Presence and Removal of Enteric Microorganisms in South East Queensland Wastewater Treatment Plants

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The Urban Water Security Research Alliance (UWSRA) is a $50 million partnership over five years between the Queensland Government, CSIRO's Water for a Healthy Country Flagship, Griffith University and The University of Queensland. The Alliance has been formed to address South East Queensland's emerging urban water issues with a focus on water security and recycling. The program will bring new research capacity to South East Queensland tailored to tackling existing and anticipated future issues to inform the implementation of the Water Strategy.

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FOREWORD

Water is fundamental to our quality of life, to economic growth and to the environment. With its booming economy and growing population, Australia's South East Queensland (SEQ) region faces increasing pressure on its water resources. These pressures are compounded by the impact of climate variability and accelerating climate change.

The Urban Water Security Research Alliance, through targeted, multidisciplinary research initiatives, has been formed to address the region's emerging urban water issues.

As the largest regionally focused urban water research program in Australia, the Alliance is focused on water security and recycling, but will align research where appropriate with other water research programs such as those of other SEQ water agencies, CSIRO's Water for a Healthy Country National Research Flagship, Water Quality Research Australia, eWater CRC and the Water Services Association of Australia (WSAA).

The Alliance is a partnership between the Queensland Government, CSIRO's Water for a Healthy Country National Research Flagship, The University of Queensland and Griffith University. It brings new research capacity to SEQ, tailored to tackling existing and anticipated future risks, assumptions and uncertainties facing water supply strategy. It is a $50 million partnership over five years.

Alliance research is examining fundamental issues necessary to deliver the region's water needs, including:

- ensuring the reliability and safety of recycled water systems.
- advising on infrastructure and technology for the recycling of wastewater and stormwater.
- building scientific knowledge into the management of health and safety risks in the water supply system.
- increasing community confidence in the future of water supply.

This report is part of a series summarising the output from the Urban Water Security Research Alliance. All reports and additional information about the Alliance can be found at http://www.urbanwateralliance.org.au/about.html.

C. Davis

Chris Davis
Chair, Urban Water Security Research Alliance

CONTENTS

Acknowledgements i
Foreword ii
Executive Summary 1
1. Introduction 2
2. Methods and Materials 3
  2.1. Wastewater Treatment Plants 3
  2.2. Sample Collection 3
  2.3 Testing for Target Microorganisms 4
    2.4.1. Faecal Indicator Bacteria and Bacterial Pathogens 5
    2.4.2. Bacteriophage 5
    2.4.3. Cryptosporidium oocysts 5
    2.4.4. Enteric Viruses 5
3. Results and Discussion 6
  3.1. Detection of Bacteria 6
  3.2. Detection of Bacteriophages 8
  3.3. Cryptosporidium oocysts 10
  3.4 Detection of Enteric Viruses 11
4. Reduction of Enteric Microorganisms in Wastewater Treatment Plants 14
5. Conclusions 16
6. Recommended Further Research 17
Appendix 1. Detection Methods for Microorganisms 19
  A1.1 Concentration of Collected Samples 19
  A1.2 Analytical Methods for Bacteria 19
  A1.3 Analytical Methods for Bacteriophages 20
  A1.4 Detection Method for Cryptosporidium oocysts 20
  A1.5 Detection Methods for Human Enteric Viruses 21
Appendix 2. Assessed Recovery Efficiencies of *Cryptosporidium* Oocysts from Primary and Secondary Effluent using Hollow Fibre Filtration and IMS 22
References 23

LIST OF FIGURES

Figure 1. Sampling sites at Luggage Point Wastewater Treatment Plant. 3

Figure 2. Sampling sites at the Oxley Creek Wastewater Treatment Plant. 4

Figure 3. Sampling sites at the Bundamba Wastewater Treatment Plant. 4

Figure 4. Average $\log_{10}$ cfu 100mL^{-1} bacteria in the sewage and treated effluent of the (a) Luggage Point; (b) Oxley Creek; and (c) Bundamba Wastewater Treatment Plants *(Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range)*. 7

Figure 5. Average, maximum and minimum $\log_{10}$ pfu mL^{-1} of (a) male specific (F+) and (b) somatic bacteriophage numbers detected in sewage and treated effluent from three different WWTPs. LP = Luggage Point WWTP; OC = Oxley Creek WWTP; and Bund = Bundamba WWTP. *(Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range)*. 9

Figure 6. Log$_{10}$ average and range of Cryptosporidium oocysts numbers per litre of sewage and treated effluent from Luggage Point (LP); Oxley Creek (OC); and Bundamba (Bund) wastewater treatment plants. *(Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range)*. 11

Figure 7. Log$_{10}$ average numbers and range of adenovirus (Adeno), polyomavirus (Polyoma) and Torque Teno Virus (TTV) PDU L^{-1} in the sewage and treated effluent at the Luggage Point, Oxley Creek, and Bundamba Wastewater Treatment Plants. (*Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range*). 13

LIST OF TABLES

Table 1. Enteric microorganisms targeted in sampling program. 5

Table 2. Average $\log_{10}$ cfu[a] 100ml^{-1} of target bacteria in the sewage and treated effluent of three SEQ wastewater treatment plants (standard deviation in parentheses), plus maximum and minimum values. 6

Table 3. Average, maximum and minimum $\log_{10}$ pfua mL^{-1}of bacteriophage detected in each of the three Wastewater Treatment Plants between March 2008 and June 2009. 8

Table 4. Average, maximum and minimum $\log_{10}$ Cryptosporidium oocysts (L^{-1}) detected in each of the three Wastewater Treatment Plants between March 2008 and April 2010[a]. 10

Table 5. Log$_{10}$ average, range and percent reductions of PDU[a] L^{-1} of enteric viruses in sewage and treated effluent at the Luggage Point, Oxley Creek, and Bundamba Wastewater Treatment Plants[b]. 12

Table 6. Average log$_{10}$ and percent reductions of enteric microbes in the three studied SEQ wastewater treatment plants. 15

EXECUTIVE SUMMARY

The production of safe and sustainable Purified Recycled Water (PRW) relies on the effectiveness of a number of treatment barriers, one of which is the wastewater treatment plants that treat raw sewage to produce a suitable quality of effluent. Although most wastewater treatment plants in the major Australian cities are modern by international standards and are designed to maximise carbon and nutrient removal (their major intended purpose), much less is known about their ability to remove the key contaminants of enteric microbial pathogens and trace organic chemicals. The research reported in this report is based on the analysis of the pathogen and indicator numbers in primary and secondary effluent of South East Queensland (SEQ) wastewater treatment plants (WWTPs). To obtain a better understanding of the treatment efficiency of SEQ WWTPs, samples were collected from three of the major WWTPs in the Brisbane and Ipswich region. Samples were collected from each treatment plant at least once every month over an 18-month period, to analyse for selected bacterial indicators and pathogens, and for bacteriophage. An additional 4-month intensive sampling period also included analysis for *Cryptosporidium* oocysts and enteric viruses, along with the enteric bacteria. Both raw sewage and secondary treated effluent were tested.

The research undertaken in this study has demonstrated that the three WWTPs tested were very efficient in removing faecal indicator bacteria, enteric bacteriophage and enteric pathogens. The plants were capable of removing up to 4.8 $\log_{10}$ of bacteria; between 2 and 4.8 $\log_{10}$ of Cryptosporidium oocysts; between 2.5 and 4 $\log_{10}$ of DNA enteric virus genomes; and up to 3 $\log_{10}$ of bacteriophages. These removal rates were determined to be generally higher than found in studies undertaken at WWTPs in other Australian States and higher than the removal rates cited in the Australian Guidelines for Water Recycling. The reason for these higher removal rates remains to be determined but could quite likely be due to differences in climatic conditions, with SEQ generally being warmer, particularly in winter, than the more southerly states of Australia.

The efficiency of the removal of microbial pathogens by WWTPs in SEQ, as demonstrated by the results in this study; also have implications for the reuse of the treated wastewater. The numbers of several of the enteric microorganisms detected in the secondary effluent in this study indicate that secondary treated effluent produced by SEQ WWTPs may pose a lower inherent health risk from microbial pathogens than may be assumed if using the guidelines values. There are also implications for the Western Corridor Recycled Water Scheme as these results demonstrate that the WWTPs can be part of the multiple treatment barrier system for pathogens and thus reduce some of the reliance on treatment from the advanced water treatment plants (AWTP) for pathogen removal.

The research undertaken in this project has also highlighted where knowledge is still lacking on microbial pathogens and their removal in WWTPs. In response, a series of potential future research topics is suggested.

1. INTRODUCTION

The production of safe and sustainable Purified Recycled Water (PRW) relies on the effectiveness of a number of treatment barriers, one of which is the wastewater treatment plant (WWTP). Although most WWTPs in the major Australian cities are modern by international standards and are designed to maximise carbon and nutrient removal (their main purpose), much less is known about their ability to remove key contaminants such as enteric microbial pathogens and trace organic chemicals.

