recovery from spiked secondary effluent measured using cell culture. % value indicates the percentage recovery calculated using the spiking stock enumerated by MPN. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive MPN result.

	HF80 + PEG (MPN/10L)	FX80 + PEG (MPN /10L)	PEG (MPN /10L)
A (Ad 2 spiked)	6.48E+02; 0.1% (4.50E+02)	0; 0% (4.50E+02)	0; 0% (7.50E+03)
B (Ad 2 spiked)	4.50E+01; 0.01% (4.50E+02)	0; 0% (4.50E+02)	0; 0% (7.50E+03)
C (non-spiked)	0 (4.50E+02)	0 (4.50E+02)	0; (7.50E+03)
Spiking stock 6.20E+05 MPN / 10L			

Concentration of viruses using direct PEG is attractive because it is relative fast, rapid and is thought to provide good recovery rates, especially compared with filtration. A limitation is that only a relatively small volume can be concentrated. Calcium carbonate precipitation is used quite successfully for *Cryptosporidium* concentration from 10L water samples. Considering that this is a precipitation technique, it has potential for virus concentration and so was trialled. 10.5 L Bolivar secondary effluent samples were spiked with Ad 2, with 10L processed by the calcium carbonate method and 500 mL processed by direct PEG. The redissolved pellet from the calcium carbonate method was pH adjusted to 7, with half further concentrated by PEG and the remainder used for direct PCR or cell culture analysis. Samples A. B and D were processed as 1 batch, samples C and E were processed as a separate batch (due to sample batch size constraints for centrifugation). The results are shown in Tables 7 and 8. Although the calcium carbonate method can process a larger volume compared with PEG, the resulting concentrate is also in a larger volume (approximately 10-fold larger than the PEG concentrate volume), so the theoretical detection limits for both concentration methods were determined to be similar (Table 21 & Table 22). The combination of the two methods allowed for a detection limit closer to that of filtration + PEG. The PCR results tended to be variable, with some spiked samples failing to amplify and others exhibiting variable amplification for the PCR replicates for that sample. Based on the numbers of viruses detected in the non-spiked controls, it appears those indigenous adenoviruses were detected in the spiked samples and that the Ad 2 were not recovered or were very poorly recovered. The calcium carbonate and direct PEG qPCR results were comparable for the non-spiked samples (within 2-fold of each other), but the number of viruses detected for the combined calcium carbonate + PEG was 10-fold lower. This suggests that the combined losses through both methods reduced the overall recovery efficiency or that the sequential precipitations cause higher losses than either method alone.

Compared with the previous recovery experiment, the numbers of Ad 2 spiked were approx. 10-fold lower as determined by qPCR, but the infectivity of these viruses was even lower than in the previous experiment by about 30-fold. The number spiked was sufficient for qPCR detection, but the culturable number of viruses was near the detection limit for the direct calcium carbonate and PEG precipitation methods, which is likely why there was no virus detected for either of these. Infectious indigenous adenoviruses were detected in 1 of the 2 non-spiked calcium carbonate + PEG samples. The numbers were similar to those detected in the spiked B and C samples, suggesting that there were no infective Ad 2 present or that Ad 2 were not recovered.

Table 21. Ad 2 recovery from spiked secondary effluent measured using qPCR. % value indicates the percentage recovery calculated using the spiking stock enumerated by qPCR and correcting for the presence of virus in the un-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive PCR result.

	Calcium Carbonate (copies/10L)	Calcium Carbonate + PEG (copies /10L)	PEG (copies /10L)
A (Ad 2 spiked)	1.47E+05[1]; 0% (5.40E+04)	1.20E+4[3]; 0.3% (6.00E+03)	0; 0% (6.20E+04)
B (Ad 2 spiked)	0; 0% (5.41E+04)	8.40E03[2]; 0.2% (6.20E+03)	2.36E+04[3]; 0% (6.40E+04)
D (non-spiked)	1.99E+05[2] (5.50E+04)	2.97E+03[3] (5.80E+03)	4.70E+05 (6.00E+04)
C (Ad 2 spiked)	1.34E+05[2]; 0% (5.51E+04)	2.68E+04[1]; 0% (5.00E+03)	1.17E+06; 20.4%; (5.60E+04)
E (non-spiked)	7.11E+05 (5.33E+04)	6.37E+04 (5.00E+03)	4.97E+05[1] (6.60E+04)
Spiking stock 2.84E+06 virus genomes / 10L			

[1] 3 out of 4 PCR replicates were positive
[2] 2 out of 4 PCR replicates were positive
[3] 1 out of 4 PCR replicates were positive

Table 22. Ad 2 recovery from spiked secondary effluent measured using MPN. % value indicates the percentage recovery calculated using the spiking stock enumerated by MPN and corrected for the presence of virus in the un-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive MPN result.

	Calcium Carbonate (MPN/10L)	Calcium Carbonate + PEG (MPN /10L)	PEG (MPN /10L)
A (Ad 2 spiked)	0; 0% (1.35E+04)	0; 0% (1.50E+03)	0; 0% (1.55E+04)
B (Ad 2 spiked)	0; 0% (1.35E+04)	1.43E+03; 8.9% (1.55E+03)	0; 0% (1.60 E+04)
D (non-spiked)	0 (1.38E+04)	0 (1.45E+03)	0 (1.50 E+04)
C (Ad 2 spiked)	0; 0% (1.38E+04)	5.50E+02, 0% (1.25E+03)	0; 0%(1.40 E+04)
E (non-spiked)	0 (1.33E+04)	1.15E+03 (1.25E+03)	0 (1.65 E+04)
Spiking stock 1.60E+04 virus MPN / 10L			

The PCR results from the 3 sets of spiking experiments are summarised in Figure 2. In general direct PEG provided a higher yield of Ad 2 compared with the other methods, except in Experiment 3 where there was a high level of variation between the PEG replicates. The recovery rate of Ad 2 as determined by PCR in these experiments was approx. 10% for direct PEG. Interestingly, the virus numbers in the non-spiked samples were consistent, suggesting that the recovery of native viruses is consistent for filtration and PEG. Overall, these results suggest that there were problems with the Ad 2 after spiking into Bolivar secondary treated effluent. The cell culture controls suggest that the Ad 2 were infectious, but after spiking the viruses were either rendered non-infectious or very poorly recovered. Disinfection experiments conducted using Ad 2 spiked into the same Bolivar effluent (winter/spring 2014) did not encounter any issues with the direct culture detection of Ad 2, although these experiments did not require sample concentration. Stability experiments are currently in process to assess the effect of storage on Ad 2 spiked into secondary effluent. Results so far suggest that storage for several days at 4°C in secondary effluent has minimal impact on virus infectivity. This was measured by direct analysis of the wastewater for using the cell culture assay. These results suggest poor recovery is the cause of virus non-detection in the current project, rather than loss of infectivity. Indigenous adenovirus could be detected by qPCR or cell culture following concentration using a variety of methods, but determination of recovery rate requires a more robust control than Ad 2.

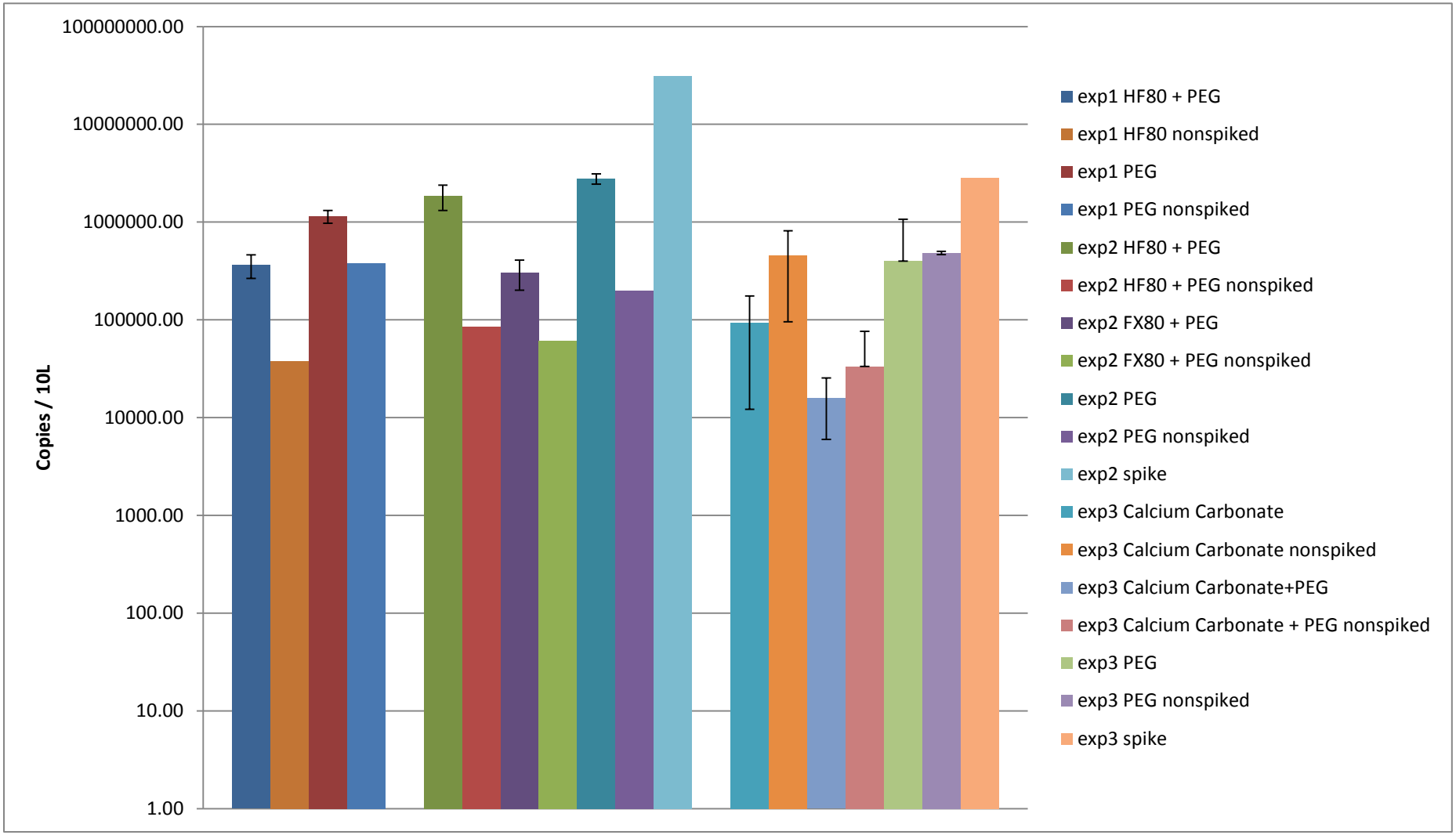


Figure 2. Comparison of Ad 2 numbers recovered by different concentration methods in spiked and un-spiked Bolivar secondary effluent. Error bars indicate %CV.

Experiments have been conducted to assess the recovery of CB5 spiked into Bolivar secondary treated effluent. The design of these experiments was similar to that for Ad 2 (spike 10.5 or 11L of wastewater, concentration 10L by filtration + PEG and the remainder by direct PEG). The recovery of infectious CB5 (Table 23, Table 24), though low, was generally better than for Ad 2. The recovery rate appeared to be better for the new ultra-filter model compared to the old filter type and was comparable with the PEG recovery rates. In these experiments, the recovery of virus copies appeared to be very low. The spiking stock had 6.20E+05 infectious virus (MPN) / 10 L but the number of virus copies determined by quantitative RT-PCR was 3.64E+08, over an order of magnitude higher. This suggests only % of virus were infective in this stock. It is possible that the non-infectious viruses are highly labile and were degraded during sample processing, resulting in a low apparent recovery rate. The number of indigenous enteroviruses appeared to be low, with only the direct PEG sample being positive for a low level of virus. Some of the spiked samples also had similar numbers, suggesting that these could be detections of indigenous virus rather than recovered spiked CB5.

Table 23. CB5 recovery from spiked secondary effluent measured using MPN. % value indicates the percentage recovery calculated using the spiking stock enumerated by MPN after subtraction of any background virus in the non-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive MPN result. The sample in red could represent detection of adenovirus.

	FX80 + PEG (MPN /10L)	HF80 + PEG (MPN/10L)	PEG (MPN/10L)
A (CB5 spiked)	1.61E+04; 2.6% (6.50E+02)	3.35E+03; 0.5% (9.00E+02)	1.19E+03; 1.6% (8.25E+03)
B (CB5 spiked)	2.42E+04; 3.9% (5.50E+02)	2.97E+04; 4.8% (6.75E+02)	7.25E+02; 0% (7.25E+03)
C (CB5 spiked)	N/A	1.26E+03; 0.2% (8.75E+02)	2.51E+04; 3.8% (6.75E+03)
D (Non spiked)	0 (6.25E+02)	0 (6.50E+02)	1.85E+03 (1.85E+04)
Spiking stock 6.20E+05 virus MPN / 10L			

Table 24. CB5 recovery from spiked secondary effluent measured using quantitative RT-PCR. % value indicates the percentage recovery calculated using the spiking stock enumerated by quantitative RT-PCR after subtraction of any background virus in the non-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive PCR result.

	FX80 + PEG (copies /10L)	HF80 + PEG (copies/10L)	PEG (copies/10L)
A (CB5 spiked)	3.30E+05; 0.9% (2.60E+03)	1.77E+05; 0.4% (3.60E+03)	1.22E+05; 0.3% (3.30E+04)
B (CB5 spiked)	2.53E+05; 0.7% (2.20E+03)	3.48E+05; 0.9% (2.70E+03)	0; 0% (2.90E+04)
C (CB5 spiked)	N/A	1.60E+04; 0% (3.50E+03)	2.98E+05; 0.8% (2.70E+04)
D (Non spiked)	0 (2.50E+03)	0 (2.60E+03)	0 (7.40E+04)
Spiking stock 3.64E+08 virus copies / 10L			

The CB5 spike experiments were repeated using FX80 filters and direct PEG precipitation (Table 25, Table 26). The performance of the FX80 filtration + PEG was similar to the previous round of testing (Table 23) for infectious virus, but the recovery rates were much higher for direct PEG. No culturable viruses were detected in the non-spiked sample. The recovery was not as high as reported previously by Keegan et al. (2012), but was a 5 to 10-fold improvement compared with the previous round. The CB5 experiments for culturable virus are summarised in Figure 3. In contrast with the previous round, the detection of virus by quantitative RT-PCR suggested a much higher recovery rate, 51 – 54% for filtration and 66% for PEG. In the case of the PEG samples, one appeared to have no recovery of virus genome copies, although the same sample had 30% recovery based on cell culture. This suggests that the RNA extraction of this sample failed. In these experiments the difference between virus MPN and copy number was only 10%.

Table 25. CB5 recovery from spiked secondary effluent measured using MPN. % value indicates the percentage recovery calculated using the spiking stock enumerated by MPN after subtraction of any background virus in the non-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive MPN result.

	FX80 + PEG (MPN /10L)	PEG (MPN/10L)
A (CB5 spiked)	1.98E+02; 0.6% (4.50E+02)	1.08E+04; 30% (7.50E+03)
B (CB5 spiked)	6.48E+02; 1.8% (4.50E+02)	6.90E+03; 19.2% (7.50E+03)
C (Non spiked)	0 (4.50E+02)	0 (7.50E+03)
Spiking stock 3.60E+04 virus MPN / 10L		

Table 26. CB5 recovery from spiked secondary effluent measured using quantitative RT-PCR. % value indicates the percentage recovery calculated using the spiking stock enumerated by quantitative RT-PCR after subtraction of any background virus in the non-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive PCR result. Red highlights a possible nucleic acid extraction failure.

	FX80 + PEG (copies /10L)	PEG (copies/10L)
A (CB5 spiked)	1.75E+05; 54.6% (1.80E+03)	8.01E+04; 0% (3.00E+04)
B (CB5 spiked)	1.66E+05; 51.7% (1.80E+03)	3.00E+05; 65.8% (3.00E+04)
C (Non spiked)	7.25E+03 (1.80E+03)	1.00E+05 (3.00E+04)
Spiking stock 3.08E+05 virus copies / 10L		

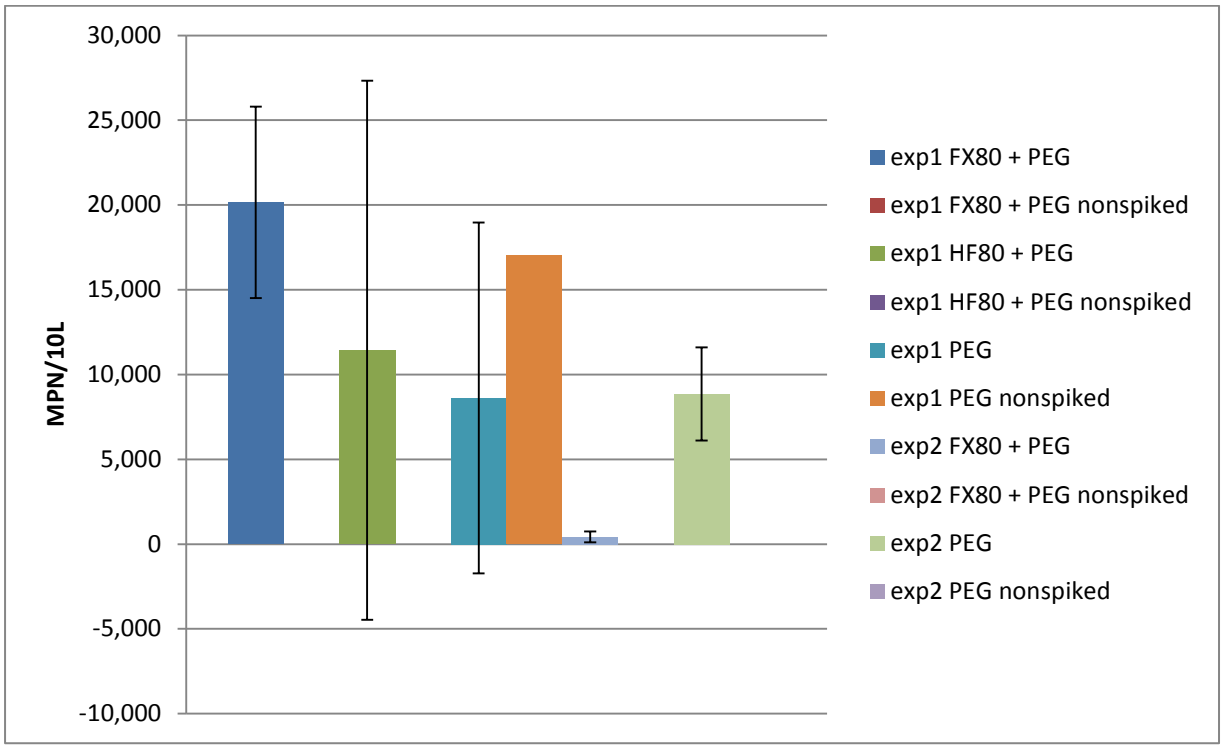


Figure 3. Comparison of infective CB5 numbers recovered by different concentration methods in spiked and un-spiked Bolivar secondary effluent. Error bars indicate %CV.

Experiments were conducted to evaluate virus recovery from primary effluent samples using spiked adenovirus. These experiments compared direct recovery of the neat sample and recovery using HF80 filtration + PEG or direct PEG for diluted primary samples. Culture results were similar to the results for secondary effluent, with indigenous adenovirus numbers in non-spiked samples similar to the numbers of adenovirus detected in the spiked samples, suggesting failure to recover the spiked Ad 2 (Table 27). Direct PEG had overall better recovery rates compared with filtration + PEG and there was no benefit from sample dilution, with recovery rates generally better for direct PEG of the undiluted primary effluent. In contrast with the cell culture results, the PCR results suggested a consistent recovery rate for the direct PEG recovery from the diluted sample (Table 28). The calculation of recovery rates for the undiluted primary effluent was complicated by the presence of high virus copy numbers in the un-spiked sample, with the levels of un-spiked virus similar to or greater than that of the spike.

Table 27. Results for 3 separate spiking trials using either HF80 ultrafiltration + PEG or direct PEG to recover Ad 2 spiked into Bolivar primary effluent. % value indicates the percentage recovery calculated using the spiking stock enumerated by MPN after subtraction of any background virus in the non-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive MPN result.

