Australian Water Recycling
Centre of Excellence

Project Report

Development of Validation Protocol for Activated Sludge Process in Water Recycling

A report of a study funded by the
Australian Water Recycling Centre of Excellence

CSIRO, December 2015

Development of Validation Protocol for Activated Sludge Process in Water Recycling

Project Leader

Dr Simon Toze
Land and Water Flagship
CSIRO
Ecosciences Precinct
42 Boggo Rd
Dutton Park Qld 4102 AUSTRALIA
Telephone: +61 7 3833 5572

Contact: Dr Simon Toze Simon.Toze@csiro.au

Partners

University of New South Wales
South East Water
Queensland Urban Utilities
WA Water Corporation
TasWater

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.

ISBN: 978-1-922202-69-7

Citation:

Sidhu, J.P.S., Ahmed, W., Hodgers, L., Smith, K., Palmer, A., Wylie, J., Low, J., Nichols, C. and Toze, S. (2015). *Development of Validation Protocol for Activated Sludge Process in Water Recycling*, Australian Water Recycling Centre of Excellence, Brisbane, Australia.

Date of publication: December 2015

Publisher:
Australian Water Recycling Centre of Excellence
Level 5, 200 Creek Street, Brisbane, Queensland 4000
www.australianwaterrecycling.com.au

This report was funded by the Australian Water Recycling Centre of Excellence through the Australian Government's National Urban Water and Desalination Plan.

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Development of Validation Protocols for Activated Sludge Process in Water Recycling

NATVAL 2.2 Sub Project 3

Jatinder Sidhu, Warish Ahmed, Leonie Hodgers, Kylie Smith, Andrew Palmer *et al.,*

20th December 2015

Australian Water Recycling Centre of Excellence

Acknowledgments

This report was funded by the Australian Water Recycling Centre of Excellence through the Australian Government's National Urban Water and Desalination Plan. We gratefully acknowledge the support of Queensland Urban Utilities, South East Water, WA Water Corporation, and TasWater for providing access to the wastewater treatment plants for sample collection and sharing their existing data on physicochemical parameters from treatment plants. In particular we would like to acknowledge the support and assistance provided by Bill Collie and Wakib Khan from Queensland Urban Utilities, Ahmet Kayaalp from WA Water Corporation, Judi Marshall, Graham Boxall and Luc Richardson from TasWater and David Smith from South East Water in Melbourne. We would also like to thank David Beale for his support with PCA analysis.

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List of Acronyms

AGWR	Australian Guidelines for Water Recycling
AS	Activated sludge
ASP	Activated sludge process
BOD	Biological oxygen demand
COD	Chemical oxygen demand
DO	Dissolved oxygen
HRT	Hydraulic retention time
ICC	Integrated cell culture
IMS	Immune-magnetic separation
LLOQ	lower limit of quantification
LRV	Log removal value
MBR	Membrane bioreactor
MLSS	Mixed liquor suspended solids
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
qPCR	quantitative PCR
RT-PCR	Reverse transcriptase-PCR
SRT	Sludge retention time
SS	Suspended solids
TOC	Total organic carbon
UASB	Up-flow anaerobic sludge blankets
WWTP	Wastewater treatment plant

Executive summary

Decreasing rainfall, frequent drought and population growth in urban environments along with an overall desire to achieve greater water sustainability have increased the demand for alternative water sources such as recycled water. This has resulted in an increased attention on the types of contaminants in wastewater and the need to protect the health of the public while implementing these water sustainability initiatives. One important means of safeguarding appropriate health standards is to ensure that contaminants are removed to appropriate levels in the treated water. This means that there needs to be the correct controls and monitoring of the treatment processes to continually meet the determined treatment requirements. One of the important initial steps is to be able to accurately validate what removal capacity a treatment process can achieve when it is operating optimally, and what conditions can cause failure in the established removal capacity.

Many water recycling schemes use wastewater treatment plants (WWTPs) as a common treatment process. WWTPs have traditionally been designed and operated to maximise the removal of nutrients and suspended solids from municipal wastewater to prevent environmental contamination. These WWTPs are now frequently required to produce high quality water (alone or in conjunction with other treatment steps) that can be recycled for a variety of direct or indirect potable reuse, agricultural irrigation, managed aquifer recharge, industrial use, recreational use and environmental enhancement. A large majority of these WWTPs utilise the activated sludge process (ASP) as a major treatment process.

Due to the inherent complexity of the activated sludge process, to date data on the level of contaminant removal has been sparse and conflicting. In addition, differences in the design of the ASP process, the types of contaminants studied, along with variations in sampling and detection methodologies have made it difficult to gain an accurate understanding of the treatment capability of ASPs. This lack of adequate data precludes the development of adequate validation steps that can assist in establishing appropriate removal credits.

The potential public health risk associated with recycled water predominantly originates from the potential presence of enteric viruses and protozoan parasites due to their high infectivity and low dose. These pathogens are also recognised to have high environmental resistance and are commonly found in higher numbers in untreated municipal wastewater than in other environmental sources. The presence of viruses in treated water used for recycling may vary according to the type of treatment process, population size, geographical location and prevalence of disease in the community. This makes it difficult to generalise what and how much treatment a WWTP must achieve (Gerba *et al*., 2013). This means that any assessment of the treatment capacity of an ASP within a wastewater treatment train needs to be assessed on an individual basis, taking into account the common microbial constituents present in that wastewater, and how well the ASP performs under local conditions.

The overall aim of this project was to collect data on pathogen removal in activated sludge plants that could be used in the development of a validation protocol to provide a standardised format for validating ASP plants in different regions across Australia. The secondary aim was to attempt to determine if there were relationships between the measured microbial log removal values and

frequently recorded (and/or easily measured) physicochemical parameters. The identification of relationships would enable, through appropriate operational monitoring and verification, the demonstration that appropriate pathogen log removals were being achieved in these biological systems. It was also hoped that such relationships could also indicate when an ASP was not operating to specifications and therefore when pathogen removal could be impacted.

The study involved sampling three activated sludge treatment plants, Oxley Creek (sub-tropical), Beenyup (mediterranean), Boneo (cool temperate) and a trickling filter plant, Rosny (mild temperate oceanic). These WWTPs represented different geographical regions of Australia and different population sizes. The selected treatment plants also varied in design and operating conditions. The performance of each plant was assessed by measuring log reduction values (LRVs) and collecting a range of physicochemical parameters, both from historical records and during the current study. The historical records provided information on the stability of the plant operation and were used to demonstrate that the plant was operating to specifications during the time when microbial LRVs were assessed.

The physicochemical parameters monitored in the influent and effluent as well as the frequency of data collection varied across the treatment plants. This made it difficult to perform a direct comparison between treatment plants. The subsequent findings indicated that the design of the plant was as important as (if not more of an influence than) the geographical location of the plant. In fact, the Rosny trickling filter plant was so different that ultimately the results from this WWTP were not used for direct comparison with the three ASP WWTPs and was examined on its own as an example of the trickling filter technology.

Despite the differences noted between the WWTPs, the analysis of physicochemical parameters (temperature, pH, DO, BOD, COD) showed little variation within an individual treatment plant indicating that all plants were operating under stable conditions. The average effluent temperature at Oxley Creek treatment plant was the highest (26.9±3.0 °C) and Rosny the lowest (18.5±3.5 °C) and this significant difference ($P < 0.05$) in operational temperature between the two plants reflected the influence of ambient climatic conditions. Sludge parameters such as sludge retention time (SRT) and mixed liquor suspended solids (MLSS) varied in response to plant design and operation, seasonal variations in wastewater inflows, and ambient temperature.

The study of microbial removal efficiencies at each plant was done using selected microorganisms from the three major pathogen groups of concern in Australia, namely bacteria, viruses and protozoa. The bacteria were represented by *E. coli* as this bacterium is the most commonly used microbial indicator and has been used historically to inform the quality of treated effluent. It was also assumed that removal efficiencies for *E. coli* would be representative of other bacterial species. Three DNA viruses (adenovirus, polyomavirus and the *Microviridae* coliphage) were tested as potential viral surrogates. Adenovirus and polyomavirus were selected as it has been previously suggested that these viruses could potentially be suitable as representative indicator pathogens. *Microviridae* was tested because somatic coliphages have often been used to represent enteric viruses, until recent advances in molecular technologies improved the detection capabilities for enteric viruses. Finally *Cryptosporidium* was chosen as the representative protozoan pathogen due to its known resistance to environmental pressures and chlorination.

The results found that *E. coli* numbers were fairly constant in the influent throughout the year at all the WWTPs ranging from 7 to 9 $\log_{10}$ L^{-1}. Effluent *E. coli* numbers were also constant in the effluent from each of the WWTPs, with mean values from 5.3 to 5.9 $\log_{10}$ L^{-1}. When these influent and effluent numbers were used to calculate LRVs, it was demonstrated that the ASP plants could consistently achieve *E. coli* removal with LRV geometric means ranging from 2.5 to 3.4 $\log_{10}$.

The virus data from all four WWTPs suggest that human adenovirus was consistently present in detectable numbers in both influent (10^6 to 10^8 L^{-1}) and effluent samples (10^3 to 10^5 L^{-1}). The LRVs determined for adenovirus in the ASP WWTPs had geometric means from 2.1 to 2.7 $\log_{10}$ indicating that adenovirus is indeed suitable for use as a conservative viral surrogate in a validation protocol. LRVs were of comparable magnitude to LRVs measured for *E. coli*, however the site-specificity for all three viruses meant that validation would need to be undertaken for each individual WWTP in order to determine appropriate virus log removal credits.

The initial attempts to detect *Cryptosporidium parvum* oocysts was found to provide numbers that were inconsistent in the influent of all WWTPs. Further research determined that this was caused by a low detection limit and issues associated with the recovery of oocysts from raw influent. Changes to the detection methodology, including the volume of sample tested (decreased from 30 mL to 15 mL) and using *Cryptosporidium* sp. genus specific primers rather than *C. parvum* species specific primers, provided results in the case of Beenyup and Boneo WWTPs. These two WWTPs respectively presented average *Cryptosporidium* sp. numbers in influent of 4.1 and 4.5 $\log_{10}$ L^{-1} and in effluent of 1.4 and 0.7 $\log_{10}$ L^{-1}. The calculated mean LRVs were 2.8 for Beenyup WWTP and 3.8 $\log_{10}$ for Boneo WWTP. These initial removal rates are similar to those determined for viruses, however these were only preliminary conclusions based on limited data. More testing would be needed from these and other WWTPs in order for more accurate conclusions to be reached on the ability of activated sludge plants to remove *Cryptosporidium* from wastewater.