Wastewater treatment processes are important for protecting humans and the environment from the contaminants in wastewater. The effectiveness of the treatment process is important in the removal of human enteric pathogens from the wastewater. The importance for the different pathogen types depend on the geographical location of the wastewater systems and the social and economic standards of the local population. While the presence of helminths and various bacterial pathogens (e.g. *Vibrio cholerae* and *Shigella dysinterae*) are important in regions with low socio-economic standards or health conditions (Pant and Mittal, 2007), regions such as South East Queensland (SEQ) have a major concern about the potential presence of specific bacterial pathogens (e.g. toxigenic *E. coli*), human enteric viruses and pathogenic protozoa in the treated effluent from wastewater treatment plants (Albinana-Gimenez *et al.*, 2006; Bofill-Mas, *et al.*, 2006; Keegan *et al.,* 2008; Nwachcuku and Gerba, 2004). In addition, improvements are needed to establish more appropriate microorganisms that can be used as indicators for the removal of the different groups of microbial pathogens from treated and untreated wastewater (Cole *et al.,* 2003; Tallon *et al.,* 2005).

The current understanding of the ability of SEQ WWTPs to remove enteric microbial pathogens is limited, with much of the information available on pathogen removal by WWTPs coming from research conducted overseas (notably the USA and Europe), much of which was published up to 30 years ago. During the development of the first stage of the new Australian Water Reuse Guidelines it was agreed that there was currently minimal data from Australia on the performance of wastewater treatment plants to remove enteric microbial pathogens and that this was impacting on the ability to undertake a comprehensive risk management of enteric microbial pathogens in recycled water (Health Working group, Stage 1 Australian Water Reuse Guidelines, 2005). Another compounding factor was the numerous designs and configurations of biological wastewater treatment plants. All of the WWTPs in SEQ vary in their design based on age and intended flow rates (based on the size of the wastewater catchments). A study of different types of WWTPs will also assist in determining if there is an optimal design, configuration or mode of operation of treatment plants for enteric pathogen reduction.

The aim of this project was to gain a comprehensive understanding of the ability of wastewater treatment plants in SEQ to remove enteric microbial pathogens.

2. METHODS AND MATERIALS

2.1. Wastewater Treatment Plants

Due to the amount of research to be undertaken, three of the six wastewater treatment plants (WWTPs) involved in the Western Corridor Recycled Water Scheme in SEQ were selected as the main sampling sites for this research. These WWTPs were three of the four larger WWTPs in the PRW scheme, being Luggage Point, Oxley Creek (at the time of sampling both operated by Brisbane Water, now Queensland Urban Utilities) and Bundamba (at the time of sampling operated by Ipswich Water, now Queensland Urban Utilities). Luggage Point directly feeds to the adjacent Luggage Point Advanced Water Treatment Plant (AWTP), while Oxley Creek and Bundamba WWTPs both supply treated wastewater to the Bundamba AWTP.

2.2. Sample Collection

Sampling of the Luggage Point and Oxley Creek WWTPs commenced in March 2008, and in September 2008 for the Bundamba WWTP. Each treatment plant was sampled monthly for up to 12 months for enteric indicator and pathogenic bacteria as well as bacteriophage up to October 2009. Following this sampling campaign, from January 2010, each of the treatment plants was sampled intensively over two four-day periods for a four-month period (Each WWTP tested every fortnight). During the intensive sampling, analysis was also undertaken for enteric viruses and *Cryptosporidium* oocysts.

Each of the WWTPs was sampled by taking grab samples. On the first day of the intensive sampling, the primary influent (sewage) was sampled (using the grab sampling method described below), once in the morning and once in the afternoon. On the second day, the treated effluent was sampled in the morning and afternoon. This was undertaken based on a hypothetical 24-hour hydraulic residence time in each of the treatment plants. This sampling procedure was repeated on days three and four. This resulted in four sewage and four effluent samples being collected over a four-day period. There was a minimum of two weeks between the sampling of each treatment plant and each treatment plant was intensively sampled on two occasions.

At the Luggage Point WWTP, samples were collected from the raw sewage entering the plant after the grit screens and from the treated wastewater channel (treated effluent) after collection from all of the clarifiers and prior to the off-take for the AWTP (Figure 1). At the Oxley Creek WWTP, samples were collected from the raw sewage after the grit screens and from the treated effluent just after the discharge from the clarifiers (Figure 2). In addition, the Oxley Creek WWTP uses UV disinfection prior to release of the clarified treated effluent, so samples were collected post UV treatment to determine the efficacy of the UV system. Note that, unless otherwise stated, all results provided in this Report for Oxley Creek treated effluent are from samples collected from the clarifiers before UV treatment to ensure comparability with the other treatment plants studied.

Figure 1. Sampling sites at Luggage Point Wastewater Treatment Plant.

Figure 2. **Sampling sites at the Oxley Creek Wastewater Treatment Plant.**

The sampling of the third WWTP (Bundamba) was similar to the other two WWTPs in that a sample was collected from the raw sewage entering the plant after the grit screens. A second sample of the treated effluent was collected after the clarifiers prior to it being pumped to the Bundamba AWTP (Figure 3).

Figure 3. **Sampling sites at the Bundamba Wastewater Treatment Plant.**

All samples were collected using a stainless steel scoop and decanted into sterile 500 mL borosilicate bottles or sterile 20 L polycarbonate carboys. The filled bottles were immediately placed in a cool box containing freezer blocks and transported to the laboratory.

2.3. Testing for Target Microorganisms

The collected samples were prepared for analysis in the laboratory within six hours of collection. The range of target microorganisms each sample was tested for is given in Table 1. The specific details of each analysis method used for each specific microorganism is provided in Appendix A.1.

For the samples collected during the intensive sampling period, they were initially used directly for the detection of the bacteria and bacteriophage. The remainder of the samples were then concentrated by tangential flow hollow fibre and centrifugal concentration devices prior to analysis for enteric viruses and *Cryptosporidium* oocysts.

Table 1. **Enteric microorganisms targeted in sampling program.**

Microbial Group	Microorganism
Bacteria	*E. coli* Enterococci *Campylobacter* *Salmonella*
Bacteriophage	Male Specific (F^+) coliphage Somatic coliphage
Virus	Adenovirus Polyomavirus Torque Teno Virus
Protozoa	*Cryptosporidium* oocysts

2.3.1 Faecal Indicator Bacteria and Bacterial Pathogens

There is a range of enteric bacteria that can be present in sewage. These can vary from the common microbial indicators such as *E. coli* and other coliforms, enterococci and *Clostridium perfringens* spores to a range of recognised bacterial pathogens. The most common pathogenic bacteria that could be expected to be found in Australian wastewater include *Salmonella,* toxic *E. coli* strains and *Campylobacter*.

All bacteria were tested for using appropriate selective isolation media and procedures. The analytical methods and media used for each bacterial type are given in Appendix 1 and Table A.2.

2.3.2 Bacteriophage

The bacteriophages tested for are viruses that infect *E. coli* only (otherwise known as coliphage). There are two different major groups of coliphage used to test water: the F^+ bacteriophage (also known as the male specific phage) and somatic phage. The F^+ bacteriophage have physical characteristics that are similar to many of the human enteric viruses and are therefore often used as surrogates for these human viruses. Somatic bacteriophages tend to be different in their characteristics to enteric viruses, but have been reported to be present in higher numbers and to be more resistant to treatment processes than the F^+ bacteriophage (Costan-Longares *et al.,* 2008). Therefore, the somatic bacteriophages are often also included as surrogate viruses for studying the potential presence of enteric viruses and the effectiveness of treatment methods for virus removal.

Both the somatic and F^+ bacteriophage were detected by culture methods using the double overlay method described by Havelaar *et al.* (1984). The analytical methods are provided in detail in Appendix A.1.

2.3.3 Cryptosporidium oocysts

Cryptosporidium oocysts were captured from the concentrated samples using immunomagnetic beads (IMS). The detection and enumeration of the captured oocysts was achieved using microscopy combined with fluorescent antibody staining. The full method is outlined in Section A1.4 of Appendix 1. The prepared sample was placed onto a microscope slide and stained oocysts identified using a fluorescent microscope. The average number of oocysts detected in 20 fields of view was then used to calculate the number of oocysts L^{-1} of sewage or treated effluent.

2.3.4 Enteric Viruses

The enteric viruses targeted in this study were adenovirus, polyomavirus and Torque Teno Virus. All three are DNA viruses and were detected using quantitative PCR (qPCR). The method for DNA extraction and the specific PCR methods and primer sets are provided in Section A1.4 and Table A.2 in Appendix 1. All PCR detections were then back calculated to obtain a number of PCR Detectable Units (PDU) L^{-1} of sewage or treated effluent.

3. RESULTS AND DISCUSSION

3.1. Detection of Bacteria

The outcomes of the detection of the enteric bacteria in sewage and treated effluent at the three wastewater treatment plants studied are given in Table 2 as an average (including standard deviation) along with maximum and minimum numbers detected. These numbers are also given graphically in the box plots in Figure 4.

Table 2. Average $\log_{10}$ cfu^{a} 100ml^{-1} of target bacteria in the sewage and treated effluent of three SEQ wastewater treatment plants (standard deviation in parentheses), plus maximum and minimum values.