	Matrix	HF80 + PEG (MPN/10L), % Recovery	PEG (MPN/10L), % Recovery
A (Ad 2 spiked)	1/10 Primary effluent	1.53E+03, 1.3% (6.25E+02)	6.90E+03, 0% (7.50E+03)
B (Ad 2 spiked)	1/10 Primary effluent	2.30E+03, 0% (2.50E+02)	2.86E+03, 4.6% (6.50E+03)
C (Ad 2 spiked)	1/10 Primary effluent	3.11E+03, 0.8% (3.38E+02)	5.98E+03, 2.5% (6.50E+03)
D (non-spiked)	1/10 Primary effluent	7.32E+02 (3.00E+02)	7.82E+03 (8.50E+03)
E (non-spiked)	1/10 Primary effluent	3,335 (3.63E+02)	0 (5.50E+03)
F (non-spiked)	1/10 Primary effluent	1.16E+03 (3.13E+02)	0 (5.50E+03)
G (Ad 2 spiked)	Neat primary effluent	ND	2.13E+04, 21.2% (6.75E+03)
H (Ad 2 spiked)	Neat primary effluent	ND	2.64E+04, 7.1% (6.00E+03)
I (Ad 2 spiked)	Neat primary effluent	ND	2.16E+04, 0% (6.75E+03)
J (non-spiked)	Neat primary effluent	ND	8.16E+03 (6.00E+03)
K (non-spiked)	Neat primary effluent	ND	2.20E+04 (5.00E+03)
L (non-spiked)	Neat primary effluent	ND	4.83E+04 (5.75E+03)
Spiking stock @ 6.20E+04 MPN/10L			

Table 28. Results for 3 separate spiking trials using either HF80 ultrafiltration + PEG or direct PEG to recover Ad 2 spiked into Bolivar primary effluent. % value indicates the percentage recovery calculated using the spiking stock enumerated by qPCR after subtraction of any background virus in the non-spiked sample. The numbers in parentheses indicate the theoretical number of viruses required in the original sample for a positive qPCR result.

	Matrix	HF80 + PEG (copies/10L), % Recovery	PEG (copies/10L), % Recovery
A (Ad 2 spiked)	1/10 Primary effluent	9.45E+04, 0% (2.50E+03)	9.28E+05, 20.7% (3.00E+04)
B (Ad 2 spiked)	1/10 Primary effluent	2.37E+05, 0% (1.00E+03)	7.52E+05, 17.2% (2.60E+04)
C (Ad 2 spiked)	1/10 Primary effluent	1.08E+06, 16.1% (1.35E+03)	1.47E+06, 35.6% (2.60E+04)
D (non-spiked)	1/10 Primary effluent	2.55E+05 (1.20E+03)	3.83E+05 (3.40E+04)
E (non-spiked)	1/10 Primary effluent	8.29E+05 (1.45E+03)	2.46E+05 (2.20E+04)
F (non-spiked)	1/10 Primary effluent	3.99E+05 (1.25E+03)	1.10E+04 (2.20E+04)
G (Ad 2 spiked)	Neat primary effluent	ND	1.97E+06, 0% (2.70E+04)
H (Ad 2 spiked)	Neat primary effluent	ND	6.33E+06, 0% (2.40E+04)
I (Ad 2 spiked)	Neat primary effluent	ND	1.05E+07, 60.7% (2.70E+04)
J (non-spiked)	Neat primary effluent	ND	9.18E+06 (2.24E+04)
K (non-spiked)	Neat primary effluent	ND	6.77E+06 (2.20E+04)
L (non-spiked)	Neat primary effluent	ND	7.96E+06 (2.30E+04)
Spiking stock @ 3.20E+06 copies/10L			

Given the potential loss of spiked infectious virus, trials were conducted to assess virus stability after spiking into Bolivar secondary effluent or into cell culture PBS or cell culture medium (MEM). After spiking, the sample was stored at 4°C and at set times an aliquot was collected and directly analysed by cell culture. The results (Table 29) suggest that the spiked adenovirus were relatively stable for up to 7 days after spiking. The same samples were extracted and adenovirus quantified by PCR. The results show similar results between the two methods, with virus copies slightly lower, possibly reflecting a difference in the accuracy of the qPCR standard or losses during DNA extraction. These results contrast with those for CB5, which suggested that only 1 – 10% of virus copies were infectious.

The stability of virus concentrates stored at -80°C was also assessed (Table 30). In general, storage over a 1 month period appeared to have little effect on the number of viruses detected by cell culture. Two data points appeared to exhibit a decrease for Ad 41 and Poliovirus. It is possible this was due to a problem with the cell culture assay at the time considering that the following sample for Poliovirus was similar to the original Poliovirus count.

Table 29. Stability experiment assessing persistence of Adenovirus virus spiked into different water types and stored at 4°C.

	PCR, 0hr (copies/mL)	0 hr (MPN/mL)	24hrs (MPN/mL)	168hrs (MPN/mL)
Bolivar secondary effluent	4.43E+07	1.22E+08	2.20E+08	7.20E+07
CC-PBS	4.10E+07	7.20E+07	1.22E+08	1.20E+08
1xMEM	4.72E+07	2.20E+08	3.20E+08	4.60E+08

Table 30. Effect of storage at -80°C on different virus concentrates measured using PLC/PRF/5 cells. Results are averages from 3 technical replicates. Results in red highlight possible outliers.

Date	Ad 2	Ad 41	Poliovirus	CB5
14/11/2014	8.70E+08			
26/11/2014			5.70E+07	1.50E+08
05/12/2014	4.80E+08	1.20E+08	1.10E+08	2.20E+08
12/12/2014	2.80E+08	1.80E+06	6.00E+06	3.00E+08
09/01/2015			7.70E+07	2.90E+08

4.3.3 *Cryptosporidium* recovery methods

Work conducted at AWQC has determined that recovery of *Cryptosporidium* from primary effluent can be improved by dilution of the primary effluent in RO water, followed by concentration. The concentration method in use at AWQC is calcium carbonate precipitation (also termed flocculation). This approach has not been trialled for filtration-based methods. To evaluate *Cryptosporidium* recovery from wastewater, primary and secondary effluent samples were collected from Bolivar WWTP. Samples (in triplicate) were spiked with ColorSeed to measure recovery rates. Secondary effluent samples were concentrated using filtration (Envirochek) or precipitation; oocysts were purified by immunomagnetic separation (IMS) and then processed using a *Cryptosporidium* infectivity assay that incorporates a step for counting oocysts (King et al. 2015). Primary effluent samples (500mL) were spiked with ColorSeed and then either concentrated by centrifugation / IMS or made up to 10L using RO water and concentrated as per secondary effluent using filtration or calcium carbonate.

Three separate rounds were conducted to compare different *Cryptosporidium* recovery methods for primary and secondary effluent: the AWQC method using calcium carbonate flocculation + Dynal IMS (neat secondary effluent or diluted primary effluent); direct centrifugation + Dynal IMS (for primary effluent only); Envirochek filtration + Dynal IMS (neat secondary effluent or diluted primary effluent). Recovery data (Figure 3) suggests that all methods performed similarly for primary effluent, with flocculation perhaps slightly better and more consistent. There appeared to be a general issue with recoveries in round three, especially for the non-flocculation samples (recoveries <10%, which is normally the rejection criteria for assay performance). It is possible that some of the round three results were impacted by a defective batch of IMS beads that was recalled by the manufacturer, this requires further investigation to determine if it is possible to match the samples with the bad batch of beads. For the secondary effluent flocculation performed consistently better than Envirochek filtration. Using the recovery rates to correct for losses, the oocyst densities in the samples were comparable irrespective of the concentration method (Figure 4). The standard deviation was higher for the secondary effluent filtered samples because the raw oocyst numbers were lower and more variable. The sample processing methods did not appear to have any impact on oocyst infectivity for rounds 1 and 3 (Figure 5), although round 2 appeared to be more variable with lower infectivity measured for the samples concentrated using calcium carbonate. Overall, the oocyst infectious fraction was similar for the same sample type irrespective of concentration method (Figure 5). It was noted while conducting the infectivity assay that filtered samples were more prone to fungal contamination compared with samples concentrated using calcium carbonate. A key step in the precipitation method is redissolving the precipitate in sulphamic acid. It is likely that this method will reduce the level of contaminating microorganisms while having no major impact on oocyst infectivity.

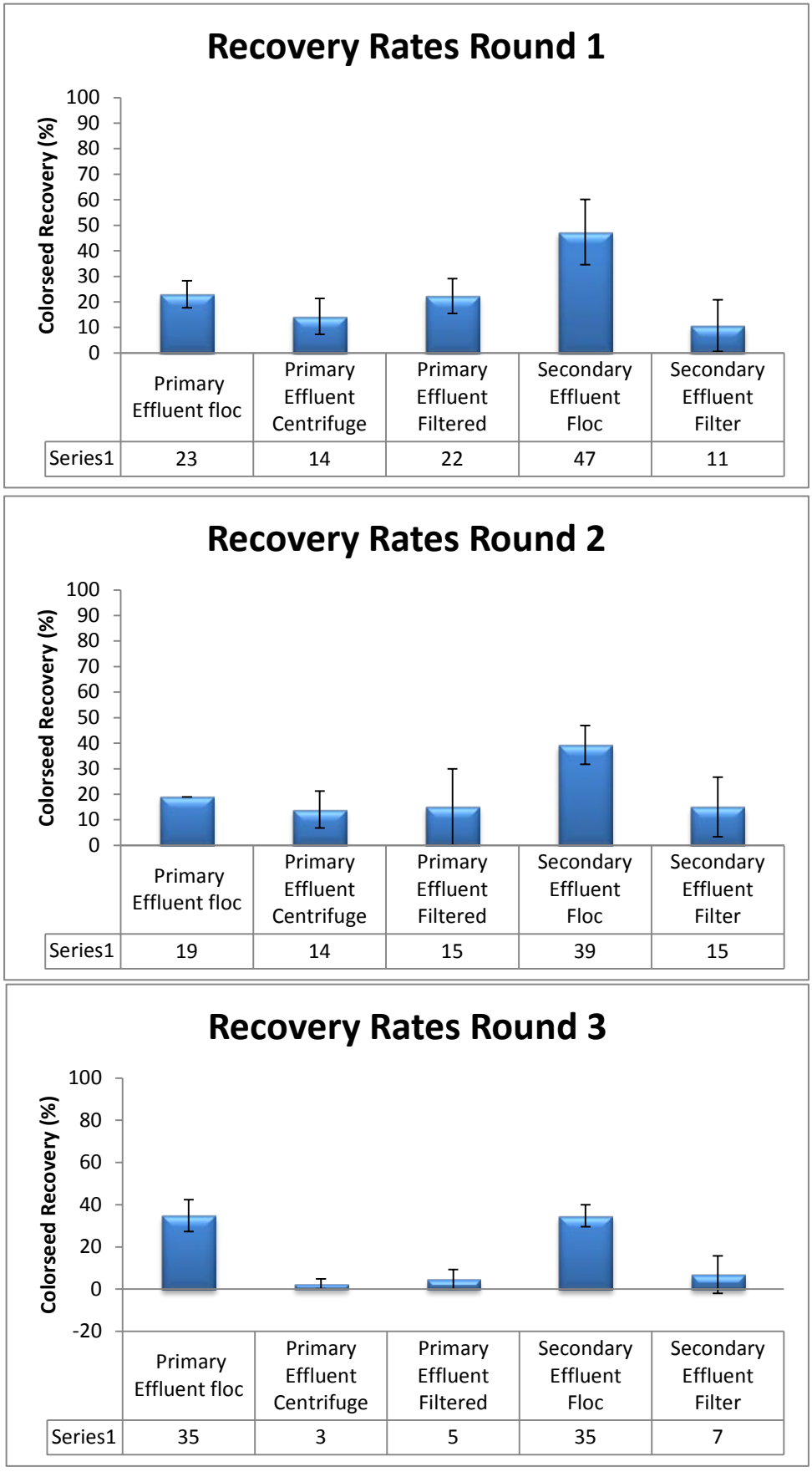
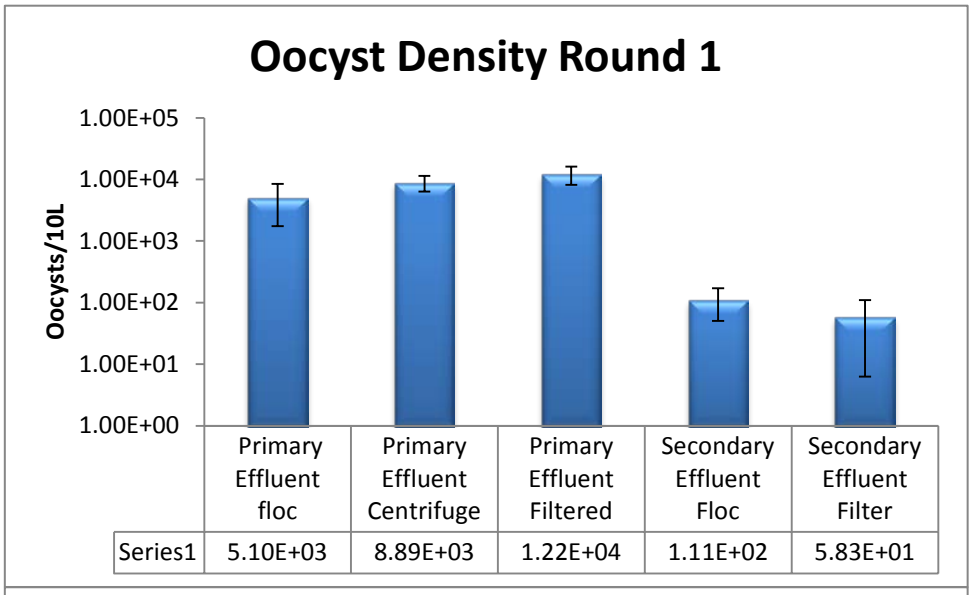
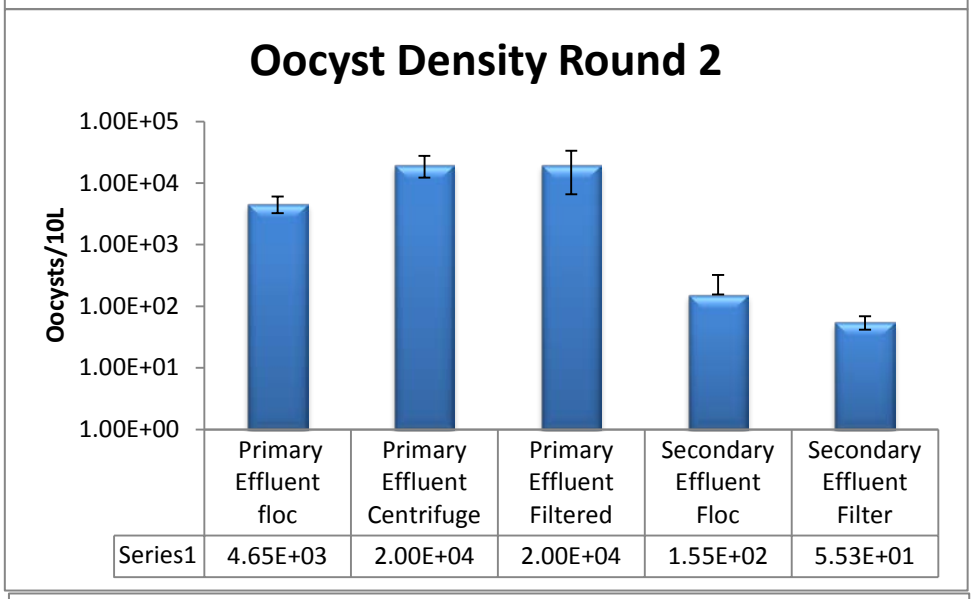


Figure 4. *Cryptosporidium* ColorSeed % recovery rates for primary or secondary effluent concentrated using different recovery techniques.





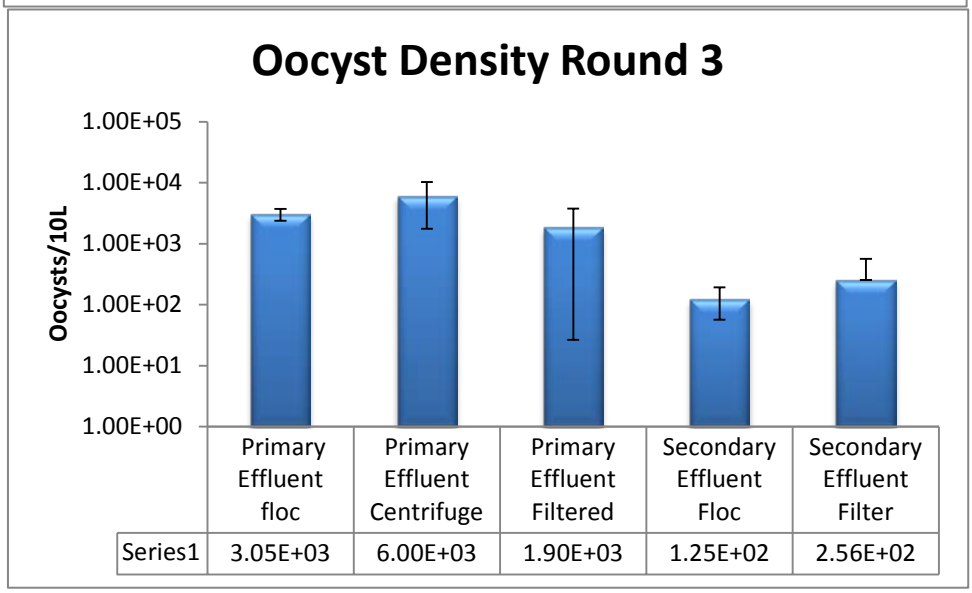


Figure 5. Oocyst density in different wastewater samples, corrected for recovery rate.

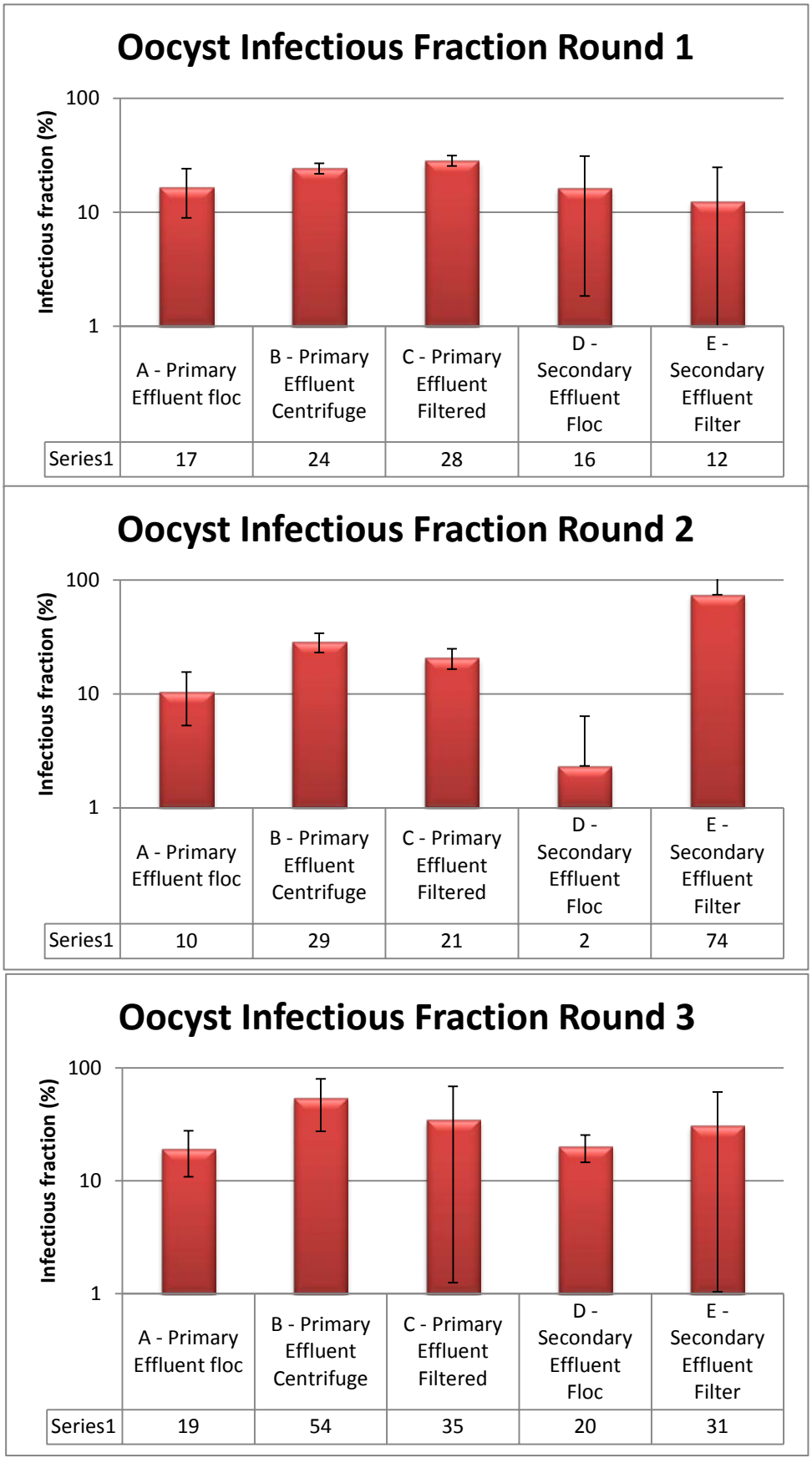


Figure 6. Percent infectivity of oocysts recovered from wastewater samples using different concentration techniques.