A standardised sampling methodology is a key component supporting the development of a validation protocol, therefore a comparison was made between undertaking simultaneous influent and effluent sampling and a paired sampling strategy based on the calculated hydraulic retention time of the plant. Using the Oxley Creek WWTP as the test site, samples were collected using both strategies and compared. The results demonstrated that there were no statistically significant differences in calculated LRVs for adenovirus, polyomavirus or *Microviridae* using either sampling method (*t* test, $P > 0.05$). This suggested that the more practical simultaneous sampling strategy could be used as the preferred sampling methodology.

It was also considered important to determine the ideal number of samples required for validation purposes. The results suggested that the analysis of 10 samples was not sufficient to capture variations in LRVs while the mean and geometric means of 20, 30, and 40 samples were statistically similar and therefore, little additional benefit was obtained by collecting more than 20 samples. This result is consistent with literature data on representative sample sizes for validation purposes.

The data on calculated microbial LRVs and measured physicochemical parameters at each plant were compared using Principal Component Analysis (PCA) to identify any potential correlations between physicochemical parameters and microbiological removal. No strong correlations or

relationships could be identified. Future improvements in sensing technology and the testing of a large number of WWTPs over a longer time period may lead to the identification of significant links, allowing the monitoring of specific physicochemical parameters to be used to demonstrate pathogen removal.

Through a collaboration with the UNSW Water Research Centre, Bayesian Belief Network models were also used to investigate potential relationships between operating conditions, monitoring parameters and microbiological removal, and assess the capacity of these models to predict ASP performance. Similar to the PCA analysis, there were limited links found between the microbial LRVs and the physicochemical parameters using the Bayesian Network modelling. The Bayesian Network analysis, however, did find potential links of low LRVs being closely associated with high concentrations of reduced nitrogen, and higher LRVs associated with much lower than average NH_4^+-N and TN concentrations. This suggests that, while these physicochemical parameters may still not be directly correlated to pathogen removal, they may be able to be associated with monitoring that demonstrates that ASP processes are performing adequately.

This study has found that activated sludge plants are able to reduce the numbers of bacteria, viruses and protozoan by 2 $\log_{10}$ or more. No seasonal impacts were observed, but design and geographical locations do have an influence on the overall efficiency of the WWTPs ability to remove microorganisms. No direct links between physiochemical parameters and microbial LRVs were identified, however, further research and data collection from a wider number of WWTPs may assist in potentially identifying suitable linkages. The additional information will also be important to further demonstrate that the LRVs of surrogates such as adenovirus can also represent the removal of other microbial pathogens, in particular RNA viruses such as norovirus and reoviruses.

1 Background

The production of safe and sustainable recycled water relies on the effectiveness of treatment barriers, one of which is frequently a conventional wastewater treatment plant (WWTP). It is therefore not uncommon for these biological systems to form one of, if not the major treatment component of a water-recycling scheme. This is particularly the case for small-scale and regional water recycling schemes. In larger water recycling schemes the biological system often provides a significant initial treatment stage within a multi-barrier scheme.

Virtually all WWTPs across Australia use a form of biological treatment such as activated sludge as a major component of the treatment of wastewater (NRMMC, 2006). The activated sludge process is a commonly used biological wastewater treatment in both Australia and around the world. The primary objective of these plants has traditionally been the removal of bio-degradable organic matter and suspended solids. Therefore, performance of the WWTP has customarily been measured on the basis of physicochemical parameters such as sludge retention time (SRT), Mixed Liquor Suspended Solids (MLSS), BOD, COD and nutrient removal (Carducci and Verani, 2013). Because of this emphasis on nutrient reduction, and the fact that the final treated effluent was discarded to the environment, the ability of activated sludge systems to reduce microbial pathogens has been of secondary concern.

The increased focus on recycling treated wastewater to improve water sustainability of communities has placed more emphasis on ensuring appropriate health standards are maintained to protect the health and wellbeing of local communities. This means that there now needs to be a greater understanding of the capability of different treatment components to reduce or remove microbial pathogens from wastewater. Due to the innate complexity of activated sludge plants, however, there remains a paucity of data on their ability to reduce pathogen numbers, how to assess any removal, or how to ensure there is a consistent level of removal. The Australian Guidelines for Water Recycling (2006) (AGWR) provide an indicative log removal value (LRV) of 0.5 to 2 $\log_{10}$ for viruses and 0.5 to 1.0 $\log_{10}$ for *Cryptosporidium* (NRMMC, 2006). This data has been derived from the literature much of which, however, is out of date (because of changes in detection capabilities) or being obtained from studies in the northern hemisphere which may, or may not be relevant to WWTPs operating in an Australian climate. In addition, there are only a limited number of international studies that have examined the efficiency of pathogen removal in activated sludge plants (Carducci *et al*., 2008; Costan-Longares *et al*., 2008; Gantzer *et al*., 1998; Rose *et al*., 1996). In Australia there is a paucity of specific information on pathogen removal in activated sludge with only a limited number of studies specifically investigating pathogen removal by biological treatment plants (Flapper *et al*., 2010; Keegan *et al*., 2013; McAuliffe and Gregory, 2010; Toze *et al*., 2012). These studies have predominantly investigated pathogen removal at a laboratory scale or within a single climatic zone with relatively small sample size.

In the absence of appropriate local LRV data the setting of default values (commonly 0.5 $\log_{10}$ for both viruses and *Cryptosporidium*) is applied to WWTPs. This can impact the ability to set up a water recycling scheme as more treatment steps may be demanded than are needed in reality. In addition, even if a recycling scheme proponent wishes to undertake a validation process in order

to claim additional log credits for an activated sludge plant, there is no recognised standard validation protocol available to achieve recognition of these log credits. It still remains to be determined what would be the best mechanism for validating biological processes in Australia.

The primary objective of the NatVal project has been to establish a national validation framework for different treatment systems commonly used in recycling schemes and this sub-project was focused on treatment by Activated Sludge Processes (ASP). There is evidence, however, suggesting that geographical regions and differences in climatic conditions can produce different removal efficiencies for biological systems in Australia (Keegan *et al*., 2013; McAuliffe and Gregory, 2010; Toze *et al*., 2012). The direct study of changes in pathogen numbers across a biological system can be complex, time consuming and prohibitively expensive, particularly for the high risk pathogens such as viruses and protozoa. There is a range of physical parameters within activated sludge plants, such as hydraulic retention times and sludge age that can also impact on the accuracy and measurement of pathogen removal rate. As a result of these complexities, the use of surrogates or indicators that can be correlated to the efficiency of pathogen removal is considered to be a suitable alternative to direct measurement of pathogens. In addition, if any physicochemical parameters (SS, BOD, COD and NO_3) could be found to consistently correlate with the presence or removal of pathogens then these could also be used for the ongoing operational monitoring and validation of treatment plants. Some physicochemical parameters could be monitored at low cost and rapidly or even measured in real-time.

A number of studies have explored the correlation between different physicochemical and microbiological parameters (Flapper *et al*., 2010; Keegan *et al*., 2013; Muela *et al*., 2011; Rolland *et al*., 1983). There is currently no consensus among the reported literature with some studies reporting some correlations between microbiological and physicochemical parameters (Flapper *et al*., 2010; Rolland *et al*., 1983) while other studies have found no relation between microorganism removal and physicochemical parameters (Carducci and Verani, 2013; Muela *et al*., 2011).The aim of this project was therefore to determine the pathogen removal efficiency of a number of ASP-based WWTPs and to provide data that could be used to develop a validation protocol for assessing pathogen removal by activated sludge plants. The data needs to enable the validation protocol to be able to be used in the different geographical regions of Australia.

Key Aims

To provide appropriate data that can be used in the development of a protocol to validate activated sludge plants for their ability to reduce microbial pathogen numbers. The data will be obtained from activated sludge plants located in different regions of Australia. The final additional aim is to attempt to determine if there are physicochemical parameters that can indicate that activated sludge plants are operating adequately and can achieve the validated log reduction values.

Objectives

The scope of the research required the work to be undertaken in 3 distinct logical stages (Figure 1) and included the following tasks:

- Undertake a survey determining the biological systems most commonly used in water recycling schemes or of importance to water utilities and water recycling operators in Australia.

- Obtain information from the scientific literature, and obtain any available internal and non-published reports on any research undertaken that can assist on assessing pathogen removal across biological systems.
- Using the outcomes of the survey, develop a draft protocol for validating biological systems that can be tested and further refined.
- Test the draft validation guidelines using a selection of established biological systems to obtain baseline information on pathogen removal and determine what target microorganisms and operational parameters (e.g., suspended solids, temperature, and dissolved oxygen) could be used for validation of these systems.