	Sewage		Treated Effluent	
Bacteria	**Average (SD)**	**Max-Min**	**Average (SD)**	**Max-Min**
		Luggage Point		
E. coli	7.7 (± 0.4)	8.9-7.2	3.6 (± 1.5)	4.5-0
Salmonella	2.9 (± 1)	4-1.5	1.5 (± 0.05)	1.6-1.5
Enterococci	7.6 (± 0.5)	9.2-7	3.8 (± 0.4)	4.4-3
Campylobacter	7.7 (± 0.3)	7.9-7	4.7 (± 0.4)	5.4-4.4
		Oxley Creek^{c}		
E. coli	7.6 (± 0.3)	8.1-7	4.3 (± 1.2)	5.4-1.6
Salmonella	3.3 (± 0.8)	4-2.4	1.6 (± 0.5)	1.9-1.2
Enterococci	7.8 (± 0.2)	8-7.4	5.2 (± 0.4)	5.6-4.4
Campylobacter	7.3 (± 0.6)	7.8-5.8	5.3 (± 0.3)	5.6-4.8
		Bundamba		
E. coli	7.8 (± 0.2)	7.9-7.6	5.1 (± 0.3)	5.3-4.6
Salmonella	ND^{b}	ND	ND	ND
Enterococci	7.5 (± 0.2)	7.9-7.2	5 (± 0.2)	5.3-4.8
Campylobacter	6.9 (± 0.6)	7.9-6.3	5.5 (± 0.1)	5.6-5.4

a cfu = colony forming units
bND = Not done
c All results given for the treated effluent form the Oxley Creek WWTP are from samples collected prior to the UV treatment system.

The results show that all of the target bacteria could be detected in the sewage and almost always in the treated effluent. The numbers detected for most of the bacteria are similar to the higher numbers quoted in the Australian Guidelines for Water Recycling (NRMMC-EPHC-AHMC 2006). The number of *Campylobacter* spp. detected, however, are higher than those quoted by the Australian Guidelines. The numbers in the guidelines are taken from the scientific literature and are predominantly sourced from studies undertaken in North American wastewater treatment plants. It should be noted that the *Campylobacter* colonies detected on the CCDA agar were not speciated, and thus the results are expressed at a genus level only while the Reuse Guidelines are specifically quoting *C. jejuni*. While *C. jejuni* can be expected to be the predominant *Campylobacter* species in urban wastewater, in must be acknowledged that the results presented in this report will include some proportion of other *Campylobacter* species such as *C. coli*. More testing should be done to further confirm the reason for the higher than anticipated *Campylobacter* spp. numbers detected in this study.

The numbers of *E. coli*, enterococci and *Campylobacter* in the sewage entering the treatment plant are consistent across all three WWTPs, at approximately 7 $\log_{10}$ per mL. The results also show that there are similarities between the numbers of each of these bacteria entering each WWTP.

Differing from *E. coli* and *Campylobacter*, *Salmonella* numbers in the sewage entering two of the treatment plants tested were in numbers of approximately 3 $\log_{10}$ (no results are available for *Salmonella* in the effluent entering the Bundamba WWTP). There was also a greater variation in the numbers of *Salmonella* detected at each of the WWTPs over time, as shown by the larger standard deviation and range compared to the other bacteria. This lower detection of *Salmonella* in treated effluent compared to other enteric bacteria, as well as greater variability in numbers, has also been observed by Howard *et al.* (2004) and Levantesi *et al.* (2010).

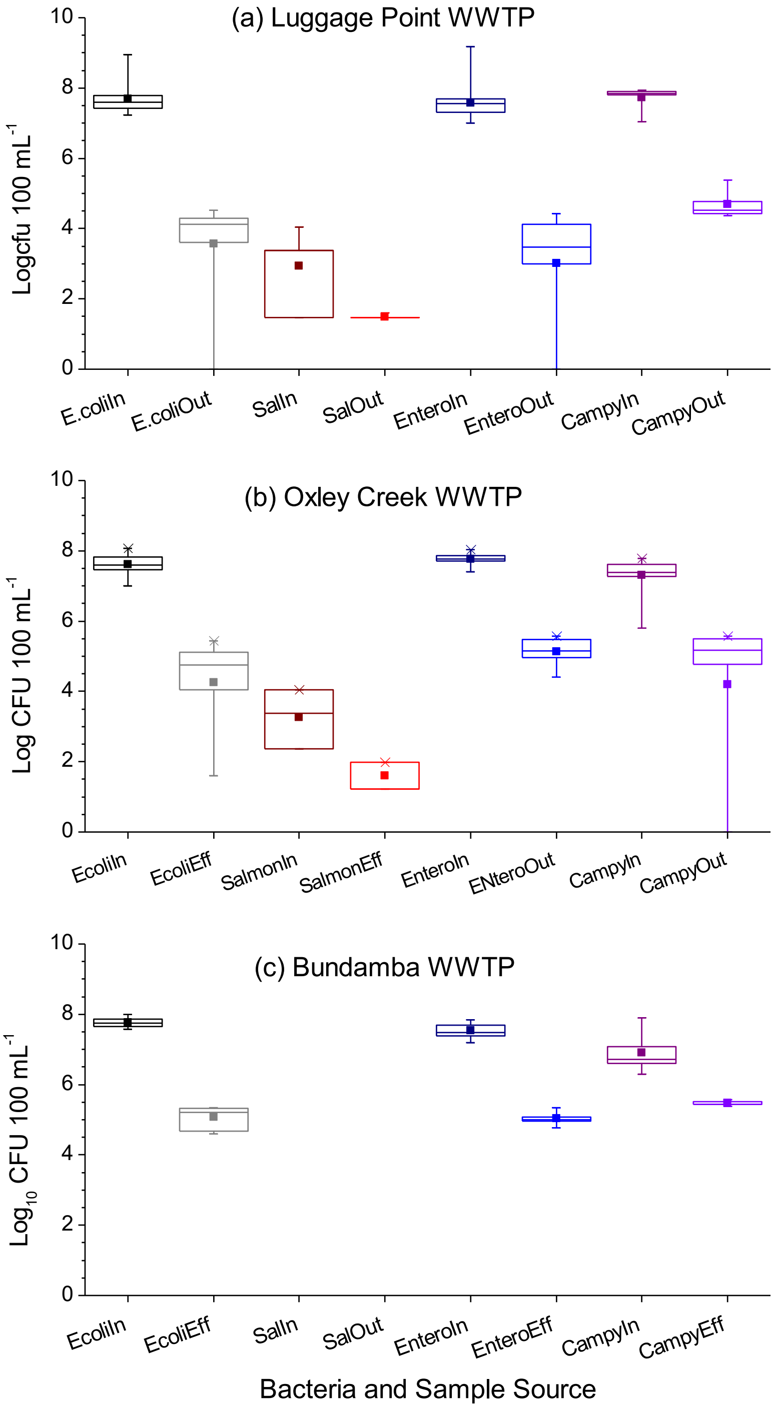

Figure 4. **Average $\log_{10}$ cfu 100mL^{-1} bacteria in the sewage and treated effluent of the (a) Luggage Point; (b) Oxley Creek; and (c) Bundamba Wastewater Treatment Plants** ***(Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range)*****.**

In contrast to the results from the sewage, the treated effluent from all three WWTPs was much more variable for *E. coli,* enterococci and *Campylobacter*, both across sampling events within one WWTP and between the three WWTPs. Again, *Salmonella* was different from the other three bacteria tested, having similar numbers and range between the two WWTPs.

A comparison of the bacterial numbers is the sewage and treated effluents entering and leaving each WWTP showed that all of the WWTPs were able to reduce the number of the target bacteria by up to 3 $\log_{10}$ (99.9% reduction). This level of treatment fits within the indicative $\log_{10}$ removals expected for secondary treatment by WWTPs give in the Australian Guidelines for Water Recycling (NRMMC-EPHC-AHMC, 2006). While the Luggage Point WWTP was able to remove 3 $\log_{10}$ of the target bacteria (apart from the previously discussed results for *Salmonella*), the Oxley Creek and Bundamba WWTP had a much more variable ability to remove the bacteria, with only *E. coli* having a 3 $\log_{10}$ removal in the Oxley Creek plant and all the bacteria having less than a 3 $\log_{10}$ removal rate in the Bundamba WWTP. The reason for this variation in log reductions of the different bacteria in the different treatment plants is unclear. More testing should be undertaken to determine if it is due to the size of the plant (for example, the Bundamba plant being smaller than the other two treatment plants), or if there is some other difference (e.g., in residence time, input sources or operational conditions) that could account for the different removal rates.

Finally, in addition to conventional secondary treatment, the Oxley Creek WWTP also employs UV treatment on the final treated effluent. An assessment was also made on the microbial quality of the treated final effluent after it had passed the UV treatment. The UV treatment of the treated effluent was effective in removing the target bacterial numbers to below detection limits (effectively 0.2 cfu $100mL^{-1}$) (results not shown). This equates to a removal efficiency for bacteria in treated final effluent of up to 5 $\log_{10}$ for the UV treatment step alone. This suggests that UV treatment could be used as an effective final polishing step for treated effluent if, at least, bacterial pathogens are considered a potential, on-going health risk.

3.2. Detection of Bacteriophages

The bacteriophage numbers detected in both the sewage and treated effluent from the three WWTPs are given in Table 3 as an average, maximum and minimum number, detected over the 18 months of sampling. The results are also provided graphically as box plots in Figure 5. Overall, there were only minor differences in the number of somatic and male specific (F^+) bacteriophage entering each of the WWTPs. The only observed difference noted was the lower average number of both types of bacteriophage in the sewage entering Luggage Point WWTP than for Oxley Creek and Bundamba WWTPs. There was much less difference between the numbers entering these other two plants.