4.3.4 **Summary of method evaluation results**

Recovery of spiked Ad 2 was variable. In secondary effluent there was detection of spiked Ad 2 by PCR but rarely by culture. In some experiments it appears that the Ad 2 were failing to be recovered but indigenous adenoviruses were being detected (ie virus detections in the un-spiked samples). The trial data using primary effluent suggest that high levels of indigenous adenovirus were present in the un-spiked effluent samples. The high numbers of indigenous adenovirus may be obscuring measurement of the recovery of spiked Ad 2, especially if the numbers of indigenous virus were similar to the numbers of Ad 2 spiked into the sample. Recovery of infectious spiked CB5 measured using cell culture was generally low and no (or very low numbers of) indigenous enteroviruses were detected. PCR analyses for CB5 were comparable with the culture results, with low levels of indigenous enterovirus detected in only 1 spiking experiment.

Stability experiments suggest that spiking Ad 2 into secondary effluent had little impact on virus infectivity. Direct analysis of the spiked samples (without any concentration) showed that the numbers of infectious Ad 2 were relatively stable for 7 days post-spike when the spiked sample was stored at 4°C. This suggests that any loss of infectious Ad 2 is due to an aspect of the sample concentration method and not due to interaction with compounds in the wastewater. Similarly, -80°C storage of the viruses used in this work did not appear to impact on infectivity.

Cryptosporidium recovery trials suggested that the Calcium Carbonate method provided more reproducible recovery rates for ColorSeed compared with filtration or direct centrifugation. However, overall oocyst counts determined by the different concentration methods were comparable when taking into account the recovery rate of ColorSeed. Some of the recovery rates for filtration / centrifugation were below the accepted 10% and would normally be treated as a test failure. A possible reason for some of the poor recoveries is the use of a defective batch of Dynal IMS beads, which were subject to a manufacturer recall after these samples had been processed / reported.

4.3.5 **Inter-laboratory method comparison**

4.3.5.1 Virus results

Primary and secondary effluent samples were spiked with laboratory-cultured Coxsackie virus CB5. The spiked virus was enumerated at the time of spiking using both cell culture and qRTPCR and it was determined that the spiked levels were 3.1×10^4 culturable virus / 10 L and 1.6×10^6 virus copies / 10 L. Primary effluent samples were concentrated by either PEG precipitation (Laboratories 1 & 3) or by molecular weight cut-off filters (Laboratory 2). Secondary effluent samples were directly concentrated by PEG (Laboratory 1) or by UF filtration followed by PEG (Laboratories 1 & 3) or by UF filtration followed by concentration through molecular weight cut-off filters (Laboratory 2). Nucleic acid (DNA and RNA) was also directly extracted from aliquots of samples prior to concentration (Laboratories 1 & 2). The results (averages for the replicate samples) for enterovirus and adenovirus are shown in Figure 7 - Figure 14, with complete results shown in Table 31 to Table 37. Overall, there was approximately a 2 $\log_{10}$ difference or greater between total viruses determined by PCR and infective viruses determined by cell culture. This was the case for the coxsackie CB5 spike, as well as for the indigenous enterovirus and adenovirus in the samples.

Similar numbers of enterovirus were detected by culture in the spiked and un-spiked Laboratory 3 primary effluent samples (Figure 7) and by qRT-PCR in the spiked and un-spiked Laboratory 1 primary samples (Figure 8). This result suggests there were significant numbers of enterovirus in the primary effluent. The numbers of background virus were similar to the numbers used as the spike, making it difficult to determine recovery rates. Based on the earlier work comparing cell lines it was anticipated that the PLC cell line would detect both enterovirus and adenovirus. However, the virus numbers detected using PLC cells were similar to the numbers of enterovirus detected using BGM cells. The numbers of enterovirus detected using the Laboratory 3 method were similar to the Laboratory 1 BGM results for the spiked sample, but in the case of the Laboratory 1 un-spiked sample the number of virus was below detection limit. For detection of enterovirus by RT-PCR, the Laboratory 2 method detected higher numbers of enterovirus in the spiked sample compared with the Laboratory 1 result (although in the same order of magnitude) but did not detect virus in the un-spiked sample. Attempts at direct detection of enterovirus by RT-PCR without sample concentration were not successful. The detection limit of this approach was determined to be 2.5×10^6 / 10 L, which was similar to the number detected after PEG concentration. It is possible that RNA extraction was less

efficient on the raw primary effluent compared with the concentrated PEG sample, resulting in a failure to detect any enterovirus by this approach.

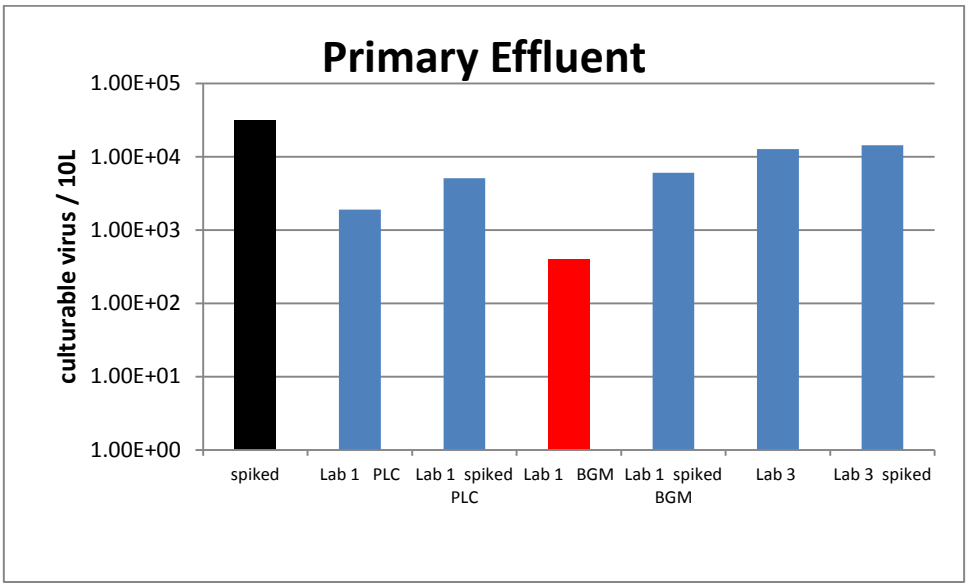


Figure 7. Comparison of the average numbers of enterovirus / 10L detected in spiked and un-spiked primary effluent samples using cell culture. PLC indicates viruses detected by MPN culture on PLC/PRF/5 cells, BGM indicates viruses detected by plaque assay using BGM cells. The black column indicates the number of viruses / 10 L spiked into the sample, the red column indicates a below detection limit result.

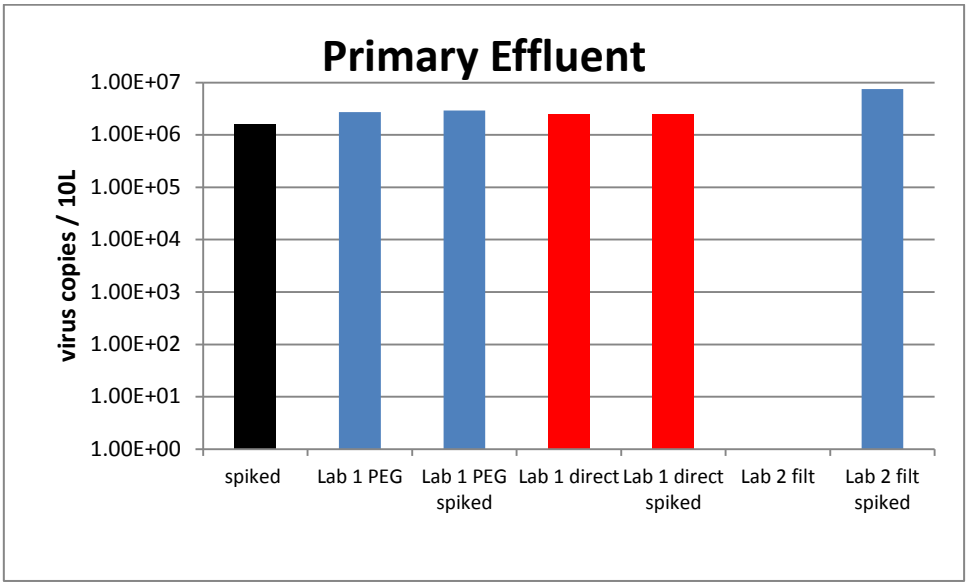


Figure 8. Comparison of the average numbers of enterovirus copies / 10L detected in spiked and un-spiked primary effluent samples using quantitative reverse transcription PCR. The black column indicates the number of viruses / 10 L spiked into the sample, the red column indicates a below detection limit result.

The detection of enterovirus was less successful in secondary effluent samples. Viruses were detected using PLC cells in the spiked Laboratory 1 samples but not in the un-spiked samples. Enteroviruses were below detection limit in the filtered + PEG Laboratory 1 samples and were only detected in the direct PEG un-spiked sample using BGM cells. In the case of the Laboratory 3 samples, the number of enteroviruses detected in the spiked sample was similar to the number of enteroviruses detected in the spiked Laboratory 1 sample. Low numbers of enteroviruses were detected in the un-spiked Laboratory 3 sample. In general, the numbers of viruses detected were near detection limit (within 10-fold) of the Laboratory 1 method, and were 1 – 3 orders of magnitude lower than the number of viruses spiked into the samples. There was no virus recovery for the Laboratory 1 samples based on the BGM culture results, using the PLC virus counts there was between 1- 10% recovery. The recovery rate for the Laboratory 3 samples was approximately 1%. Comparison of the primary and secondary effluent results suggests that there is a large decrease in the number of culturable enteroviruses following secondary treatment. Enteroviruses were not detected by RT-PCR by either Laboratory 1 or Laboratory 2 methods (Figure 10).

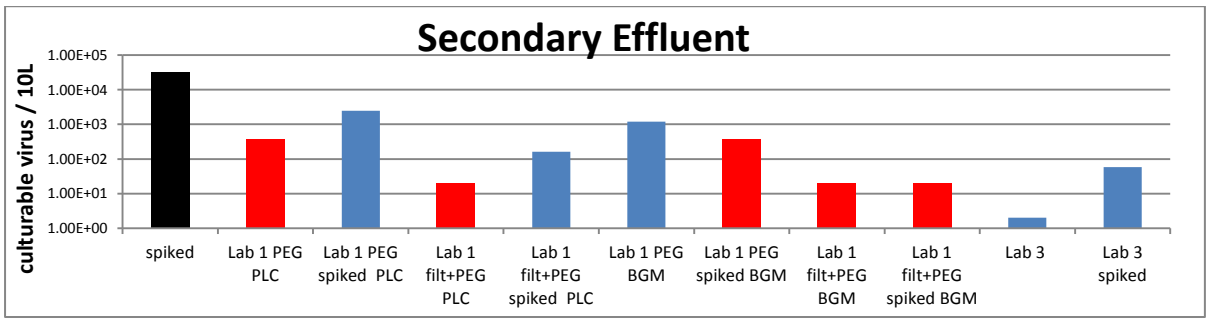


Figure 9. Comparison of the average numbers of enterovirus / 10L detected in spiked and un-spiked secondary effluent samples using cell culture. PLC indicates viruses detected by MPN culture on PLC/PRF/5 cells, BGM indicates viruses detected by plaque assay using BGM cells. The black column indicates the number of viruses / 10 L spiked into the sample, the red column indicates a below detection limit result.

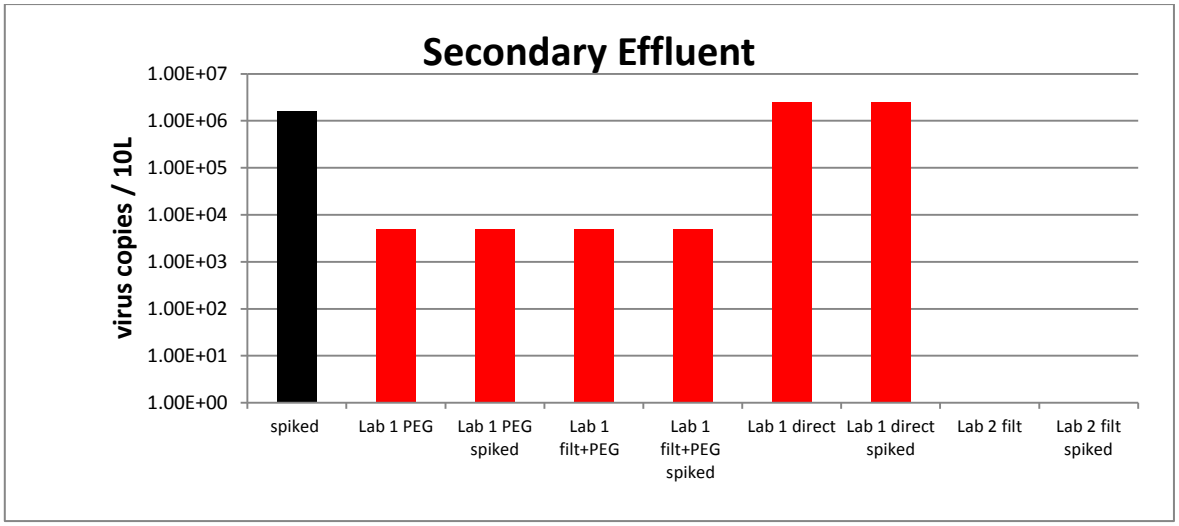


Figure 10. Comparison of the average numbers of enterovirus copies / 10L detected in spiked and un-spiked secondary effluent samples using quantitative reverse transcription PCR. The black column indicates the number of viruses / 10 L spiked into the sample, the red column indicates a below detection limit result.

The detection of culturable adenovirus in primary effluent was similar for the Laboratory 1 and Laboratory 3 methods, with the Laboratory 3 samples having more consistent virus counts (Figure 11). The number of adenovirus detected using A549 cells was an order of magnitude higher than the number of enterovirus detected, suggesting that the PLC cell culture assay was not detecting adenovirus in these primary effluent sample concentrates. The reason for the difference between the field sample results and the success of culture of the Ad 2 and Ad 41 laboratory strains of adenovirus in PLC cells is not clear. The direct detection of adenovirus without any sample concentration produced very similar results between the Laboratory 1 and Laboratory 2 analyses (Figure 12). The Laboratory 1 PEG concentrated sample gave a similar result to the direct PCR, suggesting marginal loss of virus and that direct PCR is a suitable method for adenovirus detection (being considerably simpler since there is no pre-concentration required). Filtration using molecular weight cut-off filters (Laboratory 2 filt) resulted in a loss of approximately 1 $\log_{10}$ of virus compared with direct DNA extraction and PCR.

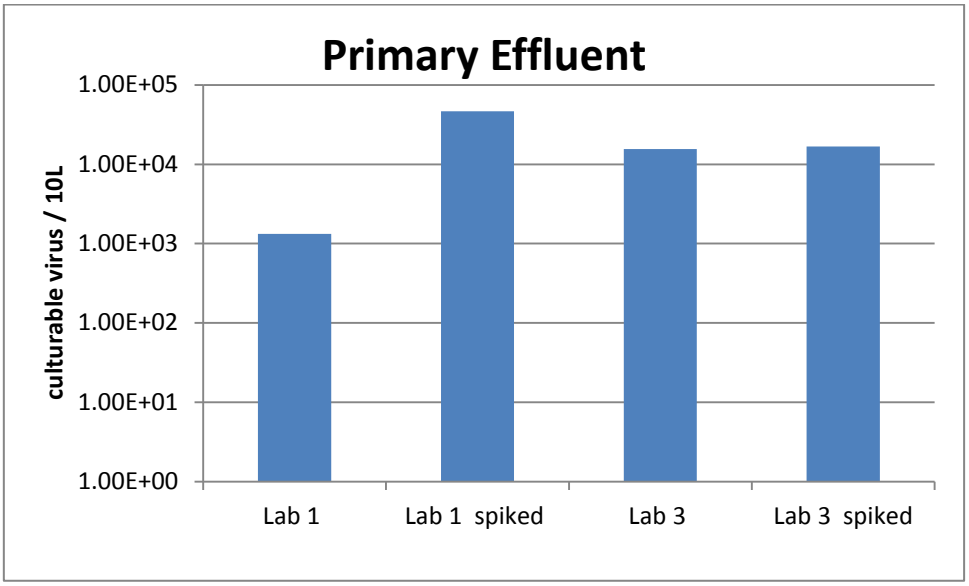


Figure 11. Comparison of the average numbers of adenovirus / 10L detected in spiked and un-spiked primary effluent samples using cell culture.

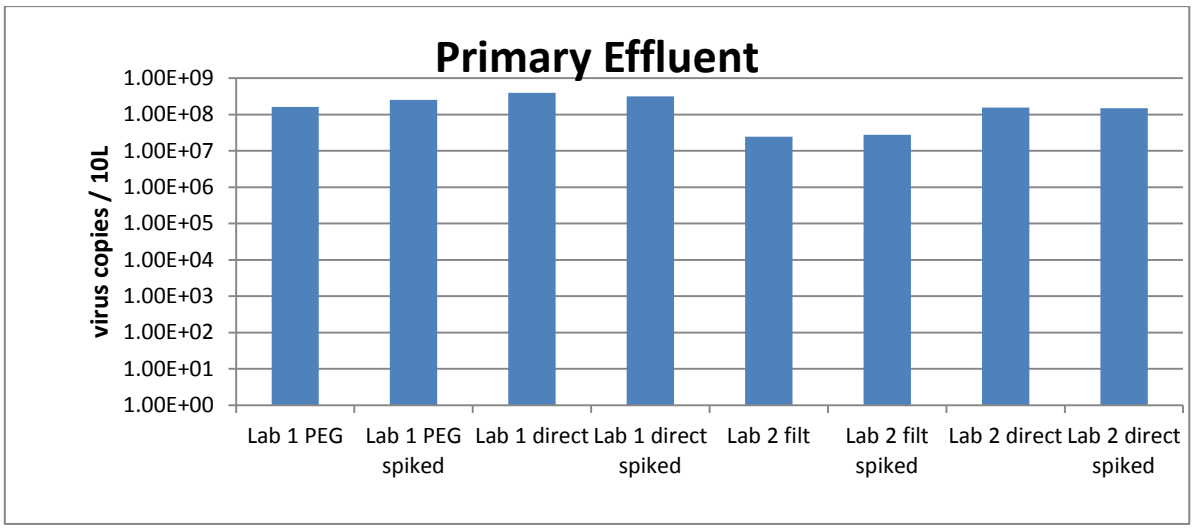


Figure 12. Comparison of the average numbers of adenovirus / 10L detected in spiked and un-spiked secondary effluent samples using quantitative real-time PCR.

The concentration / culture methods for adenovirus performed differently between Laboratory 1 and Laboratory 3 (Figure 13). The numbers of adenovirus detected by the Laboratory 3 method were lower compared with the Laboratory 1 sample results but consistent between the un-spiked and CB5 spiked samples, which is as expected considering the culture method should detect adenovirus but not enterovirus. The Laboratory 1 method, failed to detect adenovirus in the un-spiked samples. The detection of adenovirus in the Laboratory 1 spike sample was near detection limit and was only positive for one of the two samples. The detection of adenovirus was more consistent for the Laboratory 1 filtration + PEG sample. The results suggest that analysis of a greater proportion of the sample concentrate would improve the detection of adenovirus for the Laboratory 1 method. Given the low levels of adenovirus, it is possible that some of the variation between samples was due to uneven distribution of adenovirus in the secondary effluent samples. PCR analysis provided more robust results compared with the cell culture counts (Figure 14). The virus counts determined by PEG and filtration + PEG concentration (both by Laboratory 1) were comparable, although the un-spiked samples by PEG were below the assay detection limit.