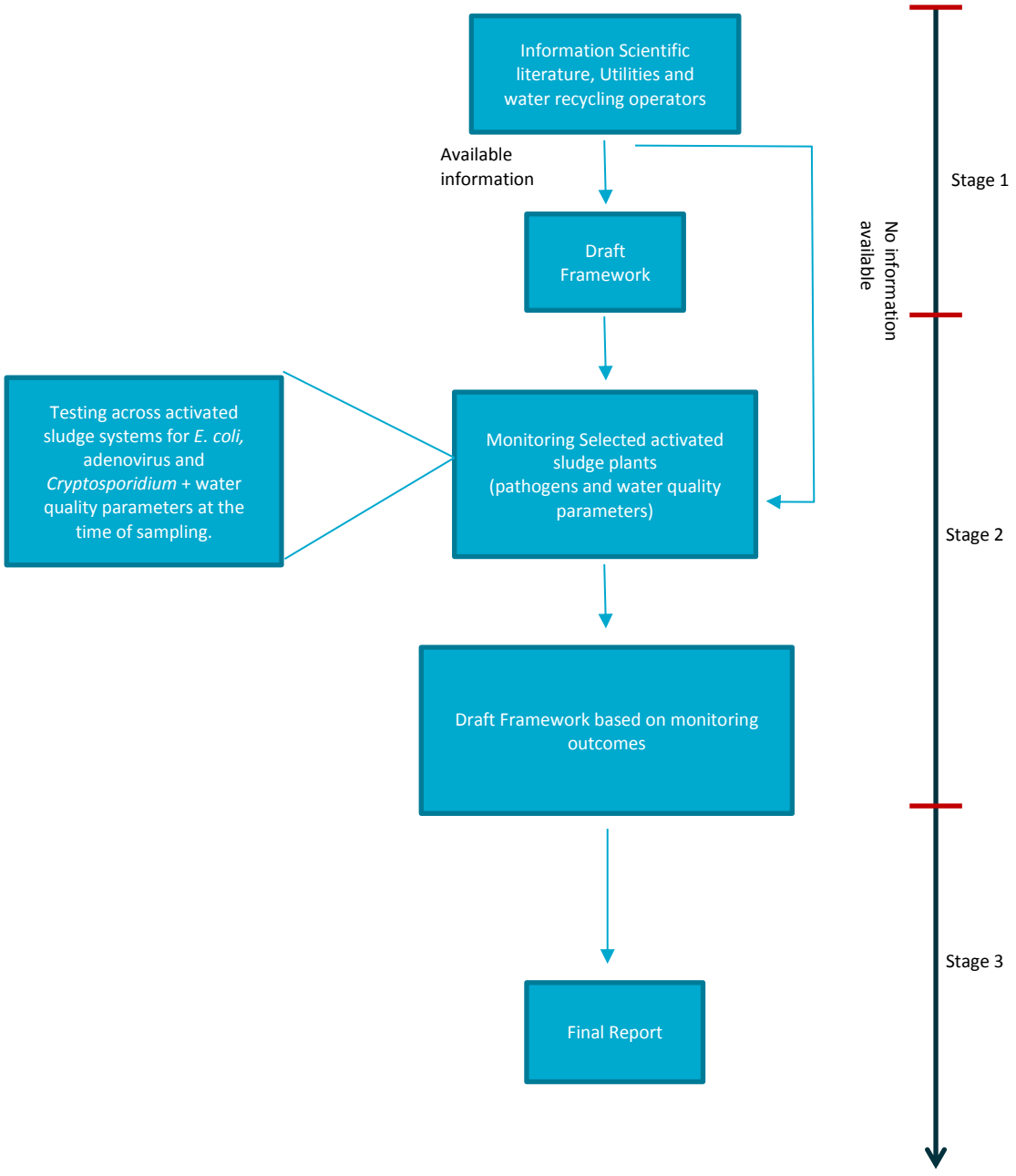

Figure 1-1. Research Stages.

2 Literature Review

Recycled water derived from wastewater treatment plants (WWTPs) is increasingly used for a variety of purposes in cities for alleviating water shortages. Inadequately treated recycled water can contain significant numbers of pathogens, which pose unacceptable health hazards. The required level of treatment is based on the intended use of recycled water, and the risk of exposure (NRMMC, 2006). Recycled water with a high probability of human contact requires the highest level of treatment due to end uses that carry a high risk of direct human exposure through intentional or incidental ingestion.

This project consisted of four major tasks:

- To carry out a literature review on pathogen and indicator reduction by ASPs;
- To collate and analyse data sets that report pathogen and indicator removal by ASPs to determine anticipated log removal values (LRVs);
- To determine mechanisms of pathogen and indicator reductions and their relationships with physicochemical parameters; and
- To determine the usefulness of physicochemical parameters for pathogen reduction by ASPs by collection and analysis of through a series of experiments and data analysis.

The literature review summarises studies and findings regarding pathogen monitoring approaches, pathogen reduction processes and their influencing factors, activated sludge data associated with bacterial, protozoan and virus reduction, and studies that have considered combined pathogen reduction, and correlation with operating parameters.

2.1 Mechanisms which influence removal of pathogens during treatment at a wastewater treatment plant

The removal of pathogenic microorganisms during wastewater treatment is variable depending upon the treatment process and factors such as hydraulic retention time, retention of solids, dissolved oxygen concentration, pH, temperature and the efficiency in removing suspended solids (Tyagi *et al*., 2011). Microbial cell adsorption onto porous media (suspended solids) is expected to be influenced by the content of organic matter, ionic strength, pH, temperature, water flow, hydrophobicity, biofilm development and bacterial numbers in the wastewater (Stevik *et al*., 2004). The mechanisms behind the removal or inactivation of pathogens are less understood or studied as compared to the mechanisms involved in retention or adsorption. Available literature on the identified pathogen removal mechanisms within the ASP is summarised below:

2.1.1 Major factors which influence removal of pathogens

In general, pathogen reduction during the ASP is influenced by three factors: (i) adsorption to suspended solids followed by settling of sludge flocs; (ii) natural decay of pathogens; and (iii) predation by other organisms such as protozoa. The first two factors play a major role whereas predation is considered not as important but, never-the-less, contributes towards removal of

bacterial pathogens and, to a certain extent, viral pathogens from wastewater matrices. The influence of these factors is expected to vary with the type of pathogen present and on plant operational conditions. During wastewater treatment, a number of plant specific physicochemical parameters such flocculation agents, pH, temperature, dissolved oxygen and turbidity may also influence pathogen removal. All these factors have complex interactions with each other and it is extremely difficult to pinpoint the exact mechanism of pathogen removal. Hence, there remains limited information in the literature on this subject. In this literature review, available information related to removal has been compiled for three microbial groups (bacteria, protozoa and viruses).

2.1.2 Bacterial removal mechanisms

The mechanisms behind the removal of *E. coli* in mixed liquor at laboratory scale (using seeded *E. coli*) was investigated by Van der Drift *et al*. (1977). They reported that a rapid adsorption of *E. coli* onto sludge flocs occurred followed by a slow removal through grazing by ciliated protozoa. Approximately one Log_{10} removal was observed in the first two hours due to adsorption, followed by an additional one log_{10} reduction after 6 hours. Similarly, 10-60% of faecal coliforms were reported by George *et al*. (2002)to be removed during the settling of wastewater sludge. However, they noted that the proportion of coliforms associated to settleable particles varied from one sample to another. In addition, they found no significant relationship between settleable particles and faecal coliform removal during settling. Despite this, they reported that the (de)nitrification step favoured removal of faecal coliforms due to protozoa grazing, competition with the indigenous microflora and sedimentation with flocs.

The influence of wastewater protozoa grazing on biofilm, on the removal of bacteria was studied in a laboratory set up by Chabaud *et al*. (2006). Protozoa in the presence of biofilm were reported to be responsible for 60% of the bacterial removal. Grazing is a common mode of feeding by ciliates such as *Vorticella*, *Opercularia* or *Carchesium* and *Paramecium*. It is most likely to occur in the biofilms where bacterial density is greater compared to the heavily aerated sludge produced during the activated sludge process.

The removal of bacterial indicators and pathogens in ASP can therefore be assumed to be a sum of the following mechanisms: die-off, sedimentation, adsorption and filter feeding.

2.1.3 Protozoa removal mechanisms

Due to the size of protozoa (oo)cysts, sedimentation is an important mechanism involved in removal. Another important mechanism is natural decay or the trophozoites within the (oo)cysts. Natural sedimentation velocities of protozoa (oo)cysts were investigated in Hanks balanced salt solution at constant temperature of 23°C and were reported to be 0.35 μm/s for *Cryptosporidium* (oo)cysts and 1.4 μm/s for *Giardia* cysts (Medema *et al*., 1998) . Medema *et al*. (1998) also determined that 30% of (oo)cysts were adsorbed to particulate matter of secondary treated effluent within 1 hour, which increased to 75% adsorbed by 24 hours. The attachment of (oo)cysts depends upon surface characteristics, pH and ionic strength of the solution. As expected, association of (oo)cysts to the smallest sized particulate matter (1-40 μm) enhanced

the sedimentation rate. Unabsorbed protozoa (oo)cysts are not expected to be removed by sedimentation alone.

Particle concentration is also reported to be a factor in the attachment of (oo)cysts. No attachment of *C. parvum* oocysts to soil particles was observed at suspended solids concentration of < 2mg/L (Dai and Boll, 2003). In comparison, in water samples of varying turbidity significant attachment of (oo)cysts has been reported to occur at 105-216 mg/L suspended solids (Feng *et al*., 2003). Feng *et al*. (2003) also reported that particle associated oocysts settled up to 50 times faster than un-attached oocysts. Similarly, Searcy *et al*. (2005) reported that in the presence of suspended charged inorganic particles (iron oxide or kaolinite), considerably higher removal of oocysts was observed compared to removal with illite suspension (as illite had a considerably more negative zeta potential than either kaolinite or iron oxide at neutral pH). They concluded that as *Cryptosporidium* oocysts are negatively charged, the electrostatic repulsive forces increased as the inorganic particles (illite) surface charge become more negative. Changes in pH and ionic strength of solution have also been found to play a significant role in the attachment for illite but not kaolinite or iron oxide (Feng *et al*., 2003). This suggests that particle type (ie, charge on the particle) is more important than solution chemistry in (oo)cyst-particle attachment.

For biological floc removal of *Cryptosporidium, r*emoval of *Cryptosporidium* oocysts at a laboratory scale (jar tests) using mixed liquor from an activated sludge plant was investigated by Suwa and Suzuki (2001). They observed a rapid initial removal of oocyst (1-2 Log_{10}) after 1 hour of aeration followed by a slow decline of a further 1 Log_{10} for 8 hour of incubation. This suggested that adsorption followed by sedimentation was the main mechanism behind the initial removal, but no significant further removal could be expected post 8h of incubation. Therefore, the authors suggested that in large scale treatment processes, there may be an accumulation of infectious oocysts in the sludge, especially during disease outbreaks. This may then eventually reduce the removal capacity of the activated sludge process by as much as 20% or more if the sludge is returned to the reactor after completion of a batch process.

The above studies suggest that adsorption to solids is an important mechanism for the removal of protozoa pathogens in the activated sludge process. However, natural decay due to environmental factors such as temperature, oxidation of (oo)cysts during aeration, and mechanical disruption of (oo)cysts are also expected to play a major role in the removal process. It has been reported that temperature is a significant factor in inactivation of metabolically active *Cryptosporidium* oocysts, with higher inactivation occurring at a temperature of 20□C or higher (King *et al*., 2005).