Table 3. Average, maximum and minimum $\log_{10}$ pfua $mL^{-1}$of bacteriophage detected in each of the three Wastewater Treatment Plants between March 2008 and June 2009.

Bacteriophage	Sewage		Treated Effluent	
	Average (±SD)	Max-Min	Average ±SD	Max-Min
	Luggage Point			
F^+ bacteriophage	2.6 (± 2.8)	3.3 - 0	0.3 (± 0.8)	1.3 - 0
Somatic bacteriophage	2.9 (± 2.5)	3.1 – 2.4	1.6 (± 1.6)	2 - 0
	Oxley Creek			
F^+ bacteriophage	3.1 (± 2.7)	3.3 – 2.7	0	0 - 0
Somatic bacteriophage	3.1 (± 2.60)	3.2 – 2.9	2.6 (± 1.9)	2.7 – 2.4
	Bundamba			
F^+ bacteriophage	3.3 (± 2.8)	3.4 – 2.9	1.5 (± 1.9)	2.3 - 0
Somatic bacteriophage	3.3 (± 3.5)	4 – 2.5	1.7 (±1.2)	1.8 - 1.3

[a] pfu = plaque forming units

The other major difference observed was the $\log_{10}$ reduction of each of the phage types in the WWTPs. Each of the WWTPs was capable of removing a greater proportion of the male specific phage (2-3 $\log_{10}$ reduction) than the somatic phage (1-2 $\log_{10}$ reduction). In fact, for both the Oxley Creek and Bundamba WWTPs, somatic phages were always able to be detected in the treated effluent. While the Luggage Point WWTP had the best average removal of somatic phage, it also had the greatest variability in the number of somatic phage detected in the treated effluent (Figure 5). Likewise, the Bundamba WWTP had the highest variability of male specific (F^+) phage in the treated effluent. It is possible that this variability could be used as a measurement of the overall performance of a wastewater treatment plant; however, this would require further study to prove or disprove.

Also of note was the ability to detect somatic phage in the Oxley Creek WWTP final treated effluent after UV treatment. It is apparent that there are at least some species of somatic bacteriophage that are more resistant to UV irradiation of treated effluent. Adenovirus is known to be the most resistant of the enteric viruses to UV radiation and more testing could be done to determine the efficacy of using somatic bacteriophage as a substitute for adenovirus or as an additional test for validating UV disinfection processes.

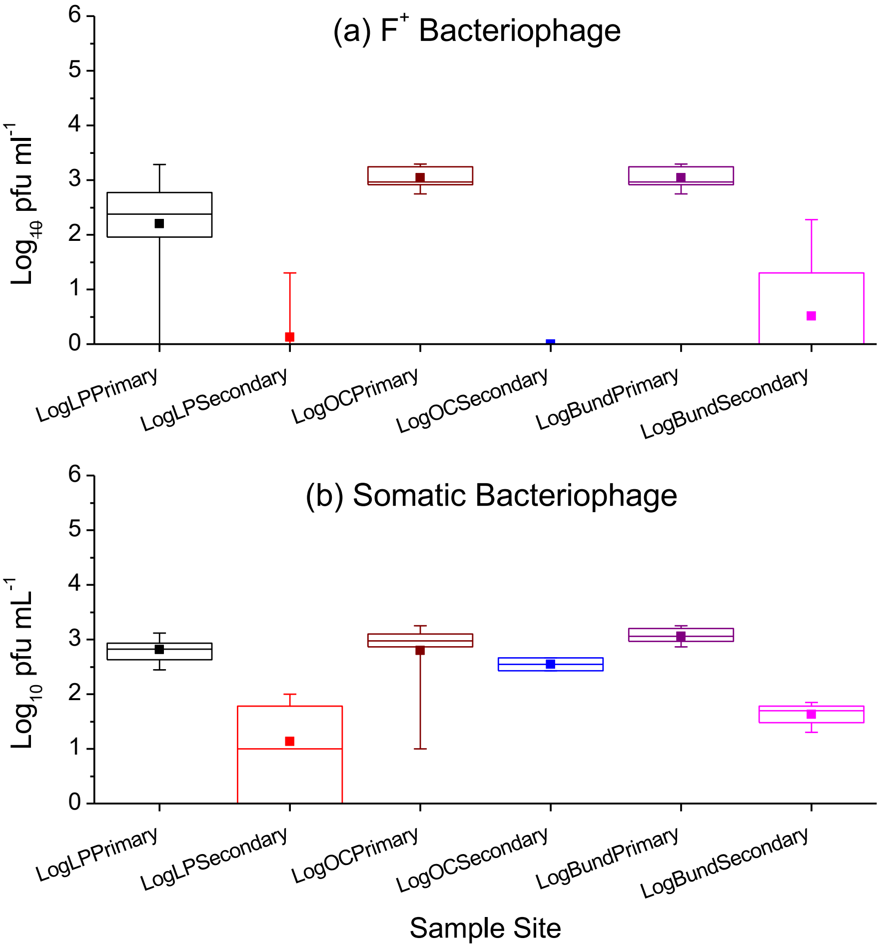

Figure 5. **Average, maximum and minimum $\log_{10}$ pfu mL^{-1} of (a) male specific (F+) and (b) somatic bacteriophage numbers detected in sewage and treated effluent from three different WWTPs. LP = Luggage Point WWTP; OC = Oxley Creek WWTP; and Bund = Bundamba WWTP.** ***(Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range).***

3.3. Cryptosporidium oocysts

Sampling of all three wastewater treatment plants showed that the average number of *Cryptosporidium* oocysts that could be detected in the sewage was consistently between 10^4 and 10^5 oocysts L^{-1} (Table 4). It should be noted that these numbers are total detected oocyst numbers and this is not a measure of viability or infectivity. The data on calculated standard deviations given in Table 4 also shows that the detection of *Cryptosporidium* has the greatest variability of all of the microorganisms studied, particularly in the Oxley Creek WWTP (both sewage and treated effluent) and the Bundamba treated effluent. This may be merely a reflection of the analytical method used (fluorescent microscopy) or may be the nature of oocysts distribution in sewage and their removal in the different WWTPs. The number of oocysts detected in the treated effluent was always much lower than in the sewage (Table 4). No oocysts were detected in any of the treated effluent samples from Luggage Point WWTP, only in the first sample event collected at the Oxley Creek WWTP, and from sample events 1 and 2 at Bundamba WWTP. The number of detected oocysts in any of the treated effluent samples was never greater than a maximum of 3.1 x 10^2 L^{-1} (2.5 $\log_{10}$) in the Bundamba WWTP.

Table 4. Average, maximum and minimum $\log_{10}$ Cryptosporidium oocysts (L^{-1}) detected in each of the three Wastewater Treatment Plants between March 2008 and April 2010[a].

Sample Event	Sewage		Treated Effluent	
	Average (±SD)	Max-Min	Average ±SD	Max-Min
	Luggage Point			
Combined day	4.8 (±0.2)	5.0 - 4.5	0 (0)	NA
Morning	4.8 (±0.2)	5.0 – 4.6	0 (0)	NA
Afternoon	4.8 (±0.2)	4.9 - 4.5	0 (0)	NA
	Oxley Creek			
Combined day	3.1 (±1.9)	4.4 - 0	0.5 (±1)	2.2 - 0
Morning	3.2 (±2.2)	4.4 - 0	0.5 (±1.1)	2.2 - 0
Afternoon	3.1 (±2.1)	4.3 - 0	0.5 (±1.1)	2.2 - 0
	Bundamba			
Combined day	4.6 (±0.3)	5.1 – 4.4	1.1 (±1.3)	2.5 - 0
Morning	4.7 (0.3)	5.1 – 4.5	1.6 (±1.4)	2.5 - 0
Afternoon	4.5 (±0.2)	4.7 – 4.4	0.7 (±1.3)	2.2 - 0

[a] n = 8 samples per site

Based on the determined recovery efficiency of oocysts from sewage of 27% from the Hemaflow™ filters and a subsequent 5% recovery using the IMS beads (see Appendix 2 for details) (meaning that the detection of one oocyst represents approximately 370 oocysts in the original sample) then the recovery corrected number of *Cryptosporidium* oocysts would be likely to be closer to 10^6 oocysts L^{-1} of sewage. These results do not take into account speciation of the detected oocysts or any assessment of viability or infectivity and it was assumed that all the detected oocysts were viable and of human origin.

Based on the determined recovery efficiency from treated effluent (Appendix 2), where it had been determined that the detection of one oocysts represented 100 oocysts L^{-1} in the original sample, this means that all three WWTPs had a capability to reduce *Cryptosporidium* oocysts by at least 2 $\log_{10}$ to 4 $\log_{10}$ (99 – 99.99%) (Figure 6). It is possible that this reduction could be even higher, especially for Luggage Point WWTP; however, the issues relating to recovery efficiencies mean that this could not be determined.

As can be seen in Table 4, there is minimal difference between the number of oocysts detected in the sewage and treated effluent collected either in the morning or afternoon at the Luggage Point and Oxley Creek WWTPs. In comparison, a larger variation in oocysts numbers was observed at the Bundamba WWTP between the morning and afternoon samples. The reasons for this variation could be the size of the plant or sludge retention time as there is not a great difference between the design or operation of the WWTPs, in particular between the Oxley Creek and Bundamba plants. However, the Luggage Point WWTP uses the addition of ethanol to improve the biological activity of the treatment process which may possibly have some impact on the better treatment at this plant compared to the other two.