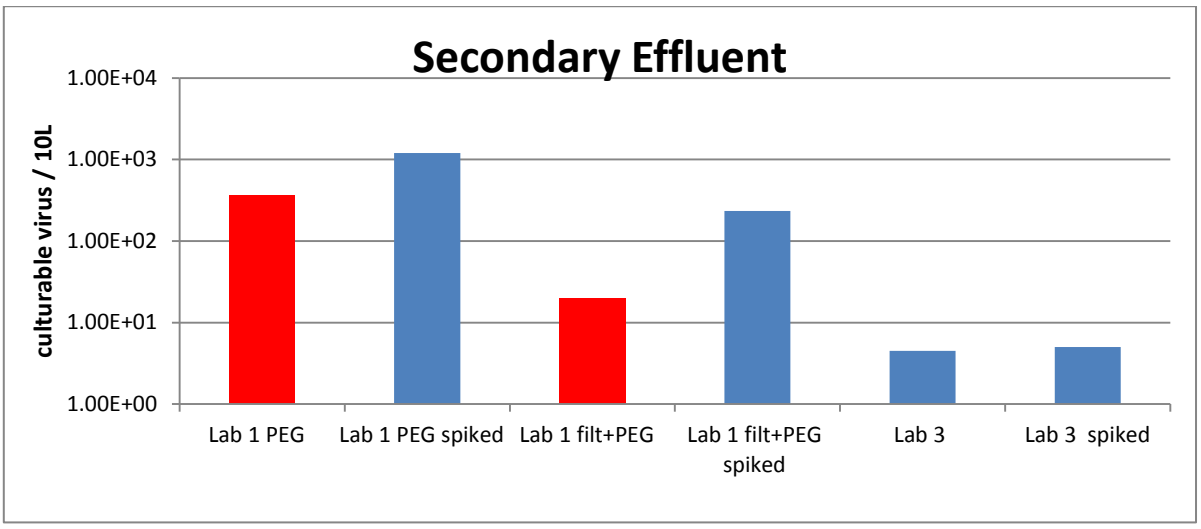


Figure 13. Comparison of the average numbers of adenovirus / 10L detected in spiked and un-spiked secondary effluent samples using cell culture. Red columns indicate results below the assay detection limit.

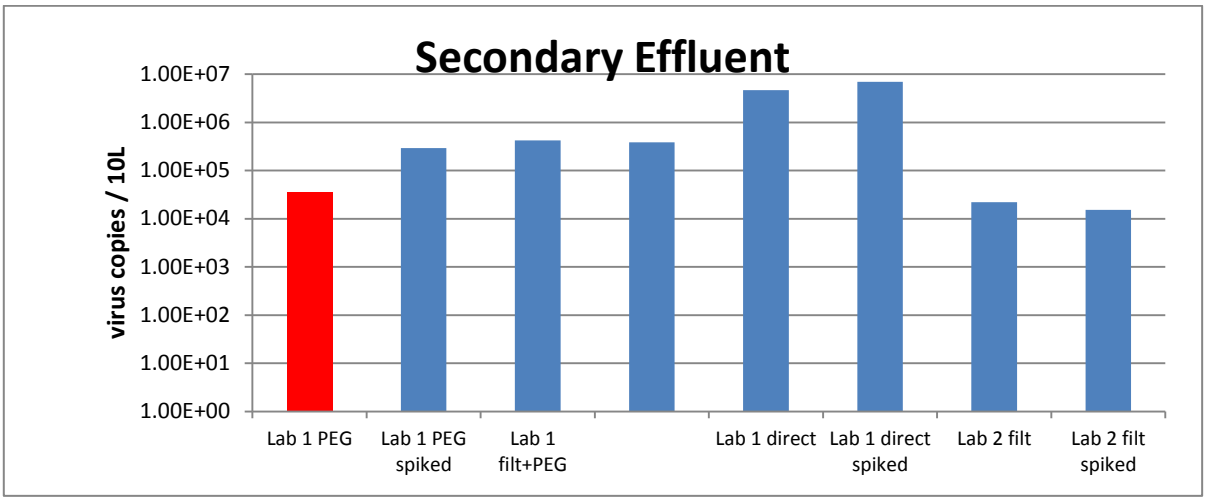


Figure 14. Comparison of the average numbers of adenovirus / 10L detected in spiked and un-spiked secondary effluent samples using quantitative real-time PCR. Red columns indicate results below the assay detection limit.

Direct detection in the secondary effluent was approximately 1 $\log_{10}$ higher compared with the Laboratory 1 concentrated samples. The Laboratory 2 filtration method detected approximately 1 $\log_{10}$ fewer adenovirus compared with the Laboratory 1 filtration method. Considering the similarity of the

PCR results between Laboratory 1 and Laboratory 2 for primary effluent, this likely reflects a poorer recovery efficiency for the Laboratory 2 filtration method, rather than any difference in the PCR assay.

Overall, there was relatively good agreement between the results from the different laboratories. The reason for the loss of the spiked coxsackie virus was not clear. Further trials would be required to verify the performance of the methods in different wastewaters, particularly to determine if the loss of the spike was due to site-specific factors in the Bolivar wastewaters.

Table 31. Cell culture enumeration of viruses using PLC/PRF/5 cells (MPN / 10 L) for CB5 spiked and un-spiked primary and secondary effluent samples.

	Laboratory 1 PEG	Laboratory 1 HF-80 + PEG
Primary spiked	6.40E+03	ND[1]
Primary spiked	3.72E+03	ND
Primary	2.40E+03	ND
Primary	4.40E+03	ND
Secondary spiked	9.36E+02	1.04E+02
Secondary spiked	3.96E+03	2.20E+02
Secondary	<3.6.00E+02	<2.00E+01
Secondary	<3.6.00E+02	<2.00E+01

Spike target is 1.6 e4 / 10L
[1]ND=not done

Table 32. Cell culture enumeration (PFU / 10 L) using BGM plaque assay (enterovirus) for CB5 spiked and un-spiked primary and secondary effluent samples.

	Laboratory 1 PEG	Laboratory 1 HF-80 + PEG
Primary spiked	5.33E+03	ND[2]
Primary spiked	6.67E+03	ND
Primary	<4.00E+02	ND
Primary	<4.00E+02	ND
Secondary spiked	<3.60E+02	<2.00E+01
Secondary spiked	<3.60E+02	<2.00E+01
Secondary	<3.60E+02	<2.00E+01
Secondary	1.20E+03[1]	<2.00E+01

Spike target is 1.6 e4 / 10L
[1]A single plaque was detected in 1 of 3 replicate wells
[2]ND=not done

Table 33. Cell culture enumeration (PFU / 10 L) using the standard A549 plaque assay (adenovirus) for CB5 spiked and un-spiked primary and secondary effluent samples.

	Laboratory 1 PEG	Laboratory 1 HF-80 + PEG
Primary spiked	5.33E+04	ND[1]
Primary spiked	4.00E+04	ND
Primary	<4.00E+02	ND
Primary	1.33E+03[2]	ND
Secondary spiked	1.20E+03[2]	<2.00E+01
Secondary spiked	<3.60E+02	<2.00E+01
Secondary	<3.60E+02	<2.00E+01
Secondary	<3.60E+02	<2.00E+01

[1]ND=not done
[2]A single plaque was detected in 1 of 3 replicate wells

Table 34. Cell culture enumeration (PFU / 10 L) using the A549 plaque assay (adenovirus) with an additional cell centrifugation step for CB5 spiked and un-spiked primary and secondary effluent samples.

	Laboratory 1 PEG	Laboratory 1 HF-80 + PEG
Primary spiked	5.33E+03	ND[1]
Primary spiked	1.33E+03	ND
Primary	6.67E+03	ND
Primary	4.00E+03	ND
Secondary spiked	<3.60E+02	2.00E+02
Secondary spiked	<3.60E+02	2.67E+02
Secondary	<3.60E+02	<2.00E+01
Secondary	<3.60E+02	<2.00E+01

[1]ND=not done

Table 35. Cell culture enumeration (virus / 10 L) for adenovirus and enterovirus from CB5 spiked and un-spiked primary and secondary effluent samples.

	Adenovirus	Enterovirus
Primary spiked	1.70E+04	1.25E+04
Primary spiked	1.65E+04	1.60E+04
Primary	1.50E+04	1.03E+04
Primary	1.60E+04	1.50E+04
Secondary spiked	<1.00E+00	3.60E+01
Secondary spiked	5.00E+00	8.10E+01
Secondary	5.00E+00	2.00E+00
Secondary	4.00E+00	2.00E+00

Table 36. PCR enumeration (copies / 10 L) for enterovirus.

	Laboratory 1 PEG	Laboratory 1 HF-80 + PEG	Laboratory 1 direct	Laboratory 2 filtered
Primary spiked	2.42E+06	ND[1]	<2.50E+06	3.83E+06
Primary spiked	3.44E+06	ND	<2.50E+06	3.67E+06
Primary	2.98E+05	ND	<2.50E+06	<[2]
Primary	5.17E+06	ND	<2.50E+06	<
Secondary spiked	<5.00E+03	<5.00E+03	<2.50E+06	<
Secondary spiked	<5.00E+03	<5.00E+03	<2.50E+06	<
Secondary	<5.00E+03	<5.00E+03	<2.50E+06	<
Secondary	<5.00E+03	<5.00E+03	<2.50E+06	<

[1]ND=not done
[2]<=below detection limit

Table 37. PCR enumeration (copies / 10 L) for adenovirus in wastewater samples spiked or un-spiked with CB5 Coxsackie virus.

	Laboratory 1 PEG	Laboratory 1 HF-80 + PEG	Laboratory 1 direct	Laboratory 2 filtered	Laboratory 2 direct
Primary spiked	2.92E+08	ND[1]	3.18E+08	3.11E+07	1.27E+08
Primary spiked	2.19E+08	ND	3.21E+08	2.46E+07	1.74E+08
Primary	3.73E+06	ND	3.72E+08	2.92E+07	1.60E+08
Primary	3.19E+08	ND	4.16E+08	2.02E+07	1.53E+08
Secondary spiked	2.12E+05	3.55E+05	<1.00E+06	1.45E+04	ND
Secondary spiked	3.72E+05	4.18E+05	6.92E+06	1.61E+04	ND
Secondary	<3.60E+04	3.46E+05	3.10E+06	2.22E+04	ND
Secondary	<3.60E+04	5.02E+05	6.27E+06	2.22E+04	ND

[1]ND=not done

4.3.5.2 *Cryptosporidium* results

The recovery rates for ColorSeed in primary effluent were similar between the Laboratory 1 and Laboratory 3 methods (Table 38). These recovery rates were within the range of the recovery rates for primary effluent in the initial recovery rate trials (19-35%, Figure 4). The recovery rates in secondary effluent were poorer for the Laboratory 3 method compared with the Laboratory 1 method. The Laboratory 1 recovery rates were also lower than in the previous three validation rounds that were conducted to compare Envirochek filtration, centrifugation and calcium carbonate precipitation. In those rounds, the calcium carbonate method recoveries were 35-47%. Interestingly, the Envirochek filtration recovery rate for those rounds was 7-15%. It is possible that there were generally poorer recovery rates due to a seasonal or other change in the water quality of the secondary effluent. The calcium carbonate method recovery was 15-20% worse compared to the previous 3 rounds of testing at Laboratory 1. If a similar performance decrease applied to the filtration methods, then this could account for the relatively low recovery for the Laboratory 3 samples.

The oocyst counts were similar between Laboratory 1 and Laboratory 3 for primary effluent (Table 39). The oocyst counts were an order of magnitude lower than in the previous 3 rounds of testing, which were conducted during autumn of 2015 and is expected considering that the prevalence of cryptosporidiosis in the community is highly seasonal with a summer/autumn peak and lowest incidence in winter. The counts for the Laboratory 1 samples were more variable compared with the Laboratory 3 samples, but this level of variability was consistent with that observed in the previous rounds of method validation testing. Additional inter-laboratory trials would be required to determine if there was a true difference in count variability between the two laboratories or if the variability is an inherent property of the heterogeneous oocyst distribution in water and wastewater samples. There was a large apparent difference between the Laboratory 1 and Laboratory 3 oocyst counts in secondary effluent. For the Laboratory 1 samples, oocyst numbers were below detection limit in the secondary effluent. The raw oocyst counts for the Laboratory 3 samples were between 0 and 4, but when the low recovery count was accounted for the resulting oocyst counts were high.

Table 38. Recovery rates for ColorSeed in Bolivar primary and secondary effluents.

	Laboratory 1	Laboratory 3
Primary 1	11.9%	22%
Primary 2	31.3%	27%
Primary 3	22.4%	13%
Average	**21.9%**	**21%**
Secondary 1	20%	4%
Secondary 2	20%	2%
Secondary 3	16%	1%
Average	**18.7%**	**2%**

Table 39. *Cryptosporidium* counts (per 10 L, corrected for recovery rate and % volume analysed) for Bolivar primary and secondary effluents.

	Laboratory 1	Laboratory 3
Primary 1	260.4	272.7
Primary 2	99.2	222.2
Primary 3	138.9	153.8
Average	**166.1**	**213.3**
Secondary 1	<5	100
Secondary 2	<5	50
Secondary 3	<6.3	<100
Average	**<5.4**[1]	**75**[2]

[1]Calculated using the average recovery rate
[2]Calculated excluding the below detection limit result

5 Summary and Conclusions

5.1 Pathogens and surrogates

Adenovirus presents benefits as an indicator due to its prevalence and the relative simplicity of the analytical method, especially PCR-based detection. However, its relatively large size (>60nm) means that it is unlikely to be considered a good indicator for processes that rely on physical exclusion such as filtration. Poliovirus has appropriate properties in terms of size and ability to measure both presence and infectivity. However, with the live vaccine no longer used it means that the vaccine strain of poliovirus can no longer be detected in wastewater and so can no longer be used as an indicator. Enteroviruses in general are in the correct size range but epidemiological studies have shown that their presence is strongly seasonal, mostly in summer and autumn. As a result, this group of viruses is at low levels or is not detected in wastewater samples during the other seasons, meaning that enteroviruses are not reliable for use as process indicators. Similarly, norovirus is mostly observed in winter and such a seasonal pattern prevents further use as an indicator. In the case of norovirus, there is no readily available infectivity assay, so it is only of use for validation of physical removal but not for validation of inactivation. Given the absence of a more suitable representative pathogen, adenovirus would be the best option as a process indicator using indigenous virus.

In terms of virus surrogates, somatic coliphages are a possibility but a more detailed literature search or additional basic research would need to be done to identify the best candidate species of somatic coliphage. The method for somatic phage is easier than for FRNA, in the case of FRNA the host bacteria must be in log phase otherwise they infection is poor and enumeration is difficult or not possible. The hosts for somatic phage are easier to prepare and so the assay is less likely to fail, especially in the hands of a novice user. However, some somatic phage are very large. For example, T4 is a somatic phage, the head is about 100nm, tail an extra 300 nm, so they are potentially poor surrogates for filtration validation. Reoviruses, which are mammalian viruses, could be potential candidates as surrogates for human enteric viruses, but further research would be required to evaluate this. F-RNA phage appear to meet all the necessary requirements in terms of size, prevalence all year round and ability to measure presence and infectivity with reliable methods.

The selection of a bacterial surrogate / representative pathogen is relatively straightforward, with *E. coli* fulfilling the criteria required for a surrogate (cost, presence, ease of detection, behaviour). One consideration for using *E. coli* or more broadly using coliforms is the nature of the matrix and environmental conditions, bearing in mind that under favourable conditions of temperature and nutrients these faecal organisms can propagate in the environment, which would confound any validation study.

There is no suitable surrogate for enteric protozoans. Spores of sulphite reducing clostridia could be used as a conservative indicator, but if the pathogen numbers are high enough it would be better to used *Cryptosporidium* oocysts to directly measure process performance. Recent advances in methods can now allow enumeration of total and infectious oocysts, making it possible to measure the effects of treatment processes on *Cryptosporidium* infectivity.

Based on the literature review the recommended surrogates / pathogens for the different sub-projects have been listed in Table 40.

Table 40. List of surrogates recommended for use in the different subprojects within NatVal 2.2.

Project	SP1	SP2	SP3	SP5
Total coliforms	?			
E coli	♦		♦	
Enterococci	?			
SRC	♦			
MS2 / F-RNA phage	?	♦		
Adenovirus	♦		♦	♦
Cryptosporidium	♦		♦	♦

5.2 Virus methods

The comparison of the different cell lines for supporting virus infection was largely consistent with the findings of Keegan et al., (2012). During this work, the laboratory did not have a plate centrifuge available to trial the effect of centrifugation on virus recovery. The addition of Ca^{2+} as a supplement to aid infectivity had the opposite effect, causing a dose-dependent reduction in infectivity, with the impact larger for adenovirus compared with Coxsackie virus. Virus recoveries were inconsistent for both of the viruses used and for the different concentration techniques (direct PEG or filtration + PEG). The adenovirus results in particular may have been impacted by the presence of indigenous viruses (especially adenoviruses) and appeared to be worse for infectivity compared with PCR. The detection of indigenous viruses and apparent loss of the spiked Ad 2 could indicate a specific process impact affecting the Ad 2 but not the indigenous viruses. The direct analysis of spiked samples (no concentration) suggests that the Ad 2 were not impacted by the samples matrix and that a step in the processing was responsible for the loss of the spiked virus (or loss of infectivity). A further consideration for interpreting the cell culture results is that the PLC cell line was used for all MPN analyses, this cell line supports a wide range of viruses and so detection in the un-spiked samples could be due to the presence of adenovirus or enterovirus. To differentiate this post-cell culture PCR analysis would be required to identify the virus causing the detected infection. The PCR analysis of the un-spiked sample concentrates suggested that indigenous adenovirus were more frequently detected, with indigenous enteroviruses detected by RT-PCR in only 1 batch of samples processed. Overall the direct PEG method gave better recoveries for both Ad 2 and CB5, although the recovery rates were not as good as that reported by Keegan et al., (2012) for samples collected from the same locations. The recovery rates for Ad 2 in primary effluent were better (although still variable) compared with secondary effluent, which is counter to what would normally be expected (based on previous results from Keegan et al., (2012) and earlier AWQC monitoring). It is possible that factors affecting the secondary effluent quality have greatly affected some of the testing conducted in this project. Future work should focus on evaluating the concentration methods using a wider range of primary and secondary effluents. The use of a surrogate such as suitably modified nanoparticles would greatly assist with method development, allowing the use of simpler enumeration methods and also eliminating any interferences from indigenous viruses, which can complicate enumeration.

5.3 Cryptosporidium methods

The comparison of methods for processing primary effluent found similar recovery rates for all 3 methods for the first 2 rounds of testing, though in the third round both direct centrifugation and filtration of diluted primary effluent appeared to perform significantly worse. The cause for this variation is unclear, but it is possible that this could coincide with a bad batch of IMS beads (that were recalled by the manufacturer after this work was completed). For secondary effluent, the calcium carbonate flocculation method was consistently better than Envirochek filtration. Although the recoveries were lower, the oocyst counts (which incorporate recovery rate and % sample processed)

were generally higher for direct filtration and filtration compared with flocculation. The infectivity of the oocysts recovered by the different methods appeared to vary. In the case of primary effluent, the infectivity of oocysts recovered also appeared to be higher in the oocysts recovered by direct centrifugation and filtration, although in some cases the variation between replicates was large for some of the samples because of the small number of oocysts applied to cell culture. Of interest, the infectivity of the oocysts in the primary and secondary effluent was different to that observed for Glenelg WWTP, with higher infectivity observed in the Bolivar primary effluent and lower infectivity observed in the Bolivar secondary effluent. Given the relative simplicity of direct centrifugation, the method performance is adequate for oocyst concentration, but the inclusion of a recovery control is essential to identify any changes in recovery performance.

5.4 Inter-laboratory comparison

Due to time constraints, it was only possible to conduct a single round of inter-laboratory comparison for virus and *Cryptosporidium* methods. The results for the primary effluent samples for both viruses and *Cryptosporidium* were comparable across the different laboratories, particularly for adenovirus detection by PCR. There was greater variation between the results from the different laboratories for secondary effluent, most likely due to differences in assay detection limit, low levels of virus present and also potentially due to differences in recovery rate. The coxsackie CB5 spiked into some of the samples did not appear to persist and there also appeared to be enterovirus within the un-spiked samples at comparable levels to the CB5 used as a spike. These factors made calculation of a recovery rate difficult and it is not clear if the obtained result was due to poor recovery of the spiked virus or due to other factors affecting the stability or culturability of the spiked CB5.