2.1.4 Virus removal mechanisms

The exact mechanisms responsible for virus removal in biological wastewater treatment are largely unknown. However, removal can be largely attributed to adsorption and subsequent settling of the sludge particles, natural inactivation or microbial predation (Kim and Unno, 1996). It is most likely that all of these mechanisms contribute towards removal, however, the effectiveness of some of the mechanisms such as adsorption could be enhanced through the use of flocculating agents, especially in the treated effluent. As occurs with bacteria and protozoa, the influence of charge is

important for the ability to remove viruses through adsorption to particles. For viruses, their isoelectric point has a major influence on adsorption. The isoelectric point is the pH at which a virus capsid has no net electric charge. Viruses are positively charged below their isoelectric point, whereas above that pH; the viruses have a net negative charge (Templeton *et al*., 2008). Each enteric virus has a different isoelectric point and due to this variability, there is no universal virus or pH point which can be used to assess viral removal or inactivation through treatment processes. It has been reported that due to different isoelectric points of tested poliovirus and bacteriophage, no single bacteriophage could accurately predict the adsorption of poliovirus to charged particles in wastewater (Moore *et al*., 1975). In general, acidic conditions (pH 5 or less) are expected to increase the adsorption of viruses to charged particles in wastewater. This suggests that in large scale activated sludge processes, pH and the presence of divalent and trivalent cations determine the adsorption of enteric virus and any differences in adsorption is due to varying isoelectric points of different enteric viruses.

Adsorption of enteric viruses to suspended solids in wastewater is a major mechanism behind the removal of virus via sedimentation. Conditions which favour adsorption of seeded poliovirus and coliphages (T2, T7 and f2) in sewage, activated sludge process and treated effluent were studied by Moore *et al*. (1975). Optimum conditions for viral adsorption to both organic and inorganic content were found to be favoured in the presence of a divalent cation (Ca^{2+}), which can act as a bridge at low pH (5.5-6.5). They also determined that in the neutral pH range (6-8), low adsorption of the phage and poliovirus to the suspended solids (1-17%) occurred in the secondary effluent. Mechanisms of poliovirus 1, coxsackievirus B-1 and coliphage removal during laboratory scale activated sludge treatment process were also studied by Glass and O'Brien (1980). They found that rapid adsorption of the viruses (20-50 min) to suspended solids occurred, followed by a slower rate of inactivation while attached to the suspended solids. An additional part of this study showed that viruses associated with the solids were found to remain infective. This suggested that virus removal through adsorption to suspended solids is not an inactivation processes, and therefore, does not reduce health risks from contact with untreated sludge.

In another study by Gerba *et al*. (1978), it was found that the percentage of coliphages associated with solids varied from between < 1 and 24% in effluents from activated sludge plants, whereas animal specific viruses were more attached (49-100%) to the suspended solids. The adsorption of bacteriophage was found to be reversible and elution of 67-70% of the coliphages was possible using glycine buffer (pH 10). However, at pH 7, less than 25% coliphage could be detached. This suggests that higher pH (> 8) may favour detachment of enteric virus attached to the charged particles in wastewater matrices. The results of this study also showed that bacteriophage and viruses were found to be predominantly attached to particles up to 8 µm diameter, which are expected to settle relatively quickly. Enteric virus type specific behaviours in adsorption have also been reported by Arraj *et al*. (2005). They showed that poliovirus and Hepatitis A virus were predominantly adsorbed onto flocks of mixed liquor, while rotavirus was only found in the liquid phase. This provides further evidence of virus specific attachment to solids in the wastewater.

Desorption of attached virus and other pathogens from sludge flocs is possible due to a sudden change in ionic strength and ionic composition, for example due to an influx of fresh

water from heavy rain and snowmelt events (Keiding and Nielsen, 1997). Similarly, ionic strength has been reported as the major factor which leads to detachment of bound bacteria to the sludge flocs (Zita and Hermansson, 1994). Omura *et al*. (1989), observed increased numbers of coliform and *Enterococcus* spp. in primary sedimentation effluent of an activated sludge plant as compared to influent. The increased numbers of bacteria in the effluent was attributed to desorption from the excess sludge returned to the primary sedimentation tank.

In a study of ASP systems in France, influent and secondary effluent samples were taken over 24, 48 and 72 hours intervals using 24 hours composite samples and 2-3 hour grab sampling (Rolland *et al*., 1983). The WWTP involved had a theoretical hydraulic retention time of approximately 6 hours. On the basis of grab samples, enteric virus removals on the four sampling days was variable ranging between 83% (0.77 log), 98% (1.7 log), 83% (0.77 log) and 87% (0.89 log) respectively, while faecal coliform removals were comparatively higher at 92% (1.09 log), 99% (2 log), 96% (1.4 log) and 99% (2 log). The authors also reported that there were limited correlations between virus numbers in the final effluent and other parameters such as chemical oxygen demand, suspended solids or faecal coliforms (calculated using the linear regression model for all flux parameters, $0.05 < P < 0.10$). Virus removal exceeded the removal of suspended solids, chemical oxygen demand and turbidity. In addition, they determined that the virus removals estimated from grab samples were very similar to automatic composite samples.

2.2 Non-adsorption factors influencing pathogen removal

The degree of removal of pathogens during activated sludge treatment (which commonly occurs at mesophilic temperatures) is influenced by a variety of interacting operational variables and conditions. These can vary between WWTPs and may often deviate from the ideal parameters. These parameters include the level of aeration, mixing and seasonal temperature variations. A number of studies have focused on understanding these factors and their influence on the removal of pathogens during wastewater treatment.

2.2.1 Temperature

Temperature of the mixed liquor is an easily monitored parameter and therefore would make an ideal performance indicator if appropriate. A study of the incubation of *E. coli* in soil at 5°C, 10°C, 20°C and 37°C showed that the best survival occurred at 5°C (Sjogren, 1995). High temperatures (> 55˚C) have been observed to be required for the rapid inactivation of pathogens such as parasite eggs, which are more resistant to heat that other microbial pathogens (Carrington *et al*., 1991). Since thermal inactivation is a function of time and temperature, better removal of pathogens can be achieved at higher temperatures and hydraulic retention time. Mesophilic anaerobic digestion (35°C) was reported to result in 2-3 $\log_{10}$ reduction in the number of faecal indicator bacteria, whereas, thermophilic anaerobic digestion (> 50°C) resulted in 4-5 $\log_{10}$ reduction (Zabranska *et al*., 2003). In the case of activated sludge treatment processes where temperature remains at approximately 35°C, other factors such as aeration, and the extent of stabilisation of organic matter may become as, if not more dominant factors in pathogen removal.

2.2.2 pH

As previously descried above, pH affects the sorption (low pH) and desorption (high pH) of viruses and bacteriophage to the matrix (Sobsey *et al*., 1980). Enteric viruses have different isoelectric points and due to this variability in the isoelectric point, variability in the removal of during the treatment processes is expected. Acidic conditions (low pH ~4) result in increased adsorption of viruses to the suspended solids. pH can also influence the sorption of bacteria. In a study on *Pseudomonas* movement through soil columns, Gammack *et al*. (1992) found higher number of cells in the effluent at pH 7.5 than at pH 4.5.

Generally, enteric pathogens are expected to survive better at near neutral pH. Rapid inactivation of bacteriophage (MS2 and Q beta) were observed to occur at pH <6 or >8, whereas both bacteriophage were found to be relatively stable at near neutral pH (6-8) (Feng *et al*., 2003). At extreme pH values, hydrogen and hydroxyl are the predominant ions and have been reported to cause oxidation of viral proteins (Feng *et al*., 2003; Nuanualsuwan and Cliver, 2003). Ward and Ashley reported that the change in pH affected the ionic states of viral capsid proteins, which ultimately determines the stability of viral cells (Ward and Ashley, 1979).

2.2.3 Suspended Solids

Viruses are negatively charged at neutral pH and tend to adsorb to suspended solids (inorganic and organic solids), and this adsorption is highly dependent on factors such as solids concentration, pH, and metal ion composition (Schaub and Sorber, 1976). In general, virus adsorbed onto suspended solids in the influent as well as virus that are secondarily adsorbed to mixed liquor-suspended solids are removed during clarification process (Hejkal *et al*., 1981). In a wetland receiving untreated wastewater, a good correlation for the removal of *Giardia* cysts and turbidity was reported by Quinonez-Diaz *et al*. (2001). Hirata and Hashimoto (1997) also examined removal of *Giardia* cysts and typically found poor correlations between various parameters and *Giardia* removal except for a correlation between turbidity reduction and a reduction of *Giardia* cysts. Stadterman *et al*. (1995) found that although parasite removal through the primary wastewater treatment process did not correlate with turbidity removal, removal was strongly correlated with turbidity reduction in the activated sludge treatment. While a strong relationship between suspended solids removal rates and parasite removal rates could be expected based on the previously discussed adsorption processes, the absence of a relationship in the literature is not surprising. Activated sludge plants (ASPs) typically operate within a relatively narrow performance band and the more extreme operating conditions necessary to clearly demonstrate this effect would not have been trialled. Therefore, it would be difficult to establish a strong relationship between suspended solids and protozoa. Furthermore the amount of suspended solids leaving the activated sludge process in the treated effluent is only a small percentage of the mass of suspended solids removed by the waste activated sludge into biosolids. There is also uncertainty from the recycling of activated sludge (and potential pathogens) into the ASP.

2.2.4 Pathogen type

Pathogen removal during wastewater treatment is pathogen type specific. In general, bacterial pathogens are more sensitive to environmental stress compared to protozoa and viral pathogens. There is even variation within pathogen types. For example, Adenoviruses 40 and 41 were found to be more resistant to inactivation than poliovirus in wastewater and to be significantly more resistant than both Hepatitis A virus and poliovirus in seawater and tap water samples (Enriquez *et al*., 1995).