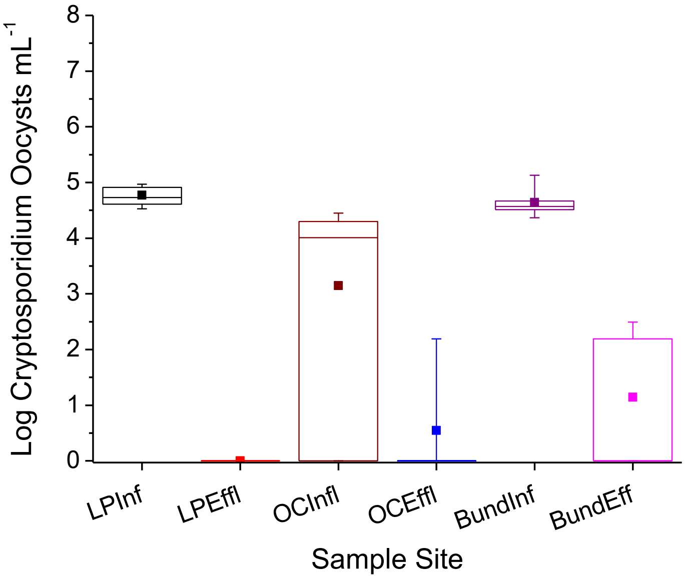

Figure 6. **Log$_{10}$ average and range of Cryptosporidium oocysts numbers per litre of sewage and treated effluent from Luggage Point (LP); Oxley Creek (OC); and Bundamba (Bund) wastewater treatment plants.** ***(Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range).***

3.4 Detection of Enteric Viruses

A dedicated survey of the DNA enteric viruses, adenovirus, polyomavirus and Torque Teno Virus, in the three WWTPs was undertaken between January and April 2010. Earlier, initial attempts had also been undertaken to detect RNA viruses in sewage and treated effluent, principally the enterovirus group and norovirus; however, poor results were obtained due to inhibition of the Reverse Transcriptase PCR reaction. As a result, all subsequent analyses focused on the DNA viruses as the quantitative PCR (qPCR) method had been shown to not be affected by contaminants in the sewage and treated effluent. The results for the sewage given in Table 5 show that all three target viruses were present in numbers ranging from 5 to almost 7 log$_{10}$ PCR Detectable Units (PDU) L^{-1} in the sewage entering the three WWTPs. While the numbers of the three viruses are similar between the three WWTPs, it can be observed that polyomavirus had a small trend to having the highest average numbers L^{-1} of the three viruses in the raw sewage. The virus numbers detected in the treated effluent were also similar between the three treatment plants, although this time, polyomavirus had the lowest detectable numbers of the three viruses in all of the treatment plants. This suggests that polyomavirus is more susceptible to the wastewater treatment processes used than adenovirus or Torque Teno Virus.

The adenovirus numbers detected in the sewage in this study are higher than the levels of 1 to 4 log$_{10}$ quoted in the Australian Guidelines for Water Recycling (NRMMC-EPHC-AHMC 2006), however, it needs to be recognised that the numbers cited in the Australian Guidelines are from published data from North American studies that used cell culture techniques. The reason for this higher detected number is most likely due to the use of qPCR in the current study which detects all viral particles whether they are infectious or not. This has been shown previously by Muscillo *et al.* (2008) who were able to detect adenovirus genomes in 31 of 43 wastewater and environmental water samples but no infectious viruses by cell culture. In comparison, cell culture techniques detect only infectious viral particles but can often underestimate the number of viruses present due to inhibition of the cell culture by contaminants in the wastewater and by not all potentially infectious viral particles causing a detectable infection (Rodriguez *et al.,* 2009). The ability to accurately detect the number of infectious viral particles in water samples such as raw and treated wastewater remains a problem and a research area for a number of laboratories around the world. The overestimation of infectious viral particles by qPCR could lead to an overestimation of the health risks posed by the wastewater if the information was used solely for determining absolute numbers in sewage and treated effluent. A major aim of this study, however, has been to investigate the ability of the wastewater treatment plants to remove viruses and other pathogens (i.e., $\log_{10}$ reduction), rather than on the actual numbers detected. Thus, it has been assumed that the log reduction in virus numbers between the sewage and treated effluent would be similar regardless whether qPCR or cell culture methods were used.

Table 5. $\log_{10}$ average, range and percent reductions of PDU[a] L^{-1} of enteric viruses in sewage and treated effluent at the Luggage Point, Oxley Creek, and Bundamba Wastewater Treatment Plants [b].

Enteric Viruses	Sewage		Treated Effluent	
	Average (±SD)	Max-Min	Average ±SD	Max-Min
	Luggage Point			
Adenovirus	5.8 (±0.3)	6.7 - 5.2	2.9 (±0.3)	3.7 – 2.5
Polyomavirus	6.0 (±0.3)	6.4 – 5.6	2.4 (±0.3)	2.8 – 1.7
Torque Teno Virus	5.6 (±0.5)	6.6 – 5.0	2.9 (±0.5)	3.9 – 1.9
	Oxley Creek			
Adenovirus	5.7 (±0.4)	6.4 – 5.0	3.0 (±0.5)	4.1 – 2.2
Polyomavirus	6.0 (±0.2)	6.4 – 5.6	2.5 (±0.4)	3.1 – 1.9
Torque Teno Virus	5.5 (±0.3)	5.9 – 4.9	2.7 (±0.5)	3.6 – 1.9
	Bundamba			
Adenovirus	5.8 (±0.4)	6.2 – 5.2	2.7 (±0.5)	3.6 – 1.9
Polyomavirus	6.1 (±0.3)	6.2 – 5.8	2.1 (±0.3)	2.7 – 1.7
Torque Teno Virus	6.0 (±0.3)	6.5 – 5.6	3.0 (±0.6)	4.1 – 2.4

[a] PDU = PCR Detectable Units
[b] n = 8 samples per site

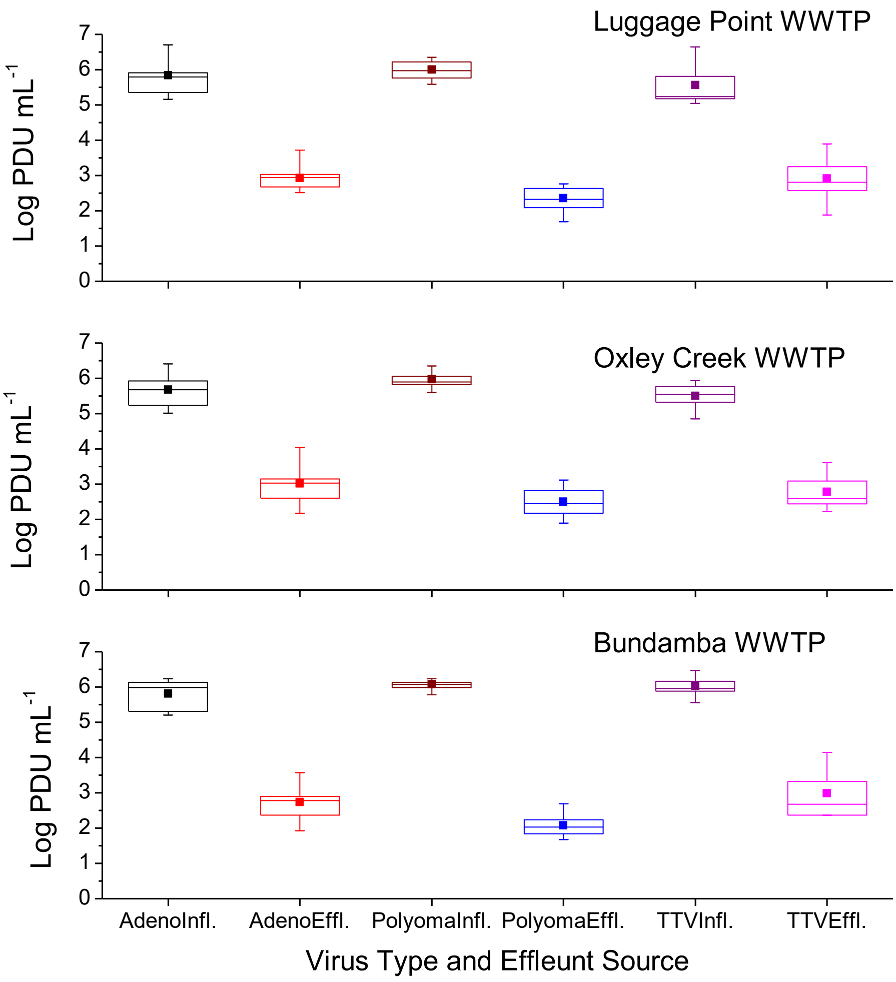

Figure 7. **Log_{10} average numbers and range of adenovirus (Adeno), polyomavirus (Polyoma) and Torque Teno Virus (TTV) PDU L^{-1} in the sewage and treated effluent at the Luggage Point, Oxley Creek, and Bundamba Wastewater Treatment Plants. (*Box plot represents in-filled square = the average, the open box = 25 – 75 percentiles, and the whiskers = maximum and minimum range*).**

4. REDUCTION OF ENTERIC MICROORGANISMS IN WASTEWATER TREATMENT PLANTS

Table 6 provides the information collated on the differences between the average numbers detected in the sewage and in the treated effluent. This difference is an indication of the average $\log_{10}$ removal that could be expected from each of the treatment plants studied. While no one treatment plant outperformed the other two plants overall, the Luggage Point WWTP gave the highest removal rates for *E. coli*, enterococci and *Cryptosporidium*; the Oxley Creek WWTP had the largest removal rates for *Campylobacter*, *Salmonella* and the F^+ bacteriophage, and the Bundamba WTTP was the most effective at removing the enteric viruses and somatic phage. It should be noted again for the Oxley Creek WWTP, that these comparisons are from microbial numbers detected in the treated effluent prior to the UV treatment stage. If this treatment step was taken into account, then the Oxley Creek WWTP would have the best removal rates for the bacteria and bacteriophage (the influence of UV on the viruses and *Cryptosporidium* oocysts is difficult to determine due to the detection methods used). It is unclear on the reasons for the better or reduced performance for the different treatment plants for removing the different enteric microorganisms and more work on issues such as plant design and operation would need to be examined to see if these factors have any influence on the relative removal rates.