For primary effluent, the number of culturable viruses versus virus genomes detected by PCR was 2 log lower for enterovirus and up to 4 log lower for adenovirus. A similar difference was observed for adenovirus but not for enterovirus in secondary effluent. In the case of enterovirus, based on the culture results it would be predicted that the number of enterovirus genomes would be $1 \times 10^4 – 1 \times 10^5$ virus copies / 10 L. However, enterovirus were below detection limit ($<5 \times 10^3$ / 10 L), which is lower than anticipated. The detection of indigenous adenovirus in the primary and secondary effluent samples used for the inter-laboratory comparison was similar to the results obtained during the initial method evaluation. In the earlier trials, PCR detected $10^4 – 10^5$ adenovirus copies / 10 L and $<10^2 – 10^3$ culturable adenovirus / 10 L in un-spiked secondary effluent samples and $10^5 – 10^6$ adenovirus copies / 10 L and $10^2 – 10^4$ culturable adenovirus / 10 L in un-spiked primary effluent. Culturable enterovirus were not detected the secondary effluent samples used for the initial method evaluation, although enterovirus were detected by RT-PCR (10^3-10^5 copies / 10 L), unlike in the case of the samples used for the inter-laboratory trial.

The PCR method for adenovirus appears to be suitable for directly measuring virus numbers without the need for sample concentration, provided the virus numbers are above 1×10^4 / 10 L. Compared with the direct detection of viruses, sample concentration appeared to result in the loss of 1 – 2 $\log_{10}$ of virus. Direct RT-PCR detection of enterovirus was not successful and was only successful for primary effluent concentrates.

Based on the limited data available, direct PCR detection of adenoviruses (without any sample concentration) is recommended as a useful and cost effective option for measuring physical removal in both primary and secondary effluents. If sample concentration is required then PEG precipitation of primary effluent samples allowed detection of both enterovirus and adenovirus for cell culture and (RT-)PCR. The results for secondary effluent concentration were equivocal for PEG versus ultrafiltration + PEG, although both methods appeared to provide better performance than ultrafiltration + molecular weight cut-off filters. A key consideration for future method improvement is improvement of assay sensitivity, which at the moment can only be achieved by analysing a larger proportion of the sample concentrate, which adds to the assay cost.

5.5 Recommendations

The literature review was used to select / recommend surrogates or pathogens for use in treatment validation studies in other sub-projects of NatVal 2. An obvious next step is to ground truth those selections by conducting some comparative trials of the relevant pathogen-surrogate pairs. Such data may be available from other sub-projects within the NatVal2 project and should be able to inform the design of any future surrogate validation experiments.

The virus method development work that was conducted in this project suggested that some cell lines were better for detection of enterovirus and adenovirus compared with others in current use. However, not all of the cell lines behaved as anticipated with real wastewater sample concentrates. Future work should compare the performance of the different cell lines for virus detection using real wastewater samples. Many of these cell lines can host multiple virus species, so any such study would need to incorporate PCR to allow for detection of specific viruses such as enterovirus or adenovirus. A cell culture / MPN assay using PCR as the virus detection endpoint is a potential assay format for such a study to more rigorously field test the cell lines used for virus culture.

The *Cryptosporidium* method comparison suggested that the calcium carbonate method combined with IMS gave the highest recovery rates for secondary effluent, and this was confirmed in the inter-laboratory comparison. This method also performed consistently well for primary effluent samples. It appeared that the infectivity of the oocysts recovered by the calcium carbonate method was lower than that of oocysts recovered by other methods. The reasons for this need to be investigated in future work. One possibility is that the calcium carbonate method has a better recovery and recovers both live and dead oocysts, whereas the other methods might have poorer recoveries because they selectively recovery live oocysts, resulting in a higher infectious fraction in those concentrates. Spiking trials using fresh oocysts in primary and secondary effluent would address this question.

The inter-laboratory comparison of methods yielded very promising results. Future work needs to be done using the same and different wastewater locations to determine how reproducible and robust the methods are. Prior to any further studies the baseline numbers of virus need to be determined for each matrix so that appropriate spike levels can be used to allow determination of recovery rates. In addition, virus stability needs to be determined for each matrix. Stability experiments with adenovirus suggested that Ad 2 was stable when spiked into Bolivar secondary effluent, yet these spiked viruses could not be efficiently recovered using the PEG of filtration concentration methods. The possibility of matrix interference needs to be investigated – is there something in the Bolivar wastewater that is particularly challenging for virus recovery or is this a general issue for wastewater?

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Appendix I: Candidate pathogens and indicators for Wastewater

CLASS: VIRUSES

Organism: Adenovirus
Type: Pathogen

Level of importance to the industry: Human adenoviruses are ubiquitous pathogens which cause a variety of diseases ranging from respiratory illness and keratoconjunctivitis to gastroenteritis. Fifty-one known human adenovirus serotypes are organized into six subgroups (A–F). Adenovirus serotypes 40 (Ad40) and 41 (Ad41) are the second most common etiological agents of gastroenteritis in children after rotaviruses (Cruz et al. 1990).

Adenoviruses are rated as highly important on the basis of their resistance to low and (to a lesser extent medium) pressure UV and their use by health regulators as one of the criteria in the assessment of the safety of re-use water. They are also on the US EPA drinking water contaminant candidate list (http://www.epa.gov/safewater/ccl/). Human adenovirus are reported to be more thermostable (Enriquez et al. 1995) and UV stable than other enteric viruses (Falabi et al. 2002). Doses in excess of 225 mJ/cm^2 are necessary for complete inactivation of the gastrointestinal adenoviruses types 40/41 (Donnellan et al. 1999, Moriyama et al. 2005), although medium pressure UV is more effective (approximately double the kill for the equivalent dose, (Linden et al. 2005). The increased UV resistance might be due to DNA repair by host cell mechanisms. They can survive in the environment for a prolonged period (Falabi et al. 2002) and consequently, their presence and survival in biosolids is a cause of major concern.

Geographical distribution: Believed to be widely distributed (Tanner et al. 2004), reports on its presence in wastewater are from USA (Falabi et al. 2002), Greece (Krikelis et al. 1985) Europe (Adrian et al. 1990) and Australia (Irving and Smith 1981). However, no single study has examined the broader geographic distribution of adenoviruses in wastewater or biosolids. Cases of infection in the general population are reported throughout the world, including South Africa, Australia and New Zealand.

Representative of other pathogens in the same class: Adenoviruses were isolated less frequently in wastewater influent compared with enterovirus and reovirus in a 9 year study (Sedmak et al. 2005). No study found on direct comparison of the behaviour of adenovirus versus other viruses during wastewater treatment or in biosolids. Based on culture dependent techniques, reduction of adenovirus (85%) by activated sludge treatment is lower compared to enteric viruses (93%) (Irving and Smith 1981, Long and Ashbolt 1994). On the basis of the high UV resistance of adenovirus (Thompson et al. 2003) it is not likely to be representative of other viruses e.g. relatively more resistant than enteroviruses and considerably more so than polioviruses and feline caliciviruses (Tree et al. 2005). It is also more thermostable compared to polio virus and HAV in tap water and sea water, although only marginally more stable in waste water (Enriquez et al. 1995). A risk assessment study of biosolids suggested that adenoviruses were amongst the most thermally resistant viruses (Falabi et al. 2002).

Presence in multiple matrices: Yes. Since they are present in most moderate-sized populations and excreted in stools, they can be expected to be present in wastewater, sludge and biosolids. There is limited literature on the detection of Adenoviruses in biosolids and sludge (Rao et al. 1986).

Pathogen numbers
In waste influent: 10^3 - $10^{5.5}$ cytopathic units / 100 L (Krikelis et al. 1985)
$10^{4.8}$ / 100 L cytopathic units (He and Jiang 2005)
$<10^2$ – 10^5 / 100 L culturable (pers. comm. M Angles, 2005; (Sedmak et al. 2005))
10^5 – 10^8 / 100 L PCR (Bofill-Mas et al. 2000)
10^8 / 100 L PCR (He and Jiang 2005)

In waste effluent: densities not known, but detected in disinfected tertiary effluent (Long and Ashbolt 1994).

Biosolids: Numbers unknown in biosolids.

Analytical method:
Type: Wastewater and biosolids - several detection and quantification approaches are available: (i) cell culture measuring Cytopathic Effect (CPE) as the infection endpoint, (ii) direct nucleic acid amplification (NAA); (iii) integrated cell culture with NAA. Cell culture with NAA targeting viral mRNA or replication intermediates will only detect the results of virus infection (Cromeans and Sobsey 2004, Moriyama et al. 2005). It has been suggested that CPE will underestimate viral numbers because it does not allow detection of all infections in cell culture (Cromeans and Sobsey 2004). Direct detection of viruses by NAA generally results in higher numbers than by cell culture (Choi and Jiang 2005, He and Jiang 2005), but since most comparisons used CPE for measuring infection it is difficult to compare the results considering the findings of (Cromeans and Sobsey 2004). Cell culture with NAA is likely to be the most appropriate method but the cost is higher to establish and maintain a cell culture facility. Direct NAA detection may be useful for measuring the UV treatment of water because it has been reported that the detection of large amplicons (2kb) correlated with infectivity after UV treatment (Wang et al. 2004).

Infectivity measurement: Wastewater and biosolids - possible for cell culture based assays, not for direct NAA.

Method performance:
Wastewater: Concentration of large volumes of raw wastewater may be problematic, but not necessary given the ubiquitous occurrence in municipal raw wastewater. Detection in treated effluent will depend on the level and type of treatment. No specific problem with tertiary treated effluent or clarified water would be anticipated.
Biosolids: Detection with NAA techniques might be problematic due to the presence of enzyme inhibitors and the potentially low numbers of virus particles in some biosolid samples. Techniques such as immunomagnetic capture coupled with RT-PCR are expected to improve the detection of viruses in these matrices (Casas and Sunen 2002).

Cost: High for cell culture and moderate for PCR.

Ability to multiply in matrix: No, requires host for multiplication.

Knowledge on pathogen: Very little is known on numbers and behaviour in wastewater and biosolids; mostly qualitative data on wastewater and environmental contamination.

Points in favour: Cell culture techniques are well established. Viral numbers in wastewater should be sufficient to allow ready detection in influent.
Points against: Not representative of other enteric viruses (particularly size and UV response). In addition there is no information regarding viral numbers in effluent or biosolids.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified.

Organism: Polyomavirus

Type: Pathogen / Viral Indicator

Level of importance to the industry: None at present. Infection is thought to occur in early childhood and is usually asymptomatic (Bofill-Mas et al. 2000). The virus can reactivate and cause disease in AIDS patients (Bofill-Mas et al. 2000) and renal transplant recipients (Nickeleit et al. 2000).

Geographical distribution: Widely distributed – there have been several studies characterizing genotypes in different countries and the JC genotype has been used to trace the global migration of humans (Sugimoto et al. 1997).

Representative of other pathogens in the same class: Not known – it is a non-enveloped dsDNA virus like Adenovirus but from a different Family. It is excreted in urine and possibly via the lungs (Eash et al. 2004). The route of infection is not well characterized – thought to be oral. No specific data available for disinfection or removal by treatment processes, except for UV. SV40 (type species for polyomavirus) has similar UV sensitivity to porcine parvovirus (Wang et al. 2004). In general, virus UV sensitivity appears to correlate with genome size irrespective of nucleic acid composition (Wang et al. 2004), so polyoma may represent viruses with genomes in the 5 kb size range.

Presence in multiple matrices: Prevalence in biosolids not known, but expected.

Pathogen numbers
In waste influent: 10^6 – 10^8 / 100 L direct PCR (Bofill-Mas et al. 2000)

In waste effluent: not known

Analytical method:
Type: Published methods for detection have been direct NAA (clinical and water) or serology (clinical). Culture has been used for propagation but is reported to be very slow in the case of bovine polyomavirus (Nairn et al. 2003).
Method performance: For direct NAA would assume that method performance would be good for effluents but that raw sewage might present difficulties with concentration / purification of virus from amplification inhibitors
Cost: moderate for NAA

Ability to multiply in matrix: No – requires a host.

Knowledge on pathogen: Little information for wastewater or biosolids; most publications in the medical area.

Correlation with pathogen presence/absence: Not known

Ease of extraction from matrix: Not known

Allows source tracking: Yes – assume that JC and BK strains are human specific.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, not listed within any risk groups so presumably could also be handled in a Class I facility.

Organism: Hepatovirus (Hepatitis A (HAV))

Type: Pathogen

Level of importance to the industry: Assume moderate importance since health regulators use Hepatitis A as one of the viruses for assessing the quality of re-use water.

Geographical distribution: Widely distributed. There are no studies that examine the geographic distribution of viruses in wastewater as such but there are numerous studies of the prevalence of HAV in various regions including North America, South America, Asia, Australia and Africa.

Representative of other pathogens in the same class: Yes. Literature is limited in terms of any comparative studies on the behaviour of HAV versus other viruses with respect to wastewater treatment. A pilot-scale activated sludge plant study suggested that HAV has similar removal rates to polio and reoviruses (Arraj et al. 2005).

Presence in multiple matrices: Yes – would expect to detect it in any matrix with human faecal contamination. Not much literature on the detection of HAV in biosolids or sludges. Most wastewater literature associated with infection risk in wastewater workers.

Pathogen numbers
In waste influent: not known

In waste effluent: not known

Analytical method:
Type: Cell culture has only been successfully used on a limited range of isolates of HAV (see 99-HHE-5UR). PCR-based detection is normally used.
Infectivity measurement: No
Method performance: For direct PCR would assume that method performance would be good for effluents but that raw sewage might present difficulties with concentration / purification of virus from PCR inhibitors
Cost: moderate

Ability to multiply in matrix: No – requires host.

Knowledge on pathogen: Little information for wastewater or biosolids, most publications related to environmental contamination.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified.

Organism: Enteroviruses (poliovirus, coxsackievirus, echovirus and enterovirus)

Type: Pathogen / Viral Indicator

Level of importance to the industry: Enteroviruses are used alongside adenovirus and HAV by health regulators as a measure of the safety of re-use water.

Geographical distribution: Widely distributed but seasonal. The vaccine strain of poliovirus is no longer prevalent in developed countries (due to switch to killed vaccine), but it may still be in use in developing countries due to lower cost. Wild polio occasionally isolated from human populations (Mulders et al. 1995). Other enteroviruses also have wide distribution – e.g. echovirus (Oberste et al. 2003) and coxsackievirus (Mena et al. 2003)

Representative of other pathogens in the same class: Likely. Sydney Water data (pers. comm. M Angles, 2005)and 2 studies (Lodder and de Roda Husman 2005, Sedmak et al. 2005) suggest similar levels of adenovirus, reovirus and enterovirus in wastewater influent. (Lodder and de Roda Husman 2005) observed similar removal in an activated sludge plant between enteroviruses and reoviruses. (Thompson et al. 2003) suggest that poliovirus has similar UV response to calicivirus and HAV. AwwaRF Report 90886 (Jacangelo et al. 2002) summary data suggests that poliovirus is similarly or more resistant to ozone, chlorine, chloramine and chlorine dioxide than adenovirus and calicivirus.

Presence in multiple matrices: Yes – detected in activated sludge (Pusch et al. 2005).

Pathogen numbers
In waste influent: $<10^0 – 10^4$ / 100 L culturable (pers. comm.. M Angles, 2005),
up to 10^6 / 100 L (Sedmak et al. 2005),
$10^3 – 10^4$ / 100L (Rose et al. 2004)

In waste effluent: Depending on treatment: $10^{-3} – 10^2$ / 100L (Rose et al. 2004)

Analytical method:
Type: Cell culture using plaque assay for poliovirus, can also use NAA-based detection with/without cell culture. CPE used for general enterovirus detection.
Method performance: Concentration of large volumes of raw sewage would be problematic. Detection in effluent will depend on level of treatment. Would anticipate no problem with tertiary treated effluent or clarified water.
Cost: moderate for NAA, high for cell culture

Ability to multiply in matrix: No – requires a host.

Correlation with pathogen presence/absence: not known how poliovirus will correlate with the presence of other enteric viruses

Ease of extraction from matrix: results from (Rose et al. 2004) suggest that enteroviruses might have some interaction with particulates since removal efficiency by filtration is higher compared with bacteriophage. Raises potential issues with isolation from biosolids.

Allows source tracking: No publications on the use of poliovirus for source tracking although presume detection of vaccine strain indicates human faecal input.

Knowledge on pathogen: Overall there are a reasonable number of publications on enteroviruses in wastewater, but most are on detection with relatively few on treatment processes or comparisons with other viruses or indicators.

Points in favour: established PCR and cell culture assays, used as a model organism for chlorine disinfection.

Points against: seasonality means that enteroviruses may not always be detected in wastewater.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified except for polio – vaccination recommended in this case.

Organism: Human Caliciviruses – norovirus, sapovirus; Hepatitis E (HEV)
Note: HEV was grouped with calicivirus but was moved to the family *Hepeviridae,* Since its physicochemical properties suggest that it might behave like a calicivirus (Smith et al. 1998) and there is limited information on HEV it has been grouped with the caliciviruses.

Type: Pathogen

Level of importance to the industry: Moderate to high. Human caliciviruses are the major cause of gastroenteritis in the United States and Europe (Lopman et al. 2002, Monroe et al. 2000). Human caliciviruses include two groups, noroviruses (formerly Norwalk-like viruses) and sapoviruses (formerly Sapporo-like viruses). Norovirus are the most common cause of acute gastroenteritis and has worldwide significance (Brown et al. 2001, Lodder et al. 1999). Immunity to Norovirus is not long lasting as is the case for rotavirus or other viruses. While norovirus is an important cause of waterborne enteric disease, it is readily inactivated by standard disinfectants. Little information is available regarding sapovirus. HEV causes a similar disease to HAV (USFDA, 2003). There is conflicting information on the risk of HEV infection to treatment plant workers via exposure to wastewater (Jeggli et al. 2004, Vaidya et al. 2003).

Geographical distribution: Widely distributed, numerous reports on worldwide outbreaks (Bon et al. 2005). Epidemiological surveillance data from US indicates that Norovirus account for 83-96% of non-bacterial gastroenteritis outbreaks (Brown et al. 2001). Similar data have been reported from France and Europe (Bon et al. 2005, Lopman et al. 2002). HEV is more associated with faecally contaminated water in parts of Asia, Africa, the Middle East and Central America (Aggarwal and Krawczynski 2000). Detected in wastewater at moderate to low frequency in Spain, France and the USA, but not as prevalent as HAV (Clemente-Casares et al. 2003).

Representative of other pathogens in the same class: Insufficient information is available to determine if caliciviruses are representative of other viruses, especially since noroviruses and sapoviruses cannot be cultured and so the effect of treatment processes cannot validly be compared with other viruses detected using cell culture techniques. Removal rates for norovirus (measured by PCR) through an activated sludge plant were similar to other viruses (enterovirus, reovirus) measured by cell culture, with both between 1 – 2 log (Lodder and de Roda Husman 2005, van den Berg et al. 2005). Similarly, 2 log reduction, in norovirus numbers during anaerobic digestion was reported by (Laverick et al. 2004). However, there is still insufficient data to definitively conclude that norovirus behaviour in wastewater and biosolids is similar to other enteric viruses. HEV was present in both the influent and effluent of 3 WWTP's in India but the detection rate in effluent was lower than enterovirus (Jothikumar and Cliver 1997).

Presence in multiple matrices: Yes, present in general population and is expected to be present in any matrix contaminated with human feces. Detected in wastewater and sludge.

Pathogen numbers
In waste influent:
$10^{4.5}$ to 10^{9} /100 L genome copies (PCR) (Laverick et al. 2004, Lodder and de Roda Husman 2005, Lodder et al. 1999, van den Berg et al. 2005).
Not known for HEV

In waste effluent:
$10^{3.6}$ – 10^{7} / 100 L genome copies (PCR) (Laverick et al. 2004, Lodder and de Roda Husman 2005, van den Berg et al. 2005).
Not known for HEV

Biosolids: Numbers unknown in biosolids, but expected to be detected.

Analytical method:
Type: Immunomagnetic capture has been reported in combination with RT-PCR (Gilpatrick et al. 2000). Molecular methods are available for the detection of norovirus, sapovirus or HEV (Jothikumar and Cliver 1997, Lodder and de Roda Husman 2005). Recently, cell culture of murine norovirus has been reported (Wobus et al. 2004), however this system used primary cultures of mouse dendritic cells which are not practical to obtain and which may not support human norovirus. Only molecular methods appear to have been used for detection in environmental samples. Cell culture can be used

for other animal caliciviruses, which have been used to model norovirus for disinfection (e.g. (De Roda Husman et al. 2004).

Infectivity measurement: At present not possible for human viruses.