2.2.5 Biological Oxygen Demand

Biological oxygen demand (BOD) is an indicator of the extent of organic matter reduction and stabilisation during wastewater treatment. Low BOD signifies well-stabilised organic matter. Efficient mixing and organic matter stabilisation have been reported to be the main factors controlling the rate of inactivation of microorganisms under mesophilic conditions (Smith *et al*., 2005). For coliphages, a positive correlation was found between coliphage removal during biological treatment and the reduction of $CBOD_5$ and total suspended solids. Rose *et al*. (1996) found no correlation, between the reduction of ammonia and coliphage. Both coliphage and enterovirus removal, however, was positively correlated to $CBOD_5$ during biological treatment. In another study of ASP at the laboratory scale, lower removal of coliforms, *E. coli*, *Enterococcus* spp., and *Cryptosporidium* were reported in the presence of high alkalinity and $CBOD_5$ (Flapper *et al*., 2010). In the same study, an opposite effect was observed, however, for the removal of bacteriophage, *Giardia* and sulphite reducing *Clostridia* (Flapper *et al*., 2010).

2.2.6 Ammonia

Free ammonia (NH_3) is very reactive and effective in the inactivation of bacteria, virus and bacteriophage (Cramer *et al*., 1983). Ammonia is produced during the degradation of nitrogen containing organic acids during wastewater treatment and its production is favoured under low pH conditions (Taylor *et al*., 1978).

2.2.7 Hydraulic retention time

Hydraulic retention time (HRT) is the average time the liquid sludge is held in the ASP system. Stabilisation of organic matter depends upon the length of HRT, with a decrease in HRT providing less time for bacterial degradation of organic matter and a subsequent reduced removal of pathogenic microorganisms. George *et al*. (2002) studied the removal of faecal coliform bacteria during wastewater treatment and reported that the most efficient removal occurred during biological treatments with high retention time (activated sludge process with nitrification and de-nitrification, lagooning). Lagooning was reported to be particularly efficient in the removal of faecal coliforms compared to other wastewater treatment processes due to the longer retention times (60+ days). Oragui *et al*. (1987) demonstrated that the retention time of wastewater in lagoons was very important in determining the treatment performance in a system. They showed that increasing the retention time lead to increased bacterial die-off. Raangeby *et al*. (1996) also concluded that retention time was the most important factor

influencing bacterial removal rates since it allows other changes in the lagoon environment, such as pH, temperature, etc. to affect bacterial die-off.

2.2.8 Solids retention time and mixed liquor suspended solids

Sludge retention time (SRT) is the average time the activated sludge solids remain in the digester. SRT is the single most important design and operating parameter affecting the performance of activated sludge systems (Metcalf, 2003). SRT is usually a function of the waste composition, reactor type, operating temperature and other process details (Buekens, 2005). In general, it can be assumed that a longer SRT will allow for more stabilisation of organic matter and pathogen inactivation compared to a shorter SRT under the same operating conditions (Loge *et al*., 2002). In a 2010 study on ASP, 1.9 $\log_{10}$ reduction of *Salmonella* was reported with a SRT of 12 days which improved to 3.75 $\log_{10}$ reduction at an SRT of 25 days at the same temperature (Chen *et al*., 2012). No further reduction in *Salmonella,* however, was observed at SRT higher than 25 days.

The mixed liquor suspended solids (MLSS) influences the SRT as it is the concentration of suspended solids in the aeration tank. MLSS is adjusted based on the inflow of wastewater to ensure that there is sufficient quantity of active biomass to digest organic matter in the aeration tank. The traditional method to control SRT during the activates sludge process is to manually adjust the sludge wasting rate based on the food-to-microorganism (F/M) ratio or mixed liquor suspended solids (MLSS) concentration. The SRT often depends upon the temperature and wastewater flow into the treatment e.g., during wet periods SRT is usually lower compared to the dry period.

2.2.9 Flocculation-aided sedimentation

The addition of chemical coagulants such as Fe_2Cl_3 is reported to improve the efficacy of both primary and secondary treatment for removing microorganisms (Zhang and Farahbakhsh, 2007). They found that the use of coagulants significantly increased the removal of bacterial indicators (faecal coliforms, *Enterococcus* spp. and sulphite-reducing clostridia) and bacteriophages by various primary and secondary wastewater treatment processes in several geographical areas. Lucena *et al*. (2004) also demonstrated that treatment processes such as lime-aided flocculation could have different influences on different microorganisms with faecal coliforms having the highest reduction and spores of sulphite-reducing clostridia and bacteriophages infecting *B. fragilis* the lowest.

2.2.10 Turbidity

Turbidity can provide both food and shelter for pathogens. Although turbidity is not a direct indicator of health risk, numerous studies have reported a strong relationship between the reduction in turbidity and the removal of protozoa. As an example, data gathered by LeChevallier and Norton (1992) from three drinking treatment plants from different watersheds indicated that for every log removal of turbidity, 0.89 log removal of *Cryptosporidium* and *Giardia* was achieved.

In a pilot plant study, the removal of particles > 2 µm was correlated to turbidity reduction and the removal of *Cryptosporidium* oocysts (Hendricks, 2000).

2.2.11 Hydrophobicity

Several non-wastewater treatment experiments have shown that hydrophobic microorganisms adhere more effectively to hydrophobic substrate than hydrophilic microbes (Fattom and Shilo, 1984; Stenström, 1989). In a study of migration through porous soils, hydrophobic bacteria were found to move slower than hydrophilic bacteria. This was shown to be due to the increased adhesion of the hydrophobic bacteria to soil particles (Lance and Gerba, 1984). Similar results have been observed in other similar experiments (McCaulou *et al*., 1994; Stenström, 1989). In contrast, Gannon *et al*. (1991) found no correlation between hydrophobicity and bacterial transport in soil porous media. Fattom and Shilo (1984) also observed that bacteria become more hydrophobic under high growth rates or during exponential growth (such as can occur in an ASP system) which could increase their removal.

2.3 Correlation between physicochemical factors and pathogen removal

Physicochemical parameters, BOD5, COD and suspended solids have been reported as useful for the control of wastewater treatment process while other parameters, such as conductivity or nitrate content have been found to be inadequate (Howard *et al*., 2004). A number of studies have tried to correlate physicochemical and microbiological parameters with varying degree of success. Atlas and Bartha (1998) identified BOD as the only parameter of wastewater quality after treatment that could be related with microbiological quality. A good correlation between BOD and SS with respect to total and faecal coliforms has also been reported in two studies by Katmi *et al*. (2008) and Williams *et al*. (1995). Conversely, in a year-long study on an activated sludge treatment plant in Spain, Muela *et al*. (2011) found no significant correlations between physicochemical parameters (COD, BOD, SS, TKN, and NO_3) and microorganism presence. Similarly, in Membrane bioreactor (MBR) and upflow anaerobic sludge blankets (UASB), no physicochemical parameter (NH_4, PO_4, COD, BOD, TOC, and SS) was able to predict the presence of enterovirus genome in the effluent (Ottoson *et al*., 2006a).

2.3.1 Bacteria removal

Rose *et al*. (1996) examined the removal of bacteria in an activated sludge treatment plant with associated nitrification in Florida. The average removal of total coliforms and faecal coliforms through biological treatment with clarification was 1.75 $\log_{10}$ and 2.06 $\log_{10}$ respectively. No correlation was observed between bacterial removal and any of the physicochemical parameters (including $CBOD_5$, organic nitrogen, total nitrogen, suspended solids or turbidity) across the activated sludge process. In a following study, Rose *et al*. (2004) investigated the microbial quality of wastewater at 6 wastewater treatment facilities in the USA. The observed bacterial removal through the activated sludge plants was 1.39-3.0 $\log_{10}$ with lower *Clostridium* removal of 1.17-2.69 $\log_{10}$. The highest removal was observed in a plant with enhanced biological phosphorus removal while the lowest removals were observed in conventional activated sludge plants. The authors concluded that a decrease in bacterial removal is proportional to increasing MLSS.

2.3.2 Enteric virus removal

In a study from France on ASP process, limited correlations were found between virus numbers in the effluent and physicochemical parameters such as chemical oxygen demand, suspended solids (Rolland *et al*., 1983). In addition, virus removals were reported to exceed suspended solids, chemical oxygen demand and turbidity removals. Similarly, in a year-long study on an activated sludge treatment plant in Spain, PCA of data indicated no significant correlations between physicochemical (COD, BOD, SS), TKN, and NO_3) and microbiological parameters (Muela *et al*., 2011). In another study, on enteric pathogens and indicators removal during membrane bioreactor (MBR) and up-flow anaerobic sludge blankets (UASB), Ottoson *et al*. (2006a) found that no physicochemical parameter (NH_4, PO_4, COD, BOD, TOC, and SS) was able to predict the presence of enterovirus genome in the effluent samples.

2.3.3 Protozoa removal

A significant link between total alkalinity, organic matter, turbidity and NO_3 and *Cryptosporidium* and *Giardia* removal was reported from a laboratory scale experiment after applying PCA analysis (Flapper *et al*., 2010). Furthermore, suspended solids, turbidity, TKN and COD were reported as major negatively correlated factors linked to LRVs. Bonadonna *et al*. (2002) investigated the occurrence of *Cryptosporidium* in secondary treated effluent samples from a municipal wastewater treatment plant. Their data analysis showed no correlation between *Cryptosporidium* numbers and measured physicochemical parameters such as pH, redox potential and total organic carbon and that of the other microorganisms.

A study by Rose *et al*. (2004) also examined the impact of loading conditions, process design, and operating parameters (MCRT and MLSS) on the removal of protozoa. Increased parasite removal biological nutrient removal facility was found to be associated with MCRT of 8.7-13.3 days. *Cryptosporidium* oocysts removal was least effective in the conventional and enhanced biological phosphorus removal facilities with MCRTs of 3-5 days and 8-16 days respectively. Conversely, most effective removal of oocysts was observed in biological nutrient removal facility with MCRT of 8.7-13.3 days. However, no correlation between process variables (MCRT and MLSS) and pathogen and indicator removals was observed.

2.4 Target pathogens and indicators/surrogates

Enteric viruses and protozoa are more significant from an Australian public health point of view than bacteria in recycled water due to their low dose, high infectivity and greater resistance to most treatment processes. Therefore, in this study viruses and protozoa are the focus as target pathogen groups for validation.

2.4.1 Criteria used for selection of representative pathogens

The three major pathogen groups considered in this study are the: bacteria, protozoa and viruses. No consideration has been given to the helminths due to their very low incidence of infection in the Australian population (and therefore extremely low incidence in wastewater). On the basis of potential risks to human health, microorganisms representing each enteric pathogen group were

evaluated for their potential to be used as representative pathogen(s). The criteria used for the selection of representative pathogens were adapted from the WERF report by Monis and Blackbeard (2010). The main selection of factors which need to be considered are listed in Table 2-1.