The calculated percent reductions given in Table 6 show that the *Cryptosporidium* oocysts, enteric viruses, *E. coli* and enterococci were all removed by 99% or more in all three of the treatment plants. *Salmonella* and somatic bacteriophage were reduced less than the other microorganisms, although still reduced by more than 90% (apart from somatic bacteriophage in the Oxley Creek plant). In addition *Campylobacter* and the F+ bacteriophage were reduced by 96% in the Bundamba treatment plant.

The $\log_{10}$ reduction data given in Table 6 shows that all of the wastewater treatment plants were able to reduce the number of the target bacteria, apart from *Salmonella,* by up to 3 $\log_{10}$. Salmonella reduction was between 1.4 and 1.7 $\log_{10}$ which may be a reflection on the lower detectable numbers in the untreated sewage rather than any lack of treatment capacity of the treatment plants. The treatment plants were also shown to have an ability to remove between 2.6 and 4.8 $\log_{10}$ of *Cryptosporidium*, 2.7 to 4 $\log_{10}$ of the enteric viruses and between 1.1 to 3.1 $\log_{10}$ of the bacteriophage (assuming the UV treatment at Oxley Creek Wastewater Treatment Plant is taken into account).

These levels of treatment generally are at least in agreement with the indicative $\log_{10}$ removals expected for secondary treatment by wastewater treatment plants given in Table 3.4 of the Australian Guidelines for Water Recycling (NRMMC-EPHC-AHMC 2006). This demonstrates that all three plants are at least operating at expected efficiencies. In some cases, notably *E. coli* and F^+ bacteriophage, the determined removal rates were slightly higher than quoted in the guidelines.

Of particular interest are the $\log_{10}$ removals for the enteric viruses and *Cryptosporidium* oocysts. The reuse guidelines quote a removal rate of 0.5 – 2.0 $\log_{10}$ for enteric viruses and 0.5 – 1.0 $\log_{10}$ for *Cryptosporidium* oocysts. In the current studies, virus removal was found to be between 2.6 and 4 $\log_{10}$ and the removal of *Cryptosporidium* between 2.6 and 4.8 $\log_{10}$. One reason for the large difference in removal of the viruses is the use of qPCR in the current study compared to cell culture techniques in the other studies cited in the Australian Guidelines. The other potential major reason is that all of the studies cited in the guidelines were undertaken in North America which mostly has a very different climate to SEQ. Studies undertaken in Melbourne and Adelaide after the development of the Australian Guidelines have indicated that removal rates in these regions of Australia fit the rates cited in the Guidelines. This adds weight to the hypothesis that climatic conditions (for example, the temperature of the water) may have an important role in the efficiency of wastewater treatment plants to remove pathogens. The impact of climate on removal efficiencies is also a likely reason for the higher removal rates in this study than those given in the Australian Guidelines.

A similar study on the removal of *Cryptosporidium* oocysts at Melbourne WWTPs found only a 0.9 $\log_{10}$ removal rate (McAuliffe and Gregory 2010). It was determined that the basic analytical methods (immunomagnetic capture and antibody staining) used were virtually the same as used in the current study, thus it can be hypothesised that the differences may be due to location dependent factors such as climate. In another study of enteric microorganisms removal using a bench scale activated sludge reactor run at 25°C, Wen *et al.* (2009) determined a removal rate of 2.4 $\log_{10}$ for *Cryptosporidium* oocysts. A comparison of these results, however, with previous results of *Cryptosporidium* oocysts removal at the Bolivar WWTP in Adelaide (which was the source of the sewage used in the batch reactor), showed that the removal of oocysts in the full WWTP was only 0.7 $\log_{10}$, which was much lower than in the laboratory reactor. Other factors, however, such as how the data is interpreted, the use of 95% data vs mean data and initial *Cryptosporidium* oocysts numbers entering the treatment plants may also have an impact on the observed differences in removal rates. It is recommended, therefore, that more analysis of *Cryptosporidium* oocysts removal in SEQ wastewater treatment plants be considered to confirm the observations above relating to the different removal rates observed in the different regions of Australia.

A closer examination of the differences between these studies (referenced and current) is warranted to further determine if issues relating to climate are the major reason for the observed differences in $\log_{10}$ removals. Wen *et al.* (2009) hypothesised that the most likely reason for this difference in removal rates observed in their study between the laboratory system and the full WWTP was temperature, with the WWTP being subjected to larger ranges of temperature than the laboratory system. Temperature has been previously considered an important factor in the removal of pathogens in wastewater treatment plants (Stevik *et al.* 2009) which could suggest the reason for the differences in the $\log_{10}$ removal rates reported above in the different wastewater treatment plants in the different Australian States (Victoria, South Australia and Queensland). If this is indeed the case, then it adds to the suggestion that the Australian Guidelines for Water Recycling may need to be updated with data from studies undertaken in the different regions of Australia to allow data to be used that is relevant to Australian conditions.

Table 6. Average $\log_{10}$ and percent reductions of enteric microbes in the three studied SEQ wastewater treatment plants.

Type of Microbe		Luggage Point		Oxley Creek		Bundamba	
	Microorganism	Log_{10} Reduction	% Reduction	Log_{10} Reduction	% Reduction	Log_{10} Reduction	% Reduction
Bacteria[a]	*E. coli*	4.1	99.99	3.3	99.95	2.7	99.80
	Enterococci	3.8	99.98	2.6	99.80	2.5	99.68
	Campylobacter	3.0	99.90	2.0	99.00	1.4	96.02
	Salmonella	1.4	96.02	1.7	98.00	ND	ND
Protozoa[b]	*Cryptosporidium*	4.8	100	2.6	99.71	3.5	99.84
Enteric virus[b]	Adenovirus	2.9	99.78	2.7	99.96	3.1	99.86
	Polyomavirus	3.6	99.97	3.5	99.95	4.0	99.98
	Torque Teno Virus	2.7	99.37	2.8	99.58	3.0	99.85
Bacteriophage[c]	F Phage	2.3	99.62	3.1	100	1.8	96.72
	Somatic Phage	1.3	92.14	0.5	61.01	1.6	96.00
	Somatic post-UV	NA	NA	1.1	87.68[d]	NA	NA

a = $\log_{10}$ 100mL^{-1}; b = $\log_{10}$ L^{-1}; c = $\log_{10}$ mL^{-1}; d = % Reduction from primary effluent

5. CONCLUSIONS

The research undertaken in this study has indicated that the three wastewater treatment plants based in SEQ are very efficient in removing faecal bacteria, bacteriophage and enteric pathogens. The treatment plants were capable of removing between 1.4 $\log_{10}$ of detectable *Salmonella and Campylobacter* cells to as much as 4.8 $\log_{10}$ of *E. coli*; between 2.6 and 4.8 $\log_{10}$ of detectable *Cryptosporidium* oocysts; between 2.7 and 4.0 $\log_{10}$ of DNA enteric viruses (measured as virus genomes); and up to 3.1 $\log_{10}$ of bacteriophage. The percent reduction of all of the microorganisms was 99% or higher apart from *Salmonella, Campylobacter* and the F^+ bacteriophage in the Bundamba treatment plant and the somatic bacteriophage in all three treatment plants. When considering removal of the pathogenic microorganisms, the worst removal was for *Salmonella* at the Luggage Point treatment plant which was still capable of a 1.4 $\log_{10}$ (96.02%) removal (from low starting numbers).

These removal rates are generally higher than have been determined in WWTPs in the southern states of Victoria and South Australia (McAulliffe and Gregory, 2010; Wen *et al.,* 2009). The reason for these higher removal rates remains to be determined but could quite likely be differences in climatic conditions, with SEQ generally being warmer, particularly in winter, than the southern states of Victoria and South Australia.