Method performance:
Wastewater: Concentration of large volumes of raw wastewater could be problematic. Detection in effluent will depend on the level of treatment. Would anticipate no problem with tertiary treated effluent or clarified water.
Biosolids: Detection by NAA could be problematic if inhibitors cannot be removed efficiently. Immunomagnetic capture coupled with RT-PCR is expected to improve the detection limit and reduce PCR inhibitors (Gilpatrick et al. 2000), but the commercial availability of reagents is not known.

Cost: Moderate for PCR, high for cell culture

Ability to multiply in matrix: No, requires host for multiplication.

Knowledge on pathogen: Very little is known regarding numbers and behaviour in biosolids, most of the published literature is on environmental contamination in water or outbreaks of disease.

Points in favour: Norovirus is a cause of major concern because it is implicated in a large number of waterborne outbreaks. It is expected to be present in wastewater and biosolids in reasonable numbers to be detected with confidence.

Points against: At present it is not possible to determine infectivity. However, due to the large number of reported outbreaks there is a case to develop more knowledge on its behaviour in wastewater and biosolids.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified.

Organism: Reoviridae (rotavirus, reovirus)

Type: Pathogen / Viral Indicator

Level of importance to the industry: Moderate. Rotavirus is an important food and waterborne agent of acute gastroenteritis worldwide, particularly in young children (Kirkwood et al. 2004), where it causes 600,000 to 800,000 infant deaths each year, predominantly in developing countries (Gentsch et al. 1996). Since infection is self limiting, the level of importance of rotavirus in adults in not clear. Reoviruses, which are included in this family, are highly prevalent but are not considered to be important as pathogens and are used as an indicator for rotavirus. There are three serotypes of reovirus, and all serotypes are found ubiquitously in water and sewage (Norman and Lee 2000). Generally detected in both raw and treated biosolids (Lodder and de Roda Husman 2005) and aerosols from wastewater treatment plants (Carducci et al. 2000). The introduction of the rotavirus vaccine will likely see a decline of rotavirus in sewage.

Geographical distribution: Widely distributed. It would be expected to find these viruses in any matrix with human faecal contamination.

Representative of other pathogens in the same class: The presence of reoviruses in wastewater is well documented, while virus numbers and behaviour in biosolids is relatively unknown. The comparison of reovirus with non-culturable viruses (eg rotavirus, norovirus) is difficult since the assay endpoints are different. Limited data suggest that reovirus and enterovirus numbers are similar in raw wastewater (Sydney Water data, pers. comm. M. Angles, 2005) and that reovirus removal by activated sludge treatment was similar to that of enteroviruses (Lodder and de Roda Husman 2005). A single report suggests that the behaviour of simian rotavirus is different from polio and HAV in activated sludge treatment as it was less strongly adsorbed to the solid phase of the sludge (Arraj et al. 2005).

Presence in multiple matrices: Yes, present in the general population and is expected to be present in any environment contaminated with human feces. However, as noted above, the introduction of a rotavirus vaccine will likely alter presence in wastewaters

Pathogen numbers
In waste influent:
10^3 to 10^7 /100 L rotavirus (NAA) (Lodder and de Roda Husman 2005)
$10^{3.6}$ – 10^5 / 100 L reovirus (culture) (Lodder and de Roda Husman 2005) M. Angles, pers. comm., 2005.
In waste effluent:
$10^{1.6}$ to 10^7 /100 L rotavirus (NAA) (Lodder and de Roda Husman 2005).
10^3 – 10^4 / 100 L reovirus (culture) (Lodder and de Roda Husman 2005)
Biosolids: Numbers unknown in biosolids, but expected to survive in the biosolids in numbers similar to effluent.

Analytical method:
Type: Cell culture measured by CPE is routine for the detection of reoviruses. Rotaviruses are more difficult to detect this way because some strains don't infect the currently used cell lines or do not produce CPE (Schwartzbrod et al. 1989). Molecular methods (eg (Lodder and de Roda Husman 2005)) are available and immunoassays have also been used for detection (eg (Kittigul et al. 2000))
Infectivity measurement: Only possible for strains which can be cultured, not for direct NAA
Method performance: Same considerations as for other viruses
Cost: Moderate for NAA, high for cell culture or cell culture and NAA combined.

Ability to multiply in matrix: No, requires host for multiplication.

Allows source tracking: No - reovirus is not human specific.

Knowledge on pathogen: Very little is known regarding numbers and behaviour in wastewater and biosolids; most of the published literature is on environmental contamination and outbreaks.

Points in favour: Expected to be present in wastewater and biosolids in reasonable numbers. Reovirus culture assays are well established. Relatively easy to culture and has been proposed as an indicator which can be spiked in biosolids to assess sanitation (Brewster et al., 2005).

Points against: Cell culture methods are present but cytopathic effect varies from one cell line to other. Not all strains can be cultured. (Lodder and de Roda Husman 2005) reported that rotavirus was less efficiently removed by activated sludge treatment compared with norovirus, enterovirus, reovirus and F-specific bacteriophage, suggesting that virus numbers could be low in biosolids.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified.

Organism: Enteric virus genomes

Type: Viral Indicator

Similar survival behaviour as pathogen:
Waste water: Limited information is available. The qualitative detection of enterovirus genomes did not correlate with the presence / absence of infectious enterovirus (Gantzer et al. 1998). Direct detection of adenovirus or enterovirus genomes in river water did not correlate with the detection of infectious viruses (Choi and Jiang 2005). Arnal et al.(Arnal et al. 1998) claim that HAV genomes persist in sea water long after loss of infectivity. Comparisons of infectious virus / genome numbers are complicated by the finding that infectious virus enumeration using cytopathic effect (or plaque formation) can fail to detect or underestimate the number of infectious virus particles present (99-HHE-5UR) (Lee and Jeong 2004). A proper comparison requires direct molecular detection versus cell culture + molecular detection of infection.

Shows similar effect of treatment to that of pathogen: Not known

Presence in multiple matrices: Yes

Wide geographical distribution: Yes

Adequate numbers for accurate detection:
In waste influent: detected but numbers not known, assume yes

In waste effluent: assume yes

Analytical method:
Type: nucleic acid amplification (NAA)-based
Method performance: matrix dependent – good provided inhibitors can be removed
Cost: moderate – low turbidity samples may still require sample concentration, which increases costs particularly for labor but concentrate can be analyzed for multiple viruses

Ability to multiply in matrix: No

Correlation with pathogen presence/absence: Yes, but not necessarily with infectious particles

Ease of extraction from matrix: reasonable

Allows source tracking: yes, depending on species of virus targeted.

Organism: PRD1 phage

Type: Viral Indicator

Similar survival behaviour as pathogen:
Waste water: Not known

Shows similar effect of treatment to that of pathogen: Not known. PRD1 has been used to as a model virus to study transport or removal in constructed wetlands (eg (Nicosia et al. 2001, Vidales et al. 2003, Woessner et al. 2001)) but there does not appear to be any literature that validates its use as a surrogate for human enteric viruses.

Presence in multiple matrices: Not known

Wide geographical distribution: Not known

Adequate numbers for accurate detection:
In waste influent: ?

In waste effluent: ?

Analytical method:
Type: Culture using *Salmonella typhimurium* as host
Method performance: ?
Cost: should be low - moderate

Ability to multiply in matrix: not known

Correlation with pathogen presence/absence: Not expected

Ease of extraction from matrix: ?

Allows source tracking: ?

Organism: Somatic coliphage

Type: Viral Indicator

Similar survival behaviour as pathogen:
Waste water: No comparative survival studies have been conducted as such.

Shows similar effect of treatment to that of pathogen: There is conflicting information for this. (Gantzer et al. 1998) found a significant correlation between the presence / absence of infectious enterovirus and the numbers of somatic coliphage. However (Harwood et al. 2005) found no correlation between coliphage and enteric viruses (in particular enteric viruses were more greatly affected by filtration and disinfection) for removal by waste water treatment processes. (Duran et al. 2003) suggest that somatic coliphage has a similar chlorine sensitivity to poliovirus.

Presence in multiple matrices: yes

Wide geographical distribution: yes (assumed so since host has wide distribution)

Adequate numbers for accurate detection:
In waste influent: 10^{5} – $10^{6.7}$ pfu / ml (Lucena et al. 2004)
$10^{3.5}$ – $10^{3.9}$ pfu / ml (Lodder and de Roda Husman 2005)

In waste effluent: $10^{1.4}$ – $10^{2.2}$ pfu / ml (Lucena et al. 2004)
$10^{2.4}$ – 10^{3} pfu / ml (Lodder and de Roda Husman 2005)

Analytical method:
Type: Culture (plaque count)
Method performance: Good
Cost: Low / moderate (depending on number of dilutions / replicates required)

Ability to multiply in matrix: Questionable – unlikely to replicate in *E. coli* in wastewater but there is potential for some coliform hosts to grow under certain environmental conditions and support phage replication. Not known how biofilms will affect host / phage interactions.

Correlation with pathogen presence/absence: see above – it may be possible to correlate phage numbers above a certain level with the presence of infectious enteroviruses. (Carducci et al. 1995) found that coliphage numbers were similar irrespective of the presence or absence of enteric viruses

Ease of extraction from matrix: Should be easier to recover than enteric viruses. (Blatchley et al. 2005) reported that phage were not associated with particles > 22µm

Allows source tracking: not reported.

Organism: F-RNA phage

Type: Viral Indicator

Similar survival behaviour as pathogen:
Waste water: As for somatic coliphage

Shows similar effect of treatment to that of pathogen: (Havelaar et al. 1993) reported significant correlations between the removal of enteroviruses and reovirus with F-RNA phage following coagulation treatment of secondary effluent, but no correlation was found in sewage of secondary effluent. (Tree et al. 2005) suggest that MS-2 phage are a conservative indicator since they are more chlorine and UV resistant than *E. coli* and poliovirus. However (Harwood et al. 2005) found no correlation between F-RNA phage and enteric viruses for removal by waste water treatment processes (in particular enteric viruses were more greatly affected by filtration and disinfection). Similarly (Simpson et al. 2003) found no correlation between traditional indicators (including F-RNA phage) and presence or inactivation of enteric viruses. (Duran et al. 2003) suggest that MS-2 phage has a similar chlorine sensitivity to poliovirus, conflicting with the report of (Tree et al. 2005). Numbers of F- RNA phage in raw sludge and anaerobically digested sludge were comparable with somatic coliphage but higher than the *B. fragilis* phage (Lasobras et al. 1999). (Lodder and de Roda Husman 2005) reported that the rate of inactivation of F-RNA phage during anaerobic digestion was greater than enteroviruses and reovirus but less than Norovirus. F-RNA phages were reported to be less resistant to heat than *B. fragilis* phage (Moce-Llivina et al. 2003). (Arraj et al. 2005) reported MS2 to be more resistant to inactivation than poliovirus, rotavirus and HAV during pilot scale anaerobic digestion.

Presence in multiple matrices: Yes, found in wastewater, sludge and biosolids.

Wide geographical distribution: yes

Adequate numbers for accurate detection:
In waste influent: $10^{3.5}$ – 10^{6} / ml (Lucena et al. 2004)
10^{2} to 10^{3} / ml (Lodder and de Roda Husman 2005).

In waste effluent: $10^{0.5}$ – 10^{2} / ml (Lucena et al. 2004)

Wastewater sludge: $10^{9.2}$ / g (Moce-Llivina et al. 2003)
$10^{10.5}$ / g (Lasobras et al. 1999)
Activated sludge: $10^{9.2}$ / g (Lasobras et al. 1999)

Analytical method:
Type: Culture (plaque count, double agar overlay method (APHA 2012)), potentially by RT-PCR. A number of different methods are used for the elution of phage from biosolids. Elution with beef extract without acidification has been reported to work well (Lasobras et al. 1999). Host strain *E. coli* HS(pFampR) was suggested to be better than *Salmonella typhimurium* WG 49 (D. Deere, personal communication).
Method performance: Good
Cost: Low / moderate (depending on number of dilutions / replicates required)

Ability to multiply in matrix: No. F-RNA phage require specific host density, host growth phase and temperature conditions (Woody and Cliver 1997).

Correlation with pathogen presence/absence: As for somatic coliphage

Ease of extraction from matrix: As for somatic coliphage

Points in favour: Numbers in raw sludge and digested biosolids sufficiently high for detection, similar to somatic coliphage and greater than bacteriophage which infect *B. fragilis*.

Points against: Reported to be less resistant to high temperature and high pH than somatic coliphage and *B. fragilis* phage.

Allows source tracking: Possibly. Schaper et al.(Schaper et al. 2002) suggest that particular genotypes of F-RNA phage were associated with human or animal waste. Genotyping techniques are available for FRNA and FDNA phage (Vinje et al. 2004).

Organism: *B. fragilis* phage

Type: Viral Indicator

Similar survival behaviour as pathogen:
Waste water: Possibly. Isolation rate for *B. fragilis* phage is lower than for somatic phage and matches closer the isolation rate of infectious viruses (Gantzer et al. 1998).

Shows similar effect of treatment to that of pathogen: (Gantzer et al. 1998) found a significant correlation between the presence of enterovirus and the detection of *B. fragilis* phage at greater than 10^2 / L. In drinking water treatment *B. fragilis* phage were found to be more resistant to treatment processes than either F-RNA or somatic phage (Jofre et al. 1995).

Presence in multiple matrices: yes

Wide geographical distribution: yes

Adequate numbers for accurate detection:
In waste influent: $10^1 – 10^2$ pfu / ml (Pina et al. 1998)
$10^1 – 10^3$ pfu / ml (Lucena et al. 2004)

In waste effluent: 10^1 pfu / ml (Lucena et al. 2004)

Analytical method:
Type: culture (plaque count)
Method performance: presume comparable to other phage methods. Method is more difficult because of more complex medium / culture conditions for host bacteria
Cost: Low / moderate (depending on number of dilutions / replicates required)

Ability to multiply in matrix: No – requires anaerobic conditions and specific nutrient requirements (for host presumably) for replication (Tartera et al. 1989).

Correlation with pathogen presence/absence: Possibly – see above with reference to (Gantzer et al. 1998)

Ease of extraction from matrix: ?

Allows source tracking: Possibly. Suggested that specific strains of *B. fragilis* did not detect phage in animal feces (Puig et al. 1999).

Class: Bacteria

Organism: *Salmonella enteritis*

Type: Pathogen

Level of importance to the industry: *Salmonella* spp. are widely spread bacterial pathogens having significant global public health implications. Laboratory-diagnosis surveillance data indicated that *Salmonella* was the most frequent cause of food-borne enteric disease in the United States (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5414a2.htm). *Salmonella* is probably not an issue for the water industry in the developed world since it is readily controlled by disinfection and less likely to cause waterborne infection than *Campylobacter*. *Salmonella* is frequently isolated from raw and treated biosolids (Sahlstrom et al. 2004).

Geographical distribution: Widely distributed - many food and water borne outbreaks are reported from around the world (Dumontet et al. 2001).

Representative of other pathogens in the same class Since *Salmonella* belongs to the family Enterobacteriaceae, which includes coliforms such as *E. coli*, behaviour is expected to be similar. Not a lot of comparative data are available. Removal rates in wastewater are similar between *Salmonella* and other enteric bacterial pathogens ((Blatchley et al. 2005) - NRC1998 summary table). Removal in wastewater is similar to indicator bacteria (Koivunen et al. 2003). An added advantage is that it is not as prevalent in the environment as coliform bacteria.

Presence in multiple matrices: Yes – present in wastewater and sludge (Zaleski et al. 2005).

Pathogen numbers

In waste influent: 10^2 – 10^4 / 100 ml (Koivunen et al. 2003)
10^6/ 100 ml (Jimenez-Cisneros et al. 2001)

In waste effluent: $<10^{0.5}$ – $10^{2.5}$ / 100 ml (Koivunen et al. 2003)
10^5/ 100 ml (Jimenez-Cisneros et al. 2001)

Wastewater sludge:

10^1– $10^{3.7}$ / g (Dahab and Surampalli 2002, Gajdusek et al. 1977)
$10^{0.2}$– 10^1 / g (Pourcher et al. 2005), t=0 for sludge mixed with straw
10^3– $10^{6.2}$ / g, *S. typhi* U.K. / USA, cited in (Jimenez et al. 2002)
10^7– 10^8 / g, *S. typhi* Mexico, cited in (Jimenez et al. 2002)

Biosolids:

$10^{-0.2}$– 10^3 / g in anaerobically digested biosolids (Gajdusek et al. 1977)

Analytical method:
Type: Culture
Infectivity measurement: Isolation by culture indicates culturability but does not strictly determine whether the isolate can infect or cause disease in a host, although this is often the inference made when a pathogen is detected by culture.
Method performance:
Wastewater: No problems anticipated since either centrifugation or membrane filtration can be used for concentration.
Biosolids: MPN based methods along with spread plating regularly used for isolation of *Salmonella* from biosolids. Biochemical tests are used for species identification.
Cost: Moderate on the basis of multiple steps including enrichment, quantification and identification.

Ability to multiply in matrix: *Salmonella* is also known to re-grow under certain conditions in stored biosolids (Gajdusek et al. 1977, Soller et al. 2002), bagged biosolids based products (Skanavis and Yanko 1994), composted biosolids (Sidhu et al. 2001) and also soils treated with biosolids (Zaleski et al. 2005).

Knowledge on pathogen: One of the most extensively studied bacteria, especially in connection with disease outbreaks. Considerably more information is available on *Salmonella spp*. compared to other bacterial pathogens in wastewater and biosolids. Less information is available in the area of wastewater concentration and removal by treatment processes.

Points in favour: Known to survive wastewater treatment process (such as anaerobic digestion) in low numbers (Sahlstrom et al. 2004). *Salmonella* monitoring is recommended by USEPA to determine the bio-safety of biosolids (US EPA 1999).

Points against: Re-grows under certain conditions in biosolids, making it difficult to assess the biosafety of biosolids. Not as prevalent as coliform bacteria or *E. coli*.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2. Vaccination and care full handling is recommended for *S. typhi*.

Organism: ***Clostridium perfringens***

Type: Pathogen / Protozoan Indicator

Level of importance to the industry: Of importance to health regulators in terms of food but of no significance to the water industry as a pathogen. It is more important as a model or faecal indicator organism. *C. perfringens* is opportunistic pathogens in immunocompromised individuals.

Geographical distribution: Assumed to be world-wide since it is used globally as a faecal indicator. Most of the species of the genus *Clostridium* are saprophytes and grow in soil, water and decomposing organic matter.

Representative of other pathogens: Possibly. Cell numbers (vegetative + spores) were reported to be similar to that of other indicator bacteria in wastewater, with similar removal rates (Rose et al. 2004), but spores will survive for longer, as well as being more heat resistant. Presumed to be removed by activated sludge and drinking water plants to a similar degree as parasitic protozoan oo/cysts (Charles et al. 2003, Payment et al. 2001), although (Harwood et al. 2005) reported no correlation. Spores appear to be poorly removed by primary treatment (Payment et al. 2001, Rose et al. 2004).

Presence in multiple matrices: Yes – would expect to detect it in any matrix with human faecal contamination. Used as an indicator in sludge (Chauret et al. 1999).

Indicator numbers

In waste influent:
10^4 – 10^{10} / 100 ml cells (Rose et al. 2004)
10^4 – $10^{4.2}$ / 100 ml spores (Rose et al. 2004)
10^2 – $10^{4.6}$ / 100 ml spores (Payment et al. 2001)

In waste effluent:
10^2 – 10^4 / 100 ml cells (Rose et al. 2004)
(1 - 10 / 100mL post disinfection)
$10^{4.5}$ / 100 ml spores (Rose et al. 2004)
(1 - 10 / 100mL post disinfection)
$10^{2.3}$ – $10^{4.7}$ / 100 ml spores (Payment et al. 2001)

Sludge: $10^{4.7}$ - $10^{4.9}$ / g in sludge (Pourcher et al. 2005)
Biosolids: $10^{6.1}$ / g (Chauret et al. 1999)

Analytical method:
Type: Culture. For *C. perfringens*, sulfite-reducing clostridia are initially isolated and then confirmation tests used to identify *C. perfringens*.
Infectivity measurement: Generally considered as a non-pathogen. Isolation by culture dependent methods shows its viability but not ability to infect or cause disease
Method performance: Based on membrane filtration so highly turbid samples (e.g. wastewater influent) could be problematic. Isolation from biosolids may be problematic
Cost: Moderate since a separate confirmation step is required.

Ability to multiply in matrix: Not reported for wastewater. Multiplication would not be expected since growth requires strict anaerobic and warm conditions. Laboratory study of sediments from wastewater outfalls suggest that numbers of total *C. perfringens* and spores remain stable, with an overall trend for the vegetative cells to die off within 7 – 30 days (Davies et al. 1995). Other species of clostridia grow in various waste matrices (e.g. hydrogen producers in wastewater sludge (Wang et al. 2003)), hence the importance to enumerate *C. perfringens*.