Table 2-1 Selection criteria for representative pathogens.

Selection Criteria
Importance to the water industries and health regulators • **Wide geographical distribution** • **Minimal seasonal variations in numbers Pathogen of human origin** • **Representative of other pathogens in the same group (*E. coli* is considered representative of enteric bacterial pathogens)** • **Applicable to as many matrices as possible** • **Survives for adequate time in matrices to be useful Ideally pathogen does not multiply in the matrix (for bacterial pathogens)**
Analytical methods performance • **Reliable** • **Reproducible** • **Quantitative** • **Able to easily detect pathogens in wastewater matrices** • **Cost effective**

Adapted from Monis and Blackbeard (2010).

2.4.2 Enteric viruses

Viral pathogens are the major cause of gastroenteritis worldwide. There are a wide variety of viruses that can be found in wastewater (Moni and Blackbeard, 2010) with the presence of specific enteric viruses and their removal during treatment processes varying depending on a range of factors including local community conditions, the WWTP design and geographical and climatic conditions.

Adenovirus

Human adenovirus are ubiquitous pathogens that can cause a variety of diseases ranging from respiratory illness and keratoconjunctivitis to gastroenteritis (Sidhu *et al*., 2013b). There are at least 57 known human adenovirus serotypes which are organized into six subgroups (A–F). Adenovirus serotypes 40 (Ad 40) and 41 (Ad 41) are the second most common etiological agents of gastroenteritis in children (Buckwalter *et al*., 2012).

Level of importance to the industry:

Adenoviruses are rated as highly important on the basis of their environmental and UV resistance. As a result they are commonly used by health regulators as one of the criteria in the assessment of the safety of the re-use of water. Human adenoviruses are reported to be more

thermostable and UV stable than other enteric viruses such as Norovirus and rotavirus (Enriquez *et al*., 1995; Gerba *et al*., 2002).

Geographical distribution:

Widely distributed in wastewater matrices (Gerba *et al*., 2002; Reynolds, 2004)

Representative of other pathogens in the same class:

On the basis of high stability in the environment, absence of adenovirus in human wastewater will indicate absence of other human pathogens. Adenoviruses have been shown to be more resistant than enteroviruses to wastewater treatment processes and other environmental factors and considerably more so than polioviruses and feline caliciviruses (Tree *et al*., 2005).

Presence in multiple matrices:

Present in most moderate-sized populations and excreted in stools. Adenovirus can therefore be expected to be present in wastewater matrices.

Numbers In influent:

$10^{2.8}$ L^{-1} cytopathic units (He and Jiang, 2005)

$<10^{0}$-$10^{2}L^{-1}$ plaque forming units (Sedmak *et al*., 2005)

10^{6} L^{-1} PCR detectable units (He and Jiang, 2005)

Numbers in effluent:

6×10^{2} L^{-1} cytopathic units (He and Jiang, 2005)

8×10^{5} L^{-1} PCR detectable units (He and Jiang, 2005)

Analytical methods:

Several detection and quantification approaches are available: (i) cell culture (ii) PCR, (iii) integrated cell culture (ICC) PCR or reverse transcriptase (RT)-PCR. Cell culture with RT-PCR only detects the results of virus infection (Ko *et al*., 2005). Cromeans and Sobsey (2004) used RT-PCR to detect replicative forms in cell culture which suggested that cytopathic effect studies underestimated infection. PCR based detection generally results in higher numbers than CPE due to the detection of all DNA from both infective and non-infective adenovirus particles (He and Jiang, 2005).

2.4.3 Protozoa

Unlike enteric bacteria and viruses, there are only two enteric protozoa (*Cryptosporidium* and *Giardia)* that are of significant concern in wastewater in Australia. The protozoan pathogen of most concern and importance to the water recycling industry is *Cryptosporidium spp.* oocysts. *Cryptosporidium* is the most resistant to inactivation, and is therefore the target protozoan pathogen for validation purposes.

Cryptosporidium parvum, Cryptosporidium hominis

Level of importance to the industry:

High, as oocysts are recognised to be more resistant to inactivation by chlorine than most other pathogens including *Giardia* cysts (Fayer *et al*., 2000). It is one of the microorganisms used by health regulators to assess the safety of recycled water. Furthermore, the infectious dose has been estimated to be as low as 30 oocysts (Fayer *et al*., 2000). Several outbreaks of cryptosporidiosis and giardiasis have been attributed to contamination of water supplies with (oo)cysts (Baldursson and Karanis, 2011).

Geographical distribution:

Widely distributed, a common parasite of humans and other mammals. *Cryptosporidium* spp. and *Giardia* spp. cause diarrheal diseases in human and animals worldwide (Rimhanen-Finne *et al*., 2004).

Representative of other pathogens in the same class:

Probably not. Generally more resistant to disinfectants compared to *Giardia*. Removal through wastewater treatment, in particular filtration, is poorly correlated with *Giardia.* Due to their smaller size, removal of *Cryptosporidium* oocysts during filtration is lower than *Giardia* (Harwood *et al*., 2005). During aerobic digestion of sludge, a higher reduction in *Cryptosporidium* numbers has been observed (2.96 Log_{10}) compared to *Giardia* (1.40 Log_{10}) (Chauret *et al*., 1999). No reduction was observed in *Giardia* numbers during anaerobic digestion of sludge (Chauret *et al*., 1999).

Presence in multiple matrices:

Yes, there are many reports of occurrence in wastewater and sludge/biosolids.

Numbers: In raw sewage:

10^1-10^2 L^{-1} (Harwood *et al*., 2005)

10^1-10^2/1 L (Montemayor *et al*., 2005)

In effluent:

10^0-10^1 L^{-1} (Harwood *et al*., 2005)

$10^{0.3}$-$10^{1.2}$ L^{-1} (Montemayor *et al*., 2005)

Detection method:

Matrix determines the primary concentration step for *Cryptosporidium* oocysts. Concentration methods have included centrifugation and filtration. The primary concentration step is usually followed by the purification by immune-magnetic separation (IMS). Direct counts can be made by fluorescent microscopy or real-time PCR. Infectivity can be measured by cell culture. Molecular tests are required for species/genotype identification.

Method performance:

Detection of *Cryptosporidium* oocysts are generally method and matrix dependent. Direct microscopic count are the most commonly used and are considered reliable. A number of cell lines and detection schemes for wastewater are in use with variable success; cell line (HCT-8) is the

most commonly used (Quintero-Betancourt *et al*., 2002). Some issues with inhibition of cell culture assays arising from sample toxicity were reported (Blatchley *et al*., 2005), although no cell culture assays inhibition was reported in another study (Harwood *et al*., 2005).

2.4.4 Criteria used for selection of appropriate microbial indicator.

Due to constraints associated with the monitoring of pathogens, indicator microorganisms are commonly used as surrogates for all pathogens. The ideal faecal indicator should fulfil each and every criterion such as consistently present in faeces, inability to multiply outside the host, be as resistant to inactivation from local environmental conditions and disinfection, have a strong association with the presence of other or all pathogenic microorganisms and allow easy detection and quantification without providing false positive results (Hurst *et al*., 2007). However, very few indicators used to monitor the presence of pathogens in wastewater matrices fulfil at least the majority of all of the above mentioned criteria.

Historically, faecal indicator bacteria such as total and faecal coliforms, *E. coli*, and *Enterococcus* spp. have been used in many countries for monitoring water quality and for prediction of presence of viral, bacterial and protozoa pathogens. In this current study, *E. coli* was used as the indicator for bacterial pathogens.

E. coli is always present in high numbers in domestic wastewater (~10^7 cfu/100 mL) and regularly used as an indicator for faecal pollution in water (WHO, 2004). The presence of *E. coli* indicates the likely presence of pathogenic microorganisms; yet it is not an unequivocal indicator of the presence of pathogens.

Based on the evidence obtained from the literature, there are no suitable indicators for enteric viruses and protozoa that fulfil all of the appropriate criteria. It has been determined that it is most probably appropriate to use adenovirus and *Cryptosporidium* as the viral and protozoan surrogates due to their established resistance to environmental and treatment processes and their use in the water recycling guidelines as reference pathogens.

2.5 Pathogen and indicators occurrence and removal during wastewater treatment

Available information from the literature was reviewed to determine pathogen removal efficiency of activated sludge treatment plants (Appendix A). This literature review aims to explore recent research on pathogen removal in activated sludge treatment plants along with detection methods used that may assist the current research. The importance of the different pathogen types is dependent on the geographical location of the wastewater systems and the social and economic standards of the local population. As such, the presence of helminths and various bacterial pathogens (*Vibrio cholerae* and *Shigella dysenteriae*) are important in regions with lower socio-economic standards but not high socio-economic regions such as Australia.

Climatic conditions as found in different geographical regions of Australia (and elsewhere around the world) are known to affect the removal efficiencies for biological treatment systems. This has been highlighted in a number of studies below.

2.5.1 Pathogen removal

Recently a small study was undertaken at a WWTP in Adelaide over six weeks to investigate virus and protozoa removal. The results indicated a minimum of 1 Log_{10} reduction of viruses and 0.5 Log_{10} reductions of protozoa for a well operated and maintained activated sludge plant. This has resulted in South Australia's Department of Health and Aging (DHA) revising their default virus reduction values (Keegan *et al*., 2013).

Abreu-Acosta and Vera (2011) investigated the occurrence and removal of pathogenic microorganisms in two wastewater reclamation systems and found that both demonstrated efficient reduction of faecal contamination indicators in the wastewater (2 Log_{10} removal). Both *Cryptosporidium* and *Giardia* (oo)cysts were found to be efficiently removed. The authors acknowledged, however, that the prevalence of *Giardia* in the local human communities and the presence of cysts in the effluents suggested that *Giardia* should be used as indicators for quality control in reclaimed effluent. This study suggested also using *E. coli* and indicators such as *Clostridium perfringens* and somatic coliphages due to a range of factors including having a direct relationship to *Giardia*, higher resistance than other bacterial indicators, simple and economical determination or a short 4 hours turnaround time for results.