The efficiency of the removal of microbial pathogens by wastewater treatment plants in SEQ, as demonstrated by the results in this study, also has implications for the reuse of the treated wastewater. The number of several of the enteric microorganisms detected in this study has differed from the numbers cited in the Australian Guidelines for Water Recycling as well as the $\log_{10}$ reductions that can be expected from a combination of primary and secondary treatment (NRMMC-EPHC-AHMC, 2006). This means that treated effluent produced by SEQ WWTPs may have a lower inherent health risk from microbial pathogens than may be currently assumed if using the Guidelines values. Also, as the Western Corridor Recycled Water Scheme did not have access to any hard data on the actual efficiency of SEQ WWTPs for removing pathogens prior to this study, a decision was made that, in the worst case scenario, pathogen removal had to be performed exclusively at the advanced water treatment plants. The results of this study suggest that, under appropriate conditions, WWTPs could be an effective treatment barrier for pathogens and, as a result, could be able to be assigned greater treatment credits than outlined in the Australian Guidelines for Water Recycling. It should be noted that a larger number of samples should be analysed than was possible in this study in order to obtain accurate 95% and 5% confidence levels for the log removal rates. Despite this, the results presented here suggest that WWTPs, at least in SEQ, could reduce the reliance on Advanced Water Treatment Plants to remove microbial pathogens, thus increasing confidence and security of water recycling schemes.

6. RECOMMENDED FURTHER RESEARCH

While the research presented in this report has shown that SEQ WWTPs are very effective in removing microbial pathogens and indicator bacteria, there remain a series of questions and issues that require further investigation, or were not completely resolved during this study. Recommended further research for consideration to further enhance the findings of this study should include:

- ***Larger data set to obtain more accurate confidence levels*****:** Due to the nature and scope of the study, the number of samples taken for the analysis of pathogens was limited, particularly for *Cryptosporidium* and the enteric viruses. The results from at least 20 samples (both for sewage and treated effluent) should be used to obtain accurate 5% and 95% confidence limits on log reduction of pathogens across the wastewater treatment plants. This amount of required data could be achieved in a targeted study over a 24-month period if sufficient research capacity could be provided using the methodologies presented in this report.
- ***Testing of WWTPS under stressed conditions***: The WWTPs were all operating optimally during the sampling period undertaken in this study. It remains to be determined how different the removal efficiencies would be, particularly for *Cryptosporidium* and viruses, when a WWTP is operating under stressed conditions. This is a difficult process, as it is sometimes difficult to identify the time when and how to effectively sample, a "stressed" treatment plant. Often, this information becomes available only when a "fortuitous" event occurs at around the time of a planned sampling event. It is recommended that consideration be given to construct a small package plant that can be used for research processes where the plant can be stressed or modified to determine what impact this has on the removal efficiencies of a range of contaminants, both chemical and biological, under less than optimal conditions.
- ***Correlations with other wastewater treatment parameters**:* Due to issues relating to capacity and capabilities of the staff undertaking the research reported in this study, there were no attempts to link pathogen numbers (and associated $\log_{10}$ reductions) with other parameters that could be used as on-line and/or real-time indicators of WWTP efficiency that could be used as a measure for assessing pathogen removal. This could be achieved via a further study or by obtaining historical data from the treatment plant operators and comparing with the detection results during individual sampling events in this study.
- ***Data on a wider range of microbial pathogens***: There should be attention given to the detection and removal of a wider range of viruses and *Giardia* cysts in WWTPs. The viruses detected in this study were all DNA viruses, which are easier to detect in raw and treated effluent when using PCR due to the greater robustness of the PCR system as opposed to the reverse transcriptase PCR (RT-PCR) used for RNA viruses. There are several RNA viruses, notably norovirus, the enterovirus group and rotavirus, that can result in major disease burden in communities and, thus, it would be highly valuable to also determine the ability for SEQ WWTPs to remove these viruses. Likewise, while *Giardia* cysts are considered to be less environmentally stable than *Cryptosporidium* oocysts (NRMMC-EPHC-AHMC, 2006), there are often more *Giardia* cysts in wastewater than *Cryptosporidium* oocysts (NRMMC-EPHC-AHMC, 2006), so more information on the removal efficiencies for *Giardia* cysts in SEQ WWTPs would be valuable.
- ***Further testing for Campylobacter and Salmonella***: Both *Campylobacter* and *Salmonella* are known to cause a significant disease burden in Australian communities and more information than was obtained in this study is required for both bacteria. In the current study, *Salmonella* results are reported from the first six months of sampling only, as there were errors in the analysis for the remaining 11 months. In addition, the detected *Salmonella* colonies were not speciated, so there is limited information on the predominance of different species of *Salmonella* and their relative removal in SEQ WWTPs. Likewise, there were laboratory errors in the analysis of *Campylobacter* during the study, hence more information on *Campylobacter* is warranted, especially as this bacterial pathogen was detected in numbers much higher than reported in the Australian Guidelines for Water Recycling. Also, like *Salmonella*, the

Campylobacter colonies were not speciated; some preliminary further work post this study has suggested that not all of the *Campylobacter* colonies isolated on the CCDA medium are the major human pathogenic species *C. jejuni* and *C. coli*. More testing of *Campylobacter* in sewage and samples across the WWTP treatment processes (including on the isolation methods used) is warranted to obtain an accurate number of the pathogenic *Campylobacter* species, which can then be used to obtain a more accurate health risk assessment for this bacterium.

- ***Improved detection of infective Cryptosporidium oocysts and enteric viruses***: The methods used in this study for the detection of *Cryptosporidium* oocysts (i.e., direct microscopy) and enteric viruses (qPCR) are accurate in determining total number of each organism. However, neither method is capable of determining the infectivity of the detected oocysts or viruses. When assessing the actual number of these microorganisms detected in a sample, these methods can lead to an over-estimation of the number of viable/infective pathogens present, which, in turn, can lead to an over-estimation of the health risks associated with that water. Despite this, the major aim of this study was to obtain the $\log_{10}$ reductions of various enteric microorganisms across SEQ wastewater treatment plants. It is probable that the calculation of log reductions is less influenced by the detection method used (assuming that changes in numbers are similar whether using detection of genomic units or cell infectivity). The lack of data on infectivity, however, means that the $\log_{10}$ reduction data could be different if the viability/infectivity status is much lower in the treated effluent than has been assumed for the qPCR results. An improved analysis that determines infectivity along with accurate quantitation may show that the WWTPs could potentially be capable of an even greater $\log_{10}$ reduction of these pathogens than has been calculated here. There are methods that are being developed, both in Australia and internationally, on the accurate and rapid determination of infective *Cryptosporidium* and viral particles. It would be of great benefit to the water industry in Queensland if these methods could be further developed to provide greater accuracy within a reasonable timeline and cost for the detection of viable or infective pathogens in wastewater samples.
- ***Efficiency of large municipal WWTPs compared with smaller regional WWTPs***: The findings presented in this study are based on research undertaken at three large municipal WWTPs in the greater Brisbane and Ipswich area. These plants treat very large volumes of sewage and are staffed 24 hours a day, 365 days a year. There is a potential that the findings on the efficiency of WWTPs to remove pathogens presented in this report may not be accurate for smaller regional plants which process smaller, more variable flows of sewage and are commonly remotely operated. A study comparing the removal efficiencies of smaller WWTPs with the larger municipal plants would be warranted as small communities are often more reliant on recycled water from these plants to irrigate sporting complexes and other recreational facilities and amenities. Thus, there may be a different health risk to these communities compared to larger SEQ urban areas using recycled water from the large WWTPs.

APPENDIX 1. Detection Methods for Microorganisms

A1.1 Concentration of Collected Samples

The concentration of collected sewage and treated effluent samples used in the *Cryptosporidium* and enteric virus analyses was undertaken using a combination of Hollow fibre, tangential flow filtration and centrifugal concentration devices. The concentration stages used hollow fibre filtration Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) followed by Jumbosep™ (Pall) centrifugal devises (using a 100,000 MWCO filter) prior to analysis for *Cryptosporidium* oocysts and enteric viruses. For the influent samples, before concentration, the samples were mixed with 4L of sterile Milli-Q water to assist with flow through the Heomflow filters.

Briefly, the water sample to be concentrated was pumped with a peristaltic pump (Masterflex: Cole Palmer Instrument Co, USA) in a closed loop with high-performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole Palmer Instrument Co.). In between sampling events, the tubing were cleaned and disinfected by soaking in 1% (v/v) bleach followed by washing with distilled water and then sterilization by autoclaving. At the end of the concentration process, pressurised air was passed through the filter cartridge from the top to recover as much water as possible. The samples were concentrated to approximately 70 mL using the Hemoflow cartridges. Further concentrations using the JumboSep devices were undertaken as per the manufacturer's instructions. A final volume of approximately 10mL was routinely achieved using the JumboSep devices. The resulting concentrate volume was then split with 1 mL used for the detection of *Cryptosporidium* oocysts and 1 mL viruses. Any remaining concentrate sample was archived by freezing at -80 °C.

A1.2 Analytical Methods for Bacteria

For the detection of the number of *E. coli*, and enterococci in both the sewage and treated effluent, samples were homogenised on a bench top vortexer for at least 60 seconds to achieve a uniform suspension and then serially diluted in sterile deionised water. 50 µL replicates of each dilution were then spread plated onto the appropriate isolation media (Table A.1) which was then incubated aerobically at 44°C for *E. coli* and 37°C for enterococci for a minimum of 20 hours.