Allows source tracking: Not reported.

Knowledge on indicator: Compared to viruses and many other bacteria there is a reasonable level of knowledge in terms of the presence of clostridia as an indicator, although there are few studies that provide comprehensive data in terms of concentrations in wastewater or removal by treatment processes (Long and Ashbolt 1994). There is more information on clostridia (perfringens or SRC) as a faecal indicator in water or an indicator for pathogen removal by processes such as constructed wetlands.

Points in favour: More resistant to inactivation as compared with other bacterial indicators, making it one of the better bacterial candidates for protozoa.

Points against: There is conflicting information on the correlation between the removal of clostridia and protozoa during wastewater treatment. Part of this confusion may be due to differences in the isolation techniques for clostridia – eg some investigators isolated total cells, others selected for spores by heat treatment.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified (except for *C. botulinum* and *C. tetani*).

Organism: ***Campylobacter jejuni***

Type: Pathogen

Level of importance to the industry: Not established yet, but it is on the USEPA emerging pathogen list. Surveillance programs have identified *Campylobacter* as the second most common cause of clinically diagnosed foodborne disease in the USA (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5414a2.htm). This pathogen should not be detected in drinking water, but is a common waterborne pathogen that causes outbreaks in Nordic countries (Hanninen et al. 2003) and in other parts of the world (e.g. (Kuusi et al. 2005, Linnemann et al. 1984). *Campylobacter* spp. may be important to health regulators in terms of food, but are less of an issue for the water industry when disinfection is used. QMRA identifies *C. jejuni* as one of the most important risks of infection in the event of a disinfection failure e.g. (Westrell et al. 2004)

Geographical distribution: *Campylobacter* is a common waterborne pathogen in many parts of the world and has caused waterborne outbreaks in Nordic countries (Hanninen et al. 2003) and other parts of the world (Frost 2001, Kuusi et al. 2005, Linnemann et al. 1984).

Representative of other pathogens in the same class: Probably not. There is a lack of data to make any comparison in terms of response to disinfectants or wastewater treatment. One study suggests that campylobacters are more sensitive to chlorine than *E. coli* (Blaser et al. 1986). There is conflicting information on the survival of *Campylobacter* in environmental samples and the viability of those surviving cells is also questionable. Since it is sensitive to oxygen conventional wisdom is that it should be inactivated quickly. However, it is known to quickly change into viable but non-culturable (VBNC) form under environmental stress that might revert to a culturable status under favorable conditions (Harvey and Leach 1998).

Presence in multiple matrices: *Campylobacter* are ubiquitous in the environment and are commonly found in surface water and sewage sludge (Dumontet et al. 2001, Sahlstrom et al. 2004).

Pathogen numbers
In waste influent: 10^3 / 100 ml (Stampi et al. 1992),
may be additional data available in (Arimi et al. 1988)
In waste effluent: 10^1 / 100 ml (Stampi et al. 1992)

In wastewater sludge:
$10^{2.4}$ / g (Stampi et al. 1999)
10^4/ 100 ml (Jones and Harrison 2004)
In biosolids: No information available.

Analytical method:
Type: Culture using membrane filtration can be used. Molecular techniques can be used for rapid identification (Alexandrino et al. 2004, Inglis and Kalischuk 2004). Use of immunomagnetic capture in combination with PCR has been reported for food and may be worth exploring for biosolids (Hu et al. 2003).
Infectivity measurement: Isolation by culture indicates culturability but does not strictly determine whether the isolate can infect or cause disease in a host, although this is often the inference made when a pathogen is detected by culture.
Method performance: Highly turbid samples (eg sewage influent) could be problematic.
Cost: Moderate as multiple steps including enrichment, quantitation and identification are required.

Ability to multiply in matrix: Unlikely. Little literature available, but it is believed that rapid inactivation of *Campylobacter* occurs in the presence of oxygen (Jones and Harrison 2004) and it has been shown that aeration of sludge leads to rapid inactivation (Stampi et al. 1999). It has also been reported that campylobacters rapidly lose culturability (within a few days) in a wastewater water microcosm (Pickert and Botzenhart 1985).

Knowledge on pathogen: Relatively little information is available on the presence and behaviour in wastewater or biosolids, with some of the information apparently contradictory. (Sahlstrom et al. 2004) reported that while wastewater treatment generally lowered the numbers of *C. jejuni* substantially, *C. jejuni* was still isolated from the composted biosolids. (Ahmed and Sorensen 1995) reported 5 log reduction in *Campylobacter* numbers in 50 days when dewatered sludge was stockpiled. Conversely,

(Iranpour et al. 2002) reported one log reduction time of 793 days for *Campylobacter* in mesophilic anaerobic digestions

Points in favour: On USEPA emerging pathogen list. Known to cause disease outbreaks.

Points against: Apparent rapid inactivation by oxygen. However, it is not clear if *Campylobacter* are inactivated or simply become VBNC. Detection methods are not adequate to reliably detect presence of *Campylobacter* in biosolids or its viability status.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified.

Organism: ***Escherichia coli***

Type: Pathogen / Bacterial Indicator

Level of importance to the industry: Important to the industry both as a cause of waterborne outbreaks and as an indicator organism. There were 31 waterborne outbreaks of *E. coli* O157:H7 in the US between 1982 – 2002, accounting for 9% of all outbreaks by this pathogen (Rangel et al. 2005).

Geographical distribution: Widely distributed both as an indicator and pathogen.

Representative of other pathogens in the same class: Yes, would expect so for other *Enterobacteriaceae*. May not be an appropriate indicator for other bacteria outside of this family as *E. coli* is more sensitive to many disinfectants e.g. chlorine, chloramine, UV, than *Legionella pneumophila* and *Mycobacterium avium* (Jacangelo et al. 2002). *E. coli* and *Aeromonas* were found at similar levels in final effluent (Yanko et al. 2004).

Presence in multiple matrices: Yes – any matrix with faecal contamination.

Pathogen numbers – limited data are available for *E. coli* in wastewater (most studies report faecal coliforms)

In waste influent: 10^2 – 10^4 / 100mL (Koivunen et al. 2003)
10^4 – 10^5 / 100mL (Sinton et al. 2002)

In waste effluent: $<10^{0.3}$ – $10^{1.5}$ / 100mL (Yanko et al. 2004)
10^1 – 10^2 / 100mL (Sinton et al. 2002) – advanced pond system

Analytical method:

Type: Culture.

Infectivity measurement: Not specifically. Isolation by culture indicates culturability but does not determine whether the isolate can infect or cause disease in a host.

Method performance: Good

Cost: low (defined substrate technology), moderate for pathogen identification.

Ability to multiply in matrix: Depends on the matrix. Reported to grow in pulp waste from a cardboard mill, although the isolates were claimed to be distinct from *E. coli* of faecal origin (Niemi et al. 2004). Faecal coliforms reported to grow in sediment (Davies et al. 1995). (Banning et al. 2003) suggest reduced survival of *E. coli* in effluent due to antagonism or competition from other microorganisms.

Knowledge on pathogen: *E. coli* is a well studied organism, although specific data regarding numbers in wastewater and process removal is minimal since most reports use faecal coliforms as an indicator. Some survival data for O157:H7 in wastewater influent, effluent and sludge (Withey et al. 2005). There appears to be more literature for *E. coli* in other (non domestic wastewater) matrices and other processes (e.g. wetlands, survival on crops, etc)

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified, except for Vero cytotoxin-producing strains (e.g. O157:H7) – gloves, only used appropriately trained and experienced staff.

Organism: *Vibrio* spp (*cholerae*, *parahaemolyticus*)

Type: Pathogen

Level of importance to the industry: Cholera is of relevance in developing countries where outbreaks still occur and where toxigenic strains have environmental reservoirs. As with other microbial enteric pathogens, it is controlled by disinfection. In developed countries it does not appear to be a problem in drinking water (as with *Campylobacter*) because there is no reservoir in the community (apart from travel acquired disease). Infection has been caused by environmental exposure (Desmarchelier et al. 1995). *V. paraheamolyticus* has been associated with outbreaks from contaminated shellfish (McLaughlin et al. 2005). Treatment of primary screened influent with lime, as practiced in some developing countries, may be relatively ineffective due to the organism's tolerance of high pH.

Geographical distribution: *V. cholerae* distribution appears to be widely distributed although there is limited information regarding the distribution of pathogenic / non-pathogenic strains in developed countries (e.g. (Desmarchelier et al. 1995)). May be present in the environment but concentrations in domestic wastewater from developed countries are not known.

Representative of other pathogens in the same class: Limited information is available. *V. cholerae* is more sensitive to chlorine than *E. coli* (Venczel et al. 2004).

Presence in multiple matrices: Not known. Assume limited presence if infection is not present in the community.

Pathogen numbers: Not known

In waste influent:

In waste effluent: Wastewater treatment is effective in eliminating *V. cholerae* as it does not survive for long under wastewater treatment conditions (AWWA 1999).

Analytical method:

Type: Culture, serology or PCR can be used for identification of pathogenic serotypes.

Infectivity measurement: Not specifically. Isolation by culture indicates culturability but does not determine whether the isolate can infect or cause disease in a host.

Method performance: Good

Cost: Low - moderate.

Ability to multiply in matrix: Claimed to reproduce in wastewater and persist for a long time (Altukhov et al. 1975). Presumably this is temperature dependent.

Knowledge on pathogen: Limited literature in the area of wastewater.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified. Toxigenic *V. cholera* are listed on the register for Security Sensitive Biological Agents and require special conditions to be legally kept or handled.

Organism: ***Aeromonas hydrophila***

Type: Pathogen

Level of importance to the industry: Probably low. *Aeromonas hydrophila* is on the US EPA candidate contaminant list. *Aeromonas spp*. are associated with opportunistic infections (Vally et al. 2004, Weber et al. 1995) and traveller's diarrhoea (Pommepuy et al. 2004).

Geographical distribution: Widely distributed e.g.. (Burke et al. 1984, Havelaar et al. 1990, Hazen et al. 1978).

Representative of other pathogens in the same class: No. Present in water in the absence of *E. coli* or coliforms (Burke et al. 1984), likely due to replication at lower temperatures. More susceptible to chlorination than *E. coli* (Sisti et al. 1998). However, a different study suggested that isolates from untreated water had the same sensitivity to chlorine as *E. coli*, but that isolates from chlorinated water immediately downstream of the water treatment plant were more resistant to chlorine (Massa et al. 1999).

Presence in multiple matrices: Yes (Poffe and Op de Beeck 1991).

Pathogen numbers: Not known

In waste influent: $10^6 – 10^8$ / 100mL *Aeromonas spp.* (Monfort and Baleux 1990)

In waste effluent: $10^3 – 10^6$ / 100mL *Aeromonas spp.* (Monfort and Baleux 1990)

Analytical method:

Type: Culture.

Infectivity measurement: Not specifically. Isolation by culture indicates culturability but does not determine whether the isolate can infect or cause disease in a host.

Method performance: Good

Cost: Low.

Ability to multiply in matrix: Can multiply in environmental biofilms (van der Kooij 1991).

Knowledge on pathogen: Limited literature in the area of wastewater.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified.

Organism: *Klebsiella* spp

Type: Pathogen / Bacterial Indicator

Level of importance to the industry: Low. Opportunistic pathogens, present in the environment. A problem in hospitals - at least one claim of infection due to operating equipment contaminated with tap water (Rudnick et al. 1996).

Geographical distribution: Broadly distributed.

Representative of other pathogens in the same class: Yes. Behaves similarly to *E. coli* (e.g. (Tanner et al. 2004) although may be slightly more UV resistant (Martiny et al. 1988).

Presence in multiple matrices: Yes (Poffe and Op de Beeck 1991).

Pathogen numbers: No specific data

In waste influent:

In waste effluent:

Analytical method:

Type: Culture.

Infectivity measurement: Not specifically. Isolation by culture indicates culturability but does not determine whether the isolate can infect or cause disease in a host.

Method performance: Good

Cost: Low - moderate.

Ability to multiply in matrix: Same as *E. coli* – has been found to multiply in pulp waste (Gauthier and Archibald 2001) and paper industry recycled water systems (Caplenas et al. 1981).

Knowledge on pathogen: Limited literature in the area of wastewater.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, no special precautions specified.

Organism: ***Pseudomonas aeruginosa***

Type: Pathogen

Level of importance to the industry: Low for the water industry. Pseudomonads pose a problem as opportunistic pathogens in hospitals, including the USA (Anaissie et al. 2002). Suggested as an indicator of health risk for water (de Victorica and Galvan 2001) or wastewater quality (Howard et al. 2004).

Geographical distribution: Widely distributed.

Representative of other pathogens in the same class: Not known. Mucoid strains of *Pseudomonas* have increased resistance to chlorine (Grobe et al. 2001), non-mucoid ones have similar sensitivity to coliforms (Palmer et al. 1995). (Korol et al. 1995) suggest similar response to chlorine and ozone for *E. coli*, *S. typhi*, *Y. enterocolitica*, *A. hydrophila* and *P. aeruginosa*.

Presence in multiple matrices: Yes. (Geuenich and Muller 1984) suggest that *P. aeruginosa* is concentrated in sludge.

Pathogen numbers: Limited data

In waste influent:

In waste effluent: 10^5 / 100mL (pre-disinfection, (Jimenez-Cisneros et al. 2001))
10^6 / 100mL (Liberti et al. 1990)

Analytical method:

Type: Culture.

Infectivity measurement: Not specifically. Isolation by culture indicates culturability but does not determine whether the isolate can infect or cause disease in a host.

Method performance: Good

Cost: Low

Ability to multiply in matrix: Shown to persist in effluent, although not part of biofilm (Banning et al. 2003). Shown to form biofilms on steel in distilled water (Boyle et al. 1991). (Geuenich and Muller 1984) suggest that *P. aeruginosa* grew during (activated sludge treatment) of wastewater.

Knowledge on pathogen: Limited literature in the area of domestic wastewater, more studies on other waste streams (particularly hospital waste). Several non-English-language reports on *Pseudomonas* in domestic wastewater.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, not listed under any particular risk group.

Organism: ***Mycobacterium***

Type: Pathogen

Level of importance to the industry: Currently not considered high priority. Listed on the US EPA candidate contaminant list. Suggested drinking water association for infections in AIDS patients (Leclerc et al. 2002). Thought to be an emerging opportunistic pathogen with increased detection due to improvements in culture / detection methods and due to changes in population composition (eg aging populations) (Falkinham 2002).

Geographical distribution: Widely distributed, ubiquitous in the environment (eg (Raizman et al. 2004, September et al. 2004).

Representative of other pathogens in the same class: No. Mycobacteria are environmental organisms that are opportunistic pathogens and with higher resistance to most disinfectants compared with enteric bacterial pathogens (Jacangelo et al. 2002, Taylor et al. 2000, Vaerewijck et al. 2005).

Presence in multiple matrices: Present in environmental waters and drinking water. Only one report of isolation from wastewater (Jin et al. 1984) and a single paper suggesting presence in sludge (Schulze-Robbecke 1993). Since they are not enteric pathogens it would not be expected that infected humans would be a major contributing source in water. They are more likely to be contributed by other sources.

Pathogen numbers: No data

In waste influent:

In waste effluent:

Analytical method:

Type: Culture or PCR.

Infectivity measurement: Not specifically. Isolation by culture indicates culturability but does not determine whether the isolate can infect or cause disease in a host.

Method performance: Not known

Cost: Moderate.

Ability to multiply in matrix: Can grow in drinking water biofilm (Lehtola et al. 2006). Can survive in reservoir water and sediment (Whittington et al. 2005).

Knowledge on pathogen: Limited for wastewater

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, requires aerosol containment and gloves for handling.

Organism: Total coliforms

Type: Bacterial Indicator

Similar survival behaviour as pathogen:
Waste water: No comparative waste water survival studies as such

Shows similar effect of treatment to that of pathogen: Yes. Koivunen et al.(Koivunen et al. 2003) found similar removal rates for total coliforms, faecal coliforms and *Salmonella* during waste water treatment (activated sludge treatment + biological nutrient removal).

Presence in multiple matrices: yes

Wide geographical distribution: yes

Adequate numbers for accurate detection:
In waste influent: $10^7 – 10^8$ / 100 ml (Koivunen et al. 2003)

In waste effluent: $10^5 – 10^6$ / 100 ml (Koivunen et al. 2003)

Analytical method:
Type: culture
Method performance: good
Cost: low

Ability to multiply in matrix: yes depending on matrix

Correlation with pathogen presence/absence: Not expected

Ease of extraction from matrix: yes

Allows source tracking: Not reported.

Organism: Faecal coliforms

Type: Bacterial Indicator

Similar survival behaviour as pathogen:
Waste water: No comparative waste water survival studies as such

Shows similar effect of treatment to that of pathogen: Yes. (Koivunen et al. 2003) found similar removal rates for total coliforms, faecal coliforms and *Salmonella* during waste water treatment (activated sludge treatment + biological nutrient removal).

Presence in multiple matrices: Yes

Wide geographical distribution: Yes

Adequate numbers for accurate detection:
In waste influent: $10^{6.4}$ – 10^{7} / 100 ml (Harwood et al. 2005)

In waste effluent: $10^{3.6}$ – $10^{5.4}$ / 100 ml (Harwood et al. 2005)

Analytical method:
Type: culture
Method performance: good
Cost: low

Ability to multiply in matrix: yes depending on matrix

Correlation with pathogen presence/absence: Not expected

Ease of extraction from matrix: yes

Allows source tracking: Possibly. (Duran et al. 2006) characterized the fatty acid methyl ester (FAME) profiles of a range of faecal coliform isolates from different sources and found that particular FAMEs were exclusively associated with isolates of human origin. The origin of a faecal coliform (human versus livestock or wildlife) could be determined by discriminant function analysis.

Organism: Enterococci / faecal streptococci

Type: Bacterial Indicator

Similar survival behaviour as pathogen:
Waste water: No? Mesocosm experiments suggest differences in the survival of enterococci and faecal coliforms (predominantly *E. coli*) (Lau et al. 2004), depending on the matrix and source of organisms. Enterococci from wastewater survived better than *E. coli* in marine water and sediment, while the converse was true for fresh water sediment.

Shows similar effect of treatment to that of pathogen: Possibly – enterococci had similar removal rates to that of coliforms / faecal coliforms (Harwood et al. 2005). Enterococci are known to be more resistant to adverse environmental conditions, such as chlorination, than coliforms and are used to monitor the efficiency of water treatment processes. Reported to be good indicator for assessing compost sanitation (Deportes et al. 1998).

Presence in multiple matrices: Yes, used as an indicator in sludges eg (Nelson et al. 2004)

Wide geographical distribution: Yes (Lau et al. 2004)

Adequate numbers for accurate detection:
In waste influent: $10^{5.7}$ – $10^{6.5}$ / 100 ml (Harwood et al. 2005)

In waste effluent: 10^{3} – $10^{4.9}$ / 100 ml (Harwood et al. 2005)

Undigested sludge: $10^{5.9}$ - $10^{6.4}$ / g (Pourcher et al. 2005)

Digested biosolids: $10^{5.6}$ /g (Chauret et al. 1999)
10^{2} - 10^{5} / g (Sano et al. 2004)

Analytical method:
Type: Culture (membrane filtration, MPN), molecular methods have been used for identification / confirmation (Rose et al. 2004).
Method performance: good
Cost: low

Ability to multiply in matrix: An initial increase in numbers of enterococci and *E. coli* were reported when sterile sediment was added to river water in lab-scale experiments, but this did not appear to be sustained growth (Desmarais et al. 2002).

Correlation with pathogen presence/absence: No, especially for marine waters it is suggested that enterococci may be poor indicators of recent faecal pollution (Charles et al. 2003).

Ease of extraction from matrix: yes

Allows source tracking: Possibly. *E. faecalis* was found to have a limited host range (humans, dogs, chickens) (Wheeler et al. 2002). FAME and antibiotic resistance analysis have also been used to characterize isolates (Genthner et al. 2005), with antibiotic resistance analysis being reported as the most accurate (yet still flawed method) for source tracking faecal streptococci (Harwood et al. 2003).
Knowledge on pathogen: Reasonable information is available on their presence in wastewater but not in biosolids as faecal streptococci are more regularly monitored in wastewater.

Points in favour: Limited non-human hosts, rarely multiply in the environment, more resistant to inactivation than faecal coliforms.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Vancomycin resistant *Enterococcus* spp. are rated Risk category 2 but no additional precautions are listed for handling.