Simmons and Xagoraraki (2011) showed an average 4.2 Log_{10} reduction of infectious viruses occurred through the wastewater treatment process in a study involving 5 full-scale WWTPs. These results are comparable to other previous studies which also reported up to 4.0 Log_{10} reductions of infectious viruses (Petrinca *et al*., 2009; Sedmak *et al*., 2005).

Castro-Hermida *et al*. (2008), investigated the ability of Spanish WWTPs to remove *Cryptosporidium* and *Giardia* (oo)cysts. They found that the (oo)cysts were present in both the influent and effluent of all the samples from the WWTPs with minimal removal of (oo)cysts by the treatment process. In fact, it was noted that the number of (oo)cysts in the effluent from several of the WWTPs were actually greater than detected in the influent. The residence time of the wastewater in the treatment plants was taken into consideration when sampling. No specific information was given on actual log removal capacity of any of the treatment plants but the averaged results suggest that the LRV was less than 2 Log_{10}.

Castro-Hermida *et al*. (2008) studied the contribution of treated wastewater to the contamination of recreational river areas with *Cryptosporidium* spp. and *Giardia duodenalis*. Both *Cryptosporidium spp* and *Giardia duodenalis* were found in the influent and final effluent samples of 12 WWTPs in Spain (Castro-Hermida *et al*., 2008). The numbers of *Giardia* in the influent were found to be significantly higher than *Cryptosporidium*. Numbers in the final effluent ranged from 2-390 and 79-2469/L for *Cryptosporidium* and *Giardia*, respectively. The highest numbers of parasites were observed at all WWTPs in spring and summer. Da Silva *et al*. (2007) examined the removal of noroviruses in French WWTPs with different treatment types (stabilization ponds, a small and large activated sludge systems and membrane bioreactors). They observed that the removal efficiency varied from 1 Log10 to as high as 3 Log_{10} depending on the WWTP. None of the WWTPs tested completely removed noroviruses all of the time. The small activated sludge and membrane bioreactor systems were found to give the highest removal efficiencies.

Human adenoviruses have been reported to be 10 times higher in numbers than enterovirus in wastewater (Reynolds, 2004), and are known to survive better than enterovirus during wastewater treatment (Bofill-Mas *et al*., 2006). High numbers (10^5 gene copy numbers/L) of adenoviruses have been reported for both sewage and primary sludge (Albinana-Gimenez *et al*., 2006; He and Jiang, 2005). However, the cell culture technique has resulted in reports of lower numbers (10^2 pfu/L) (He and Jiang, 2005). The discrepancy is possibly due to overestimation of infectious viral numbers by qPCR, while cell cultures assays tend to underestimate virus numbers (He and Jiang, 2005) indicating that it is importance of a standardised detection method.

Adenoviruses are known to be more UV and thermos-stable compared to other enteric viruses and can survive in the environment for a long time (Enriquez *et al*., 1995; Gerba *et al*., 2002). Several studies have investigated the removal and correlations between bacterial indicator micro-organisms and viruses (Keegan *et al*., 2013; Muela *et al*., 2011; Petrinca *et al*., 2009). Petrinca *et al*. (2009) found that high removal rates of bacteria were contrasted with limited removal of viruses determining that the bacteria were not predictive of enteric virus presence. In another study, adenovirus was reported to have a minimal correlation between adenovirus and indicator microorganisms (sulphite-reducing clostridia and F-RNA bacteriophage) (Keegan *et al*., 2013). This led the authors to conclude that where possible, pathogens should be used for assessing plant pathogen reduction performance.

Seasonal variations in pathogens have also been suggested to be important. However, an eight month study found no significant seasonal differences in adenovirus numbers in the effluent from four WWTPs in the USA (Kuo *et al*., 2010). The presence of infectious human viruses in non-disinfected effluent regardless of the treatment method used has been reported with adenovirus, norovirus and enterovirus found to be always present in primary influent in small, medium and large WWTPs (Hewitt *et al*., 2011). Norovirus infection were found to be associated with peaks in the winter season in Japan, Norway and The Netherlands (Mounts *et al*., 2000). In contrast, no clear seasonal peak for norovirus infection was found in New Zealand (Hewitt *et al*., 2011). Reported norovirus reductions in wastewater treatment plants ranged from 0.0 to 3.6 Log_{10} (Kuo *et al*., 2010; Lodder and de Roda Husman, 2005; Nordgren *et al*., 2009; Ottoson *et al*., 2006a; van den Berg *et al*., 2005).

Van den Berg *et al*. (2005) reported high numbers (10^5/L) of noroviruses in raw sewage with limited inactivation during wastewater treatment (10^3/L). Similar limited inactivation of noroviruses has also been reported elsewhere (Hewitt *et al*., 2011; Laverick *et al*., 2004). Norovirus cannot be cultured, therefore comparison of data on noroviruses with other enteric virus numbers and inactivation in wastewater determined using culture based methods is theoretically not possible. In one study, removal rate of norovirus was compared with other viruses during wastewater treatment using PCR as a detection method for all the viruses, a similar removal for norovirus (0.2-2.1 Log_{10}), reovirus (0.9-1.4 Log_{10}) and enterovirus (0.7-1.8 Log_{10}) but a greater removal rate than rotaviruses (0.003-1.1 Log_{10}) was reported (Lodder and de Roda Husman, 2005).

In a literature review, enterovirus numbers were reported to range from 10^2 to 10^4 /gm dry weight in raw sludge and 300/gm in anaerobically digested sludge (Straub *et al*., 1993). Monpoeho *et al*.

(2004) reported that enterovirus numbers in raw sludge varied between 37-288 cytopathic units/gm (cell culture), whereas qPCR based quantification resulted in higher detection of 10^5 copy numbers/gm. Similarly enteric viruses were found in raw wastewater samples at concentrations between 10^2 to 10^4 MPN/100 L in a study in Brazil (Hachich *et al*., 2013).

Data from a study in the US has found that *Cryptosporidium* oocysts were present in low numbers in many wastewaters as well as in the effluents being discharged from the studied WWTP (McCuin and Clancy, 2006). However, the assays used in this study were unable to differentiate between live and dead oocysts and therefore further research would be required to determine the infectivity of the detected oocysts and thereby assess the associated health risks. Cheng *et al*. (2012) reported positive correlations between the abundance of enterococci and *E. coli* and the abundance of *Cryptosporidium* spp. and *Giardia* spp. This study also noted a strong correlation between *Giardia* and *Cryptosporidium*. Fu *et al*. (2010) compared faecal coliforms and somatic coliphages numbers with *Cryptosporidium* and *Giardia* in untreated wastewater and secondary treated effluent and found that the somatic coliphages correlated better than the faecal coliforms with the protozoa. It was noted however, that the concentrations of pathogenic protozoa could not be determined by detecting concentrations of somatic coliphages.

2.6 Influence of wastewater treatment operational parameters on the removal of the pathogen numbers from the wastewater

Information on the physical and chemical parameters (operational parameters) of WWTPs that can be used for developing a validation protocol is very limited. Available information on the WWTP specific information and information on the LRV's is presented in Appendix A. In this section, we have explored the available literature to extract information on the operational parameters of wastewater treatment and their influence on the LRV for pathogen and indicators.

The report by Flapper *et al*. (2010) is one of the few reports which has investigated the physicochemical variables of the treatment process and tried to model removal of pathogens. The modelling of pathogen removal was done by applying a Principal Components Analysis (PCA) approach to predict pathogen removal during the wastewater treatment process. The outcomes suggested that parameters such as COD, turbidity and nitrate should be further explored for their link to pathogen occurrence and removal from wastewater (Flapper *et al*. 2010).

Rose *et al*. (2004) investigated the presence and removal of pathogens and indicators at six wastewater treatment plants in the USA. They examined the impact of loading conditions, process design, and operating parameters on the removal of pathogens. The biological treatment plants included conventional activated sludge, a biological nitrification-denitrification plant, and an enhanced biological phosphorus removal plant. Samples were taken at a number of points during the treatment process (influent, secondary effluent, filtration and disinfection). Bacterial removal through the activated sludge plants were found to be between 1.39-3.0 Log_{10}. The highest bacterial removal was in enhanced biological phosphorus removal plant while the lowest removals were observed in the conventional activated sludge plants. The removal of *Giardia* cysts had an average 2 Log_{10} removal, while *Cryptosporidium* oocyst removal was more variable with an average 1.5 Log_{10} removal.

In an earlier study by the same group, pathogen reduction at the Upper Occoquan Sewage Authority activated sludge plant was examined. Raw sewage and secondary treated effluent was collected monthly for a year. Average removals of 97% (1.51 Log_{10}) and > 79% (0.68 Log_{10}) for *Giardia* and *Cryptosporidium* (oo)cysts, respectively were reported. Removal rates of faecal coliform of 99% (2.05 Log_{10}), coliphage 99.5% (2.3 Log_{10}), Enterovirus 98% (1.65 Log_{10}) and *C. perfringens* 88% (0.92 Log_{10}) were reported (Rose *et al*., 2001). However, limited details of the plant characteristics or plant specific removal data were provided.

Hewitt *et al*. (2011) investigated the removal of adenoviruses, enteroviruses and norovirus entering WWTPs in New Zealand serving different-sized communities (large, medium and small), and the effectiveness of different treatment processes in reducing virus numbers. Virus numbers and type were found to be generally independent of population size and treatment process (moving bed biofilm reactors, activated sludge, waste stabilisation ponds). The numbers were more stable in the large WWTPs, potentially due to the population size. They determined that the numbers of adenoviruses and enteroviruses in wastewater were more variable in small community size plants (< 4000) and medium-sized (10,000-64,000) WWTP than in large-sized (> 130,000) plants. The range of virus numbers was narrower for adenoviruses and enteroviruses in both influent and effluent in the larger plants compared to the smaller plants. Smaller plants generally had variable virus numbers and sporadic spikes in numbers in both the influent and effluent. The occurrence of noroviruses (GI and GII) was sporadic in both influent and effluent in all WWTPs. The norovirus numbers in effluent samples were independent of the treatment processes and often unrelated to the influent.