Detection of *Campylobacter* spp. involved plating 100 µL samples onto modified CCDA agar. These inoculated plates were incubated at 37°C for up to 48 hours in gar jars using Oxoid Campy gas sachets to create a microaerophilic environment.

The detection of *Salmonella* spp. involved an enrichment step. This involved enriching 5mL samples of sewage or secondary effluent in buffered peptone water overnight at 37°C. After this enrichment 10 mL, 1 mL and 100µL samples were incubated at 37°C overnight in 90 mL, 9 mL and 0.9 mL respectively of RV Broth as an MPN test. Samples that showed positive growth in any of the MPN dilutions were then spread plated onto XLD agar for confirmation of the presence of *Salmonella* spp. cells.

After incubation all inoculated media plates with filters were visually inspected for the presence of colonies that had characteristics representative of the target microorganisms (Table A.1) growing on the surface of the media. Comparison to positive controls of media inoculated with standard strains was also done on each occasion to aid in identifying presumptive positive colonies. The number of bacterial colonies displaying the characteristics of the target bacterial strain was counted as presumptive positive results. Representative presumptive positive colonies on each plate were subcultured and further tested via PCR to confirm that they were isolates of the target bacterium. The recorded results for numbers of detected bacterial strains was then amended if necessary based on the outcomes of the confirmation tests.

Table A.1. Isolation media used to detect enteric bacteria in sewage and treated effluent

Enteric Bacteria	Isolation Medium	Characteristics of Representative Bacterial Colonies
E. coli	Chromocult® coliform Agar (Merck) or mfc agar (Oxoid)	Violet colonies (Chromocult) Pink colonies surrounded by yellowing media (mfc)
Enterococcus spp.	Chromocult® enterococci Agar (Merck) or Bile Aesculin agar (Oxoid)	Pink Colonies (Chromocult) Brown colonies (Bile Esculin)
Salmonella spp.	RV enrichment broth with XLD Agar (Difco) for confirmation	Growth in the enrichment broth indicating presence of salmonella. For XLD media Black colonies surrounded by yellowing of the media
Campylobacter spp.	CCDA + selective supplement (Oxoid)	Cream coloured colonies

A1.3 Analytical Methods for Bacteriophages

Both the somatic and F^+ bacteriophage were detected by culture methods using the double overlay method described by Havelaar *et al.* (1984) using TYGA medium supplemented with antibiotics. Before plating, the sewage was diluted tenfold in to steps to 1:100. 100 µL (0.1 mL) of the 1:10 and 1:100 dilutions are then used as the inoculum source. The treated effluent was used neat with no dilution (again as a 100 µL volume). In brief, this involves the mixing of a diluted or non-diluted sample with the appropriate *E. coli* host in 3 mL of melted semi-solid agar of the TYGA isolation medium and pouring this suspension over the surface of a petri dish containing TYGA medium. The semisolid agar was allowed to set and the plates incubated at 37°C overnight. Five replicates of each sample were plated. The principal of the double overlay method is that the *E. coli* host grows as a lawn in the semisolid agar. The presence of any coliphage is indicated by a small circular area of clearing in the *E. coli* lawn (known as plaques) due to lysis of the *E. coli* host cells by the phage.

After overnight incubation the inoculated plates were visually examined for the presence and number of bacteriophage plaques in the *E. coli* lawn. These were recorded as the number of plaque forming units and dilutions factored in for back calculating to final numbers per mL of original sample.

A1.4 Detection Method for *Cryptosporidium* oocysts

Detection of *Cryptosporidium* oocysts in both sewage and treated effluent was achieved using immunomagnetic capture and antibody staining. This method was based on the principals of the USEPA method 1623 but has been modified in the lab to suit working with wastewater where the in-line filters recommended by the USEPA method would not work due to clogging issues.

Prior to concentration of the sewage samples, due to the heavy loading of suspended solids these samples were centrifuged at slow speed (800 xg) for 10 minutes to pellet the solids while maintaining the oocysts in suspension (the recovery efficiency tests done outlined in Appendix 2 determined that there was minimal loss of oocysts during this slow centrifugation). The supernatant was then decanted for concentration through the Hemoflow filters. 10 mL of sterile distilled water was also added to the pelleted solids, the resulting suspension was sonicated for 10 minutes in a sonication bath and 1 mL of the supernatant removed and tested for *Cryptosporidium* oocysts using immunomagnetic capture.

Following concentration, a 1mL volume was taken from the concentrate and treated with anti-*Cryptosporidium* IMS beads (Dynabeads™) as per the manufacturer's instructions. The eluate from the IMS beads was then placed onto a well slide, dried and stained with fluorescein labelled anti-*Cryptosporidium* antibody stain (EasyStain™ BTF) as per manufacturer's instructions and viewed under a fluorescent microscope using x100 magnification. A total of 20 fields of view were examined for the presence of stained *Cryptosporidium* oocysts. The number of detected oocysts in the 20 fields of view were then averaged and used to calculate the number of oocysts per litre in the original sample.

A1.5 Detection Methods for Human Enteric Viruses

The detection method specifically involved extracting viral DNA from a 200 µL portion of the 1mL of sample concentrate collected and processed as described above in section A1.1. Viral DNA was extracted from the water concentrate using the Blood and Tissue DNA Extraction Kit (Qiagen) using the manufacturer's instructions.

The extracted viral DNA was then amplified using quantitative PCR using adenovirus and polyomavirus specific primers to determine the number of these viruses in the primary and secondary wastewater samples. The primers used for the detection of adenovirus were the primer set Heim I and Heim II (Heim et al., 2003) (this primer set detects all 51 types of the human adenoviruses genus). Detection and quantification of the amplification products was achieved using the DNA-binding dye SYBR Green. Polyomavirus was detected using the primer set detailed by McQuaig et al. (2009) with detection and quantification of the specific amplification product achieved through the use of a specific Taqman probe. The Torque Teno Virus target DNA was amplified using the published Taqman probe set (Tokita *et al.,* 2002) The sequence of all primer sets and probes is given in Table A.2.

Quantitative PCR reactions were performed on Bio-Rad iQ5 (Bio-Rad Laboratories, California, USA), using iQ Supermix (Bio-Rad). Each 25µL PCR reaction mixture contained 12.5 µL of SuperMix, 120 nM of each primer, and 3 µL of template DNA. Bovine serum was added to each reaction mixture to a final concentration of 0.2 µg μL^{-1} to remove PCR inhibition (Kreader 1996). For each PCR experiment, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included.

Table A.2. PCR primers used for the detection of adenovirus and Polyomavirus.

Primer	Adenovirus	Polyomavirus	Torque Teno Virus
Forward	5′- GCC ACG GTG GGG TTT CTA AAC TT - 3′	5′- AGT CTT TAG GGT CTT CTA CCT TT - 3′	5'- CGG GTG CCG **D**AG GTG AGT TTA CAC-3'
Reverse	5′- GCC CCA GTG GTC TTA CAT GCA - 3′	5′- GGT GCC AAC CTA TGG AAC AG - 3′	5'-GAG CCT TGC CCA T**R**G CCC GGC CAG-3'
Probe	5'-(FAM)-TGC ACC AGA CCC GGG CTC AGG AGG TAC TCC GA -(BHQ1)-3'	5' - (FAM)-TCA TCA CTG GCA AAC AT-(TAMARA) - 3'	5'-(FAM)-AGTC AAG GGG CAA TTC GGG CTCG GGA-(TAMRA)-3'

Thermal cycling conditions for adenovirus were as outlined in Sidhu et al., (2010). Detection of the A melt curve analysis was performed after the PCR run to differentiate between actual products and primer dimmers, and to eliminate the possibility of false-positive results. The melt curve was generated using 80 cycles of 10 s each starting at 55°C and increasing in 0.5°C intervals to a final temperature of 95°C.

Thermocycling conditions for polyomavirus were an initial denaturation at 95ºC for 5 min, followed by 40 cycles at 95ºC for 20 s, 55ºC for 15 s, 60ºC for 60 s. As detection of the amplicon was achieved through the use of a Taqman probe no melt curve was used. As for adenovirus, the melting temperature(Tm) for each amplicon was determined using the iQ5 software.

Thermocycling conditions for Torque Teno Virus were an initial denaturation at 95ºC for 5 min, followed by 40 cycles at 95ºC for 20 s, 62ºC for 20 s, and 72ºC for 20 s. As detection of the amplicon was achieved through the use of a Taqman probe no melt curve was used.

The melting temperature(Tm) for the amplicon of each virus was determined using the iQ5 software.

APPENDIX 2. Assessed Recovery Efficiencies of *Cryptosporidium* Oocysts from Primary and Secondary Effluent using Hollow Fibre Filtration and IMS

Table A 3. Recovery efficiency of concentration methods for the detection of *Cryptosporidium* oocysts.

Treatment Step	Sewage		Treated Effluent	
	Recovery Efficiency (%)	Hypothetical oocyst numbers	Recovery Efficiency (%)	Hypothetical oocyst numbers
Hypothetical Minimum oocysts Viewed under microscope		1		1
IMS recovery efficiency[1]	**5**	20	**5**	20
Minimum concentrate volume (5mL)		100		100
Hemaflow recovery efficiency	**27**		**100**	
Number in original volume[2]		**370**		**100**

[1] Recovery from 1 mL of concentrate
[2] Based on the original 1 oocyst detected.

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