Organism: Bifidobacteria

Type: Bacterial Indicator

Similar survival behaviour as pathogen:
Waste water: ?

Shows similar effect of treatment to that of pathogen: ?

Presence in multiple matrices: ?

Wide geographical distribution: yes

Adequate numbers for accurate detection:
In waste influent: ?

In waste effluent: ?

Analytical method:
Type: culture / NAA for specific identification
Method performance: ?
Cost: ? moderate

Ability to multiply in matrix: ?

Correlation with pathogen presence/absence: not predicted

Ease of extraction from matrix: presume yes

Allows source tracking: Yes (Bonjoch et al. 2004, Long et al. 2005).

Organism: ***Bacteroides fragilis***

Type: Bacterial Indicator

Similar survival behaviour as pathogen:
Waste water: No? Suggested to die off faster than *E. coli* (Allsop and Stickler 1985).

Shows similar effect of treatment to that of pathogen: ?

Presence in multiple matrices: Yes

Wide geographical distribution: Yes

Adequate numbers for accurate detection:
In waste influent: ?

In waste effluent: ?

Analytical method:
Type: Culture, NAA has been used for identification (Bernhard and Field 2000)
Method performance: ? presume good
Cost: ? low - mod

Ability to multiply in matrix: not known

Correlation with pathogen presence/absence: ?

Ease of extraction from matrix: ?

Allows source tracking: Potentially, (Allsop and Stickler 1985, Bernhard and Field 2000).

Organism: Aerobic spores

Type: Protozoan Indicator

Similar survival behaviour as pathogen:
Waste water: ?

Shows similar effect of treatment to that of pathogen: No. Galofre et al. (Galofre et al. 2004) suggest that aerobic spore-formers can regrow on filters.

Presence in multiple matrices: ? limited literature available

Wide geographical distribution: assume yes

Adequate numbers for accurate detection: assume yes but no specific data available
In waste influent: ?

In waste effluent: ?

Analytical method:
Type: culture
Method performance: good
Cost: low

Ability to multiply in matrix: at least 1 report of growth on filters in a WTP (Galofre et al. 2004)

Correlation with pathogen presence/absence: ?

Ease of extraction from matrix: presume good

Allows source tracking: ? no reports.

Class: Protozoa

Organism: *Cryptosporidium* spp (*hominis*, *parvum* + others)

Type: Pathogen

Level of importance to the industry: High, as oocysts are known to be more resistant to inactivation by chlorine and other commonly used disinfectants (Fayer et al. 2000). It is one of the organisms used by health regulators to assess the safety of re-use water. Furthermore, the infectious dose has been estimated to be as low as 30 oocysts, although this is dependent on the species and strain (Fayer et al. 2000). Numerous outbreaks of cryptosporidiosis and giardiasis have been attributed to contamination of water supplies with (oo)cysts (Leclerc et al. 2002, Slifko et al. 2000). Spreading of biosolids on agricultural land may potentially lead to health risks as runoff water may contaminate surface water and food products (Fayer et al. 2000, Slifko et al. 2000). Monitoring of raw and drinking water for *Cryptosporidium* is a worldwide practice to ensure safety of drinking water (McCuin and Clancy 2005).

Geographical distribution: Widely distributed, a common parasite of humans and other mammals (Monis and Thompson 2003).

Representative of other pathogens in the same class: Probably not. Generally *Cryptosporidium* is more resistant to disinfectants compared with *Giardia* (Jacangelo et al. 2002). Removal through wastewater treatment, in particular filtration, is poorly correlated with *Giardia (Harwood et al. 2005)*. During aerobic digestion of sludge, a higher reduction in *Cryptosporidium* numbers occurs (2.96 log) compared to *Giardia* (1.40 log). No reduction occurs in *Giardia* numbers during anaerobic digestion of sludge (Chauret et al. 1999).

Presence in multiple matrices: Yes, many reports of occurrence in wastewater and sludge/biosolids.

Pathogen numbers:
In waste influent:
$10^{3.2}$ - $10^{4.5}$ / 100 L (Harwood et al. 2005, Montemayor et al. 2005)

In waste effluent:
$10^{1.6}$- $10^{3.2}$ / 100 L (Harwood et al. 2005, Montemayor et al. 2005)
$<10^{2}$ – 10^{6} / 100 L (Medema and Schijven 2001)

In Biosolids:
$10^{0.3}$- $10^{0.4}$ / g in composted biosolids (Rimhanen-Finne et al. 2004)
$10^{0.4}$- $10^{0.7}$ / g of dewatered anaerobic sludge (Chauret et al. 1999)

Analytical method:
Wastewater: The matrix determines the primary concentration step (centrifugation or flocculation / filtration). Oocysts are purified by immunomagnetic separation (IMS) to improve recoveries and aid direct counting by fluorescent microscopy or real-time NAA. Fluorescent *in situ* hybridization (FISH) can be used in conjunction with fluorescent antibodies to aid in confirmation / species identification of oocysts by either microscopy or flow cytometry (eg (Charles et al. 2003)). Molecular analysis is required for species/genotype identification.
Biosolids: NaCl flotation and differential density flotation with sucrose in combination with IMS. Counting as above.
Infectivity measurement: Cell culture infectivity is the most reliable and reproducible, with animal infectivity originally the gold standard (Keegan et al. 2003). Dye exclusion and FISH are known to overestimate viability.
Method performance: The efficiency of oocyst recovery is generally method and matrix dependent. Direct counting by fluorescence microscopy is the industry standard. For the detection of infectious oocysts, a number of cell lines and detection schemes for wastewater are in use with variable success (Quintero-Betancourt et al. 2002). Some issues with sample inhibition were reported by (Blatchley et al. 2005), although (Harwood et al. 2005) reported no such problems.

Cost: High with cell culture methods, moderate - high for standard IMS / microscopy

Ability to multiply in matrix: Not reported, although a report of cell free replication bears future consideration if it can be independently replicated (Hijjawi et al. 2004).

Knowledge on pathogen: One of the most extensively studied protozoan pathogens.

Point in favour: The resistance of oocysts to disinfectants and some treatment processes makes them a conservative indicator of treatment efficacy.

Points against: Generally lower in numbers in wastewater and sludge compared to *Giardia*. Also, rate of inactivation during anaerobic digestion and sludge storage appears to indicate lower survival potential than *Giardia* in biosolids.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2

Organism: *Giardia* spp, *G. duodenalis*

Type: Pathogen

Level of importance to the industry: Moderate to high. As *Giardia* cysts are more susceptible to disinfectants than *Cryptosporidium* oocysts, they are of less concern in developed countries. However, giardiasis is more prevalent than cryptosporidiosis in the community. Several outbreaks of giardiasis attributable to contamination of water supplies with cysts have been reported (Slifko et al. 2000). (Caccio et al. 2003) reported that *Giardia* cysts are more frequently isolated throughout the year from wastewater as compared to *Cryptosporidium* oocysts, which have a seasonal distribution. In addition, *Giardia* cyst numbers are generally higher in the wastewater and sludge as compared to *Cryptosporidium* oocyst numbers (Payment et al. 2001).

Geographical distribution: Widely distributed. *Cryptosporidium* spp. and *Giardia* spp. are a common parasite of humans and mammals worldwide causing diarrhoeal diseases (Monis and Thompson 2003, Slifko et al. 2000).

Representative of other pathogens in the same class: Not representative of *Cryptosporidium* or amoeba cysts. Giardia cysts are larger than *Cryptosporidium* oocysts and can be filtered out more easily (Harwood et al. 2005). In addition they are less resistant to commonly used disinfectant as compared to *Cryptosporidium* oocysts (Jacangelo et al. 2002). *Acanthamoeba* cysts are also more resistant to chlorine (Storey and Ashbolt 2003), while *Naegleria* cysts are more sensitive to chlorine than *Giardia (Engel et al. 1983). Giardia* cysts have similar chlorine, ozone and UV sensitivity to microsporidia (Lewis et al. 1983). No comparative data for enteric protozoa (other than *Cryptosporidium* or *Giardia*) in terms of water treatment or behaviour.

Presence in multiple matrices: Yes – many reports of presence in wastewater and sludge/biosolids.

Pathogen numbers:
In waste influent: $10^{4.5} – 10^{5.5}$/ 100L (Harwood et al. 2005)

In waste effluent: $10^{1} – 10^{3.6}$/ 100L secondary effluent (Harwood et al. 2005)

Wastewater sludge:
$10^{4.5} – 10^{5.9}$ / g (Gajdusek et al. 1977)

In Biosolids: $10^{4.6} – 10^{5.1}$ / g (Gajdusek et al. 1977)
$10^{0.5} – 10^{0.8}$ / g composted biosolids (Rimhanen-Finne et al. 2004)
$10^{-0.6} – 10^{1.5}$ / g dewatered anaerobic sludge (Chauret et al. 1999)

Analytical method:
Type: Methods similar to *Cryptosporidium* monitoring with matrix determining primary concentration step.
Infectivity measurement: Yes, when using an animal model. Vital dye staining appears to correlate with viability but depends on mode of inactivation.
Method performance: Detection of cyst generally method and matrix dependent. Direct microscopic count generally used and is reliable. For “dirty” samples, IMS improves recovery and detection by microscopy.
Cost: High.

Ability to multiply in matrix: No, needs a host for multiplication. *Giardia* is an obligate parasite lacking many biosynthetic pathways (e.g. de novo synthesis of nucleotides and lipids) and relies on scavenging from the host for these compounds (Adam 1991).

Knowledge on pathogen: Extensive. Literature is available on the presence of *Giardia* in wastewater and biosolids. During aerobic digestion of sludge a higher reduction in *Cryptosporidium* numbers occurs (2.96 log) compared to *Giardia* (1.40 log). However, no reduction in *Giardia* cyst numbers occurs during anaerobic digestion (Chauret et al. 1999). *Giardia* cysts remain intact in biosolids stored for more than a year (Hu et al. 1996), although retention of viability was not reported.

Points in favour: Numbers in wastewater and biosolids are higher and less seasonal than *Cryptosporidium* oocysts and *Giardia* appears to survive better in biosolids.

Points against: Known to be less resistant to chlorination, UV and ozone compared to *Cryptosporidium.*

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2.

Organism: ***Entamoeba histolytica***

Type: Pathogen

Level of importance to the industry: Probably low. *Entamoeba* may be of historical interest in drinking water judging by the age of most of the literature, but there appears to be little recent research activity. A Mexican study associated *Entamoeba* infection in children with exposure to wastewater (Cifuentes et al. 1991). It appears to be more of an issue in developing countries, particularly where there is poor water treatment.

Geographical distribution: Widely distributed, especially in developing countries (Marshall et al. 1997).

Representative of other pathogens in the same class: Not known. WHO guide on water treatment (http://www.who.int/water_sanitation_health/dwq/en/watreatpath3.pdf) suggests *Entamoeba* has a similar chlorine resistance to *Giardia*.

Presence in multiple matrices: Detected in solid municipal waste in Africa (Adeyeba and Akinbo 2002) and wastewater sludge (Paulino et al. 2001). Limited data on presence in wastewater.

Pathogen numbers:

In waste influent:

In waste effluent: $10^{1.9}$ / 100L *E. histolytica* (Argentina, (De Luca et al. 1997))
$10^{2.7}$ / 100L *E. coli* (Argentina, (De Luca et al. 1997))

Analytical method:

Type: Clinical detection by microscopy, no literature on detection in water other than (Chang and Kabler 1956). PCR can be used for differentiating *E. histolytica* from *E. dispar*.

Infectivity measurement: Animal model available using gerbils (eg (Shibayama et al. 1997))

Method performance: Poor. Recovery can lead to damage causing false negatives (USFDA, 2003). Would assume problematic in the absence of any IMS to assist with sample purification

Cost: Anticipated to be moderate to high.

Ability to multiply in matrix: Require a human or primate host for multiplication (http://www.cfsan.fda.gov/~mow/chap32.html).

Knowledge on pathogen: Limited

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Yes, risk group 2, gloves recommended for handling.

Organism: Microsporidia, *Cyclospora*, *Isospora*

Type: Pathogen

Level of importance to the industry: Possibly emerging pathogens. Microsporidia are listed on the US EPA's candidate contaminant list. Limited incidences of waterborne outbreaks for microsporidia (one by (Cotte et al. 1999) but this was disputed by (Hunter 2000)). The first report for waterborne transmission of *Cyclospora* in the USA was (Huang et al. 1995), There is also a claim for waterborne transmission in a village in Egypt (el-Karamany et al. 2005). Food borne outbreaks of *Cyclospora* appear to be more common. No reports of outbreaks attributed to *Isospora*.

Geographical distribution: Widely distributed (Marshall et al. 1997).

Representative of other pathogens in the same class: Microsporidia have similar disinfection sensitivity compared to *Giardia* (Jacangelo et al., 2003;(Lewis et al. 1983) (Wolk et al. 2000)). (Wright and Collins 1997) claim that *Cyclospora* is resistant to chlorine.

Presence in multiple matrices: Microsporidia detected in a variety of water sources (Dowd et al. 1998a). No data for the other organisms. Isospora detected in wastewater sludge (Paulino et al. 2001).

Pathogen numbers: microsporidia detected by PCR in wastewater effluent (Dowd et al. 1998a), *Cyclospora* have been detected in wastewater (Sherchand et al. 1999, Sturbaum et al. 1998).

In waste influent:

In waste effluent:

Analytical method:

Type: Varies depending on organism. *Cyclospora* have been isolated by filtration / flotation and detected by microscopy / PCR (Sturbaum et al. 1998).

Infectivity measurement: Yes – cell culture techniques can be used to measure infectivity (Quintero-Betancourt et al. 2002).

Method performance: No known. Would assume problematic in the absence of any IMS to assist with sample purification

Cost: Probably moderate to high

Ability to multiply in matrix: No – they are obligate intracellular parasites (Curry and Smith 1998).

Knowledge on pathogen: Limited

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Not currently listed in any risk group category.

Class: Helminth

Organism: ***Ascaris***

Type: Pathogen / Helminth Indicator

Level of importance to the industry: High in the developing world, but of limited importance in the developed world. It may be an issue for reuse water and disposal of biosolids. Helminth infections are a major concern in the developing world where a lack of effective sanitation results in the spread of infections. In South Africa, helminth infection rates among school age children is high (58%) with the majority of the infections caused by Ascaris (24.8%) and *Trichuris* (50.6%) (Tree et al. 2005). Most helminth eggs in wastewater are concentrated in sludge due to their high settling velocities (Nelson et al. 2004). *Ascaris lumbricoides* has been reported to be more common than *Ancylostoma* and *Hymenolepis diminuta* in sludge stored in ponds for 6 months (von Sperling et al. 2005).

Geographical distribution: Widely distributed especially in the underdeveloped countries with poor sanitation (Sanguinetti et al. 2005).

Representative of other pathogens in the same class: Ascaris eggs have been reported to be more resistant to inactivation compared to *Trichuris* and *Toxocara* (Nelson et al. 2004), although an earlier report suggested that survival of *Ascaris* eggs in sludge was similar to that of *Toxocara* eggs (O'Donnell et al. 1984). Ascaris is used as a model for inactivation of other helminths in sludge (Abu-Orf et al. 2004, Capizzi-Banas et al. 2004).

Presence in multiple matrices: Yes.

Pathogen numbers:
In waste influent: $10^{1.3}$ – $10^{2.4}$ / L (von Sperling et al. 2003)
In waste effluent: $10^{0.6}$ – $10^{1.5}$ / L (von Sperling et al. 2003)

Undigested sludge: ?
Digested biosolids: 10^{5} - 10^{6} / g of dry weight sludge (Barbier et al. 1990)

Analytical method:
Type: A number of different procedures for quantification and viability assessment of helminth eggs in wastewater and biosolids have been developed eg. (Bowman et al. 2003, de Victorica and Galvan 2003). However, none of the methods are universally accepted for regular use for detection of helminth eggs in wastewater and biosolids. Concentration by sedimentation is claimed to be less selective for different species (Bouhoum and Schwartzbrod 1989). Currently, the recommended methods for the detection of helminth eggs from biosolids involve the isolation of eggs by centrifugal flotation, culturing of eggs to allow them to hatch eggs and develop into infective larvae; then identification and quantification (USEPA 1999). A variation of this method, the 'Tulane Method' (Bowman et al. 2003), is also used for the quantification and estimation of viability of eggs.
Infectivity measurement: (i) Requires an animal model. (ii) (Lewis et al. 1983) suggest a method for viability estimation by monitoring embryo development in eggs. US EPA also recommends culture dependent method to monitor larval development (USEPA 1999). The larval culture methods are tedious, time consuming and require technical skill for larval differentiation. (iii) Staining procedure (vital dyes) to determine the viability of helminth eggs without culturing has also been proposed, it has been shown to have good correlation with the standard USEPA recommended method (de Victorica and Galvan 2003). However, the reliability of this method with biosolids based samples is unknown.
Method performance: Potential difficulty in concentrating eggs from biosolids matrices. Vital dyes assays to monitor viability might not work well if the sample is not sufficiently clean.
Cost: moderate - high: culture techniques to monitor infectivity are time consuming and so likely to be more expensive.

Ability to multiply in matrix: No, needs a host.

Knowledge on pathogen: Most of the studies are on the persistence of *Ascaris* in stored sewage sludge/biosolids.

Points in favour: Other helminths are not as widely distributed as *Ascaris* and their numbers in biosolids are generally lower than *Ascaris*. Prolonged survival in biosolids, soil and slurries is well documented. Due to its wide distribution as compared to other helminths, valuable information can be derived from comparing data on *Ascaris* monitoring at different locations.

Points against: Some work on method development for monitoring is required. Generally low level risk in developed countries, removed into sludges where long retention time when lagooning is utilized. Potentially more problematic for sludge use.
Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Risk category 2, direct skin contact to be avoided.

Organism: Hookworm (*Ancylostoma duodenale*)

Type: Pathogen

Level of importance to the industry: In developing nations the majority of the helminth infections are caused by helminths other than hookworm (Tree et al. 2005). However, a quarter of the world's population is infected by hookworm (Gilles 1985). Hookworm is more prevalent in the tropical areas of Australia, especially Western Australia. Presence of *Ascaris* in these areas is rare. Under these conditions hookworms can be used as model for other helminths.

Geographical distribution: Know to exist in worldwide but numbers are generally low as compared to *Ascaris* and *Trichuris* which are more prevalent (Tree et al. 2005, von Sperling et al. 2003).

Representative of other pathogens in the same class: Generally *Ascaris* eggs are used as indicators and very little is known about survival of hookworm eggs versus eggs of other helminths.

Presence in multiple matrices: Yes.

Pathogen numbers:
Undigested sludge: no information available
Digested biosolids: No information available

Analytical method:
Type: Methods used are similar to *Ascaris*.
Infectivity measurement: Similar approach to *Ascaris*.
Method performance: Potential difficulty in concentrating eggs from biosolids matrix. Vital dyes assays to monitor viability might not work well if sample is not sufficiently clean.
Cost: Moderate - high.

Ability to multiply in matrix: No, needs a host.

Knowledge on pathogen: Very limited; numbers and behaviour in biosolids are unknown.

Points against: Assumed to be less resistant to inactivation than *Ascaris*. Numbers are low in wastewater compared to other helminths. Methods need to be further developed for viability assessment.

Can be handled in a Class II (AS/NZS 2243.3:2002) laboratory: Risk category 2, direct skin contact to be avoided.

Class: Miscellaneous

Measure: Particle profiling

Type: Bacterial / Protozoan / Helminth Indicator

Similar survival behaviour as pathogen:
Waste water: NA

Shows similar effect of treatment to that of pathogen: Chavez et al.(Chavez et al. 2004) found a correlation between the removal of particles <8 microns and the removal of faecal coliforms and *Salmonella spp* (R^2 = 0.8217 and 0.7148 respectively). A correlation was also found between the number of helminth ova / L and the volume of particles in the 20 – 80 micron size range (R^2 = 0.98). No information was presented for protozoa but it would be worth investigating further.

Presence in multiple matrices: yes

Wide geographical distribution: yes

Adequate numbers for accurate detection: assume yes
In waste influent:

In waste effluent:

Analytical method:
Type: particle profiler. AWQC has access to a A LISST-100 Particle Size Analyser Type C (Sequoia, Redmond WA USA), which can profile 2 460 micron particles. Do plants have the equivalent installed for monitoring process performance?
Method performance: ?
Cost: ? Low for the analysis, capital required for equipment

Ability to multiply in matrix: NA

Correlation with pathogen presence/absence: No

Ease of extraction from matrix: Yes

Allows source tracking: No.