Reinoso and Becares (2008) investigated parasite removal (*Cryptosporidium*, *Giardia* and helminths) in two conventional activated sludge treatment plants for pig slurry in north-western Spain. These parasites numbers in the pig slurry were 10^4-10^5 (oo)cysts/L for *Cryptosporidium*, 10^3 cysts/L for *Giardia* and 10^2–10^3 eggs/L for helminths. The overall removal of parasites ranged from 86.7% to over 99.9% (0.88-4 Log_{10} removal). The activated sludge process was the most efficient process in the treatment chain in reducing protozoa and helminths with 78–81% (0.65-0.72 Log_{10} removal) for *Cryptosporidium* oocysts and over 99.9% (> 3 log_{10}) for helminth eggs. The combined use of ferric chloride with a polyacrylamide (PAM) cationic polymer also improved the efficiency of parasite removal by 40–44%.

Chauret *et al*. (1999) investigated the removal of *Cryptosporidium*, *Giardia* and a number of indicators in an Ottawa treatment facility treating approximately 500 ML/d of sewage through an activated sludge plant (ASP). Average removals of 2.96 Log_{10} and 1.40 Log_{10} for *Cryptosporidium* and *Giardia* respectively were observed although this was determined from a small number of samples (n = 4). The primary treatment was shown to have a limited effect on protozoa removals being 0.1 Log_{10}. Less overall reduction was observed for both *C. perfringens* spores and vegetative cells (0.89 Log_{10} and 0.96 Log_{10}, respectively). The other bacteria (total coliforms, faecal coliforms, *Enterococcus sp.* and *Aeromonas sp.*) reduced by $\geq$ 3.50 Log_{10}. During mesophilic anaerobic sludge digestion, no statistically significant reduction was observed for *C. perfringens*, *Enterococcus* spp., *Cryptosporidium* oocysts, and *Giardia* cysts.

Ottoson *et al*. (2006a), investigated log reduction of enteroviruses, noroviruses, *Cryptosporidium* and *Giardia* in four full scale Swedish activated sludge plants. At Klangshamn, the effluent passed through a rapid sand filter and in Ryaverket, additional nitrogen removal was included. Paired samples were taken from the inlet and outlet of each WWTP. The average reduction at all four full-scale plants for *Cryptosporidium* and *Giardia* was 1.3 ± 0.46 Log_{10} and 3.3 ± 0.46 Log_{10} respectively. Noroviruses and enteroviruses were removed by 0.9 and 1.3 Log_{10} respectively. There was no correlation between the removals of pathogens and indicators ($p > 0.05$). Montemayor *et al*. (2005) investigated *Cryptosporidium* oocysts in five WWTPs in north-east Spain. The results showed that all WWTPs had higher levels of *Cryptosporidium* oocysts in raw sewage during the spring months, with lowest levels during summer. Three activated sludge plants were studied for *Cryptosporidium* removal with geometric means of log removal from raw sewage to secondary effluent of 1.7, 1.9 and 1.4 (mean 1.7±0.27).

None of these studies have provided adequate information on the relationship between physicochemical and microbiological parameters or their correlation with pathogen removal in activated sludge process. This indicates the importance for continuing this investigation within the NatVal project to study and determine the most appropriate methods and surrogates for developing a validation protocol that can be used nationally across Australia.

2.7 Conclusions

The pathogen numbers in the influent and removal depend upon a number of parameters as evident from the literature summary. These include:

- Infection prevalence in the community, which can be seasonal.
- A variable fraction, as high as 50% of the enteric virus present in the raw sewage, has been reported to associate with solid fraction. Solids removal from wastewater could be a key pathogen reduction step for activated sludge treatment process.
- Attachment of pathogens to solid particles is driven by the net charge of specific viruses and particulate matter and the pH of wastewater. In general, low pH favours adsorbed virus and at high pH free virus in the liquid phase.
- Pathogen removal could be driven by type of treatment, size of WWTPs, hydraulic retention times and type of coagulant used.

3 Large Scale Activated Sludge Treatment Plant Physicochemical Data

3.1 Activated sludge treatment process

The activated sludge treatment process is an aerobic biological treatment process for primarily reducing soluble dissolved organic matter (BOD). In brief, the treatment process involves the removal of grit followed by primary sedimentation which is followed by transfer of settled sludge into aeration tanks (Figure 3.1).

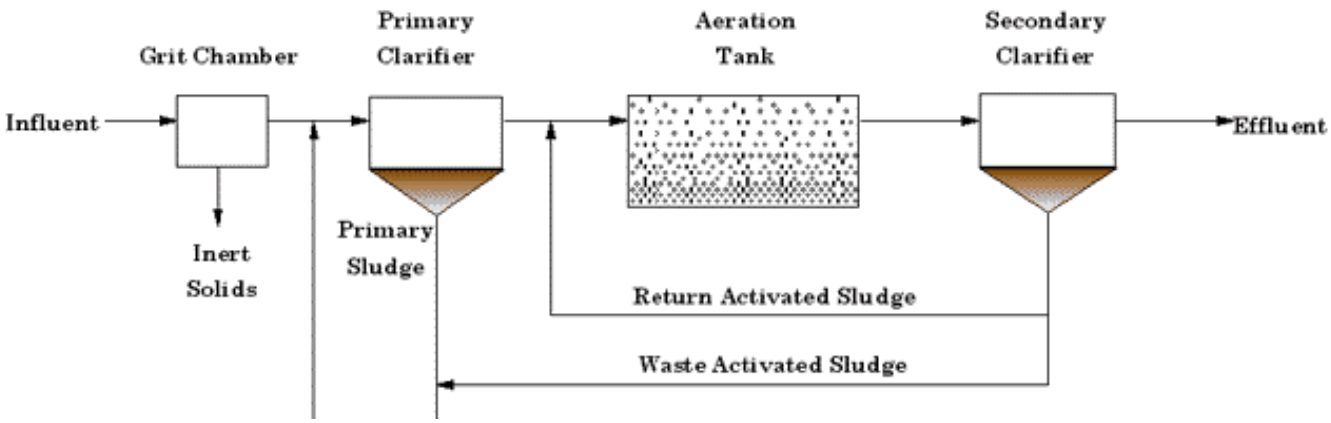

Figure 3-1. Schematic diagram of an activated sludge treatment plant.

The different stages of a WWTP commonly comprises a pre-treatment to remove about 50–60% of the suspended solids and 30–40% of the BOD (Metcalf, 2003). The settled primary sludge contains mainly water (between 97% and 99%) and separates mostly organic matter that is highly putrescible.

The pre-treatment is followed by a biological step where aerobic microorganisms remove the remaining (or nearly total) BOD and suspended solids. Nitrogen (N) and phosphorus (P) are commonly removed simultaneously, although N is more usually and easily targeted first. A secondary clarifier produces the dischargeable effluent as overflow and a bottom sludge (98–99% water). The bottom sludge is partly recycled to maintain the concentration of the microorganisms and biodegradable organic matter at the required level. If a pre-treatment process is present, primary and secondary sludge are often combined and thickened sludge undergoes further treatment.

3.2 Selection of activated sludge treatment plants

During the initial stages of the literature review and from information available in the Australian Guidelines for Water Recycling (AGWR) it became clear that there was minimal data from Australia on the performance of WWTPs to remove enteric microbial pathogens (NRMMC, 2006). In addition, there are a range of designs and configurations of biological WWTPs currently used throughout Australia. This makes it difficult to set a standard for validation of ASP without strong

data on the effects issues such as variations in design and operation, geographical and climatic, as well as source inputs on the operating efficiencies of ASPs. Furthermore, WWTPs also vary across Australia in their design based on age and intended flow rates (based on the size of the wastewater catchments). It was agreed that the activated sludge process (ASP) would be the primary focus of this study.

The main criteria used for the selection of the WWTPs for this study was a wide representation of treatment plants (size and operational conditions) and varying climatic zones of Australia. Four treatment plants were identified in four different climatic zones of Australia representing sub-tropical to temperate conditions (Table 3.1). Selected treatment plants include Oxley Creek (Sub-tropical), Beenyup (Mediterranean), Boneo (cool temperate) and Rosny (mild temperate oceanic). The specifics of each treatment plant are provided in Table 3-2.

Table 3-1. Selected wastewater treatment plants in four climatic conditions of Australia.

Climatic conditions	Location	WWTP	Hydraulic Retention Time (hours)	Samples collated
Warm, humid (Sub tropical)	Brisbane	Oxley Creek	55	48
Warm dry summer, cool wet winter. (Mediterranean)	Perth	Beenyup	24	22
Cool temperate climate	Melbourne	Boneo	37-39	20
Mild temperate oceanic climate	Hobart	Rosny	2	24

3.2.1 Collection of operator supplied plant specific operational data

In the initial stage of the project, participating water utilities were contacted to obtain their input and experience on the biological systems they operate. These participating water utilities were requested for any available documentation or reports that provide information on studies undertaken on the detection and removal of pathogens and indicator microorganisms in their biological systems plants. In addition, information on relevant measured water quality parameters, information on plant performance and impacts were sought. Where data was provided, attempts were made to ensure at least one complete 12 month cycle of routine physicochemical parameters for the influent and effluent (post clarifiers) was obtained to assess the influence of seasonal variations on the individual treatment plants performance.

Table 3-2. Detailed information on the selected wastewater treatment plants for the study.

Parameter	Oxley Creek	Boneo	Beenyup	Rosny
Climate	subtropical	Cool temperate, Warm to hot summer, cool winter	Mediterranean: Hot summer, cold winter,	Cool temperate
Annual Temperature range (from ASP process)	22 – 30 ˚C	17.3 - 25.1˚C	18 – 27˚C	8.3-16.9˚C
% domestic & industrial wastewater	~20% industrial ~80% domestic	100% domestic	100% domestic	~90% domestic ~10% industrial
Population equivalent load or ML/d	270,000 ~65 ML/day	~75 ML/d	~135 ML/ day	~7.5-15ML/d
Age of treatment plant	9 years, Upgraded in 2006		7 years, Upgraded 2005	65 years upgrades
Influent Flow	~46 ML/day	8.64 ML/d	9.17 ML/d	~6.13 ML/day
Effluent Flow		9.40 ML/d		
Primary treatment eg. grit removal, screen, primary treatment (may be more than one process)	Rotary drum screens → grit removal→ Primary sedimentation polymer aided flocculation	Screening →grit removal →Primary sedimentation → polymer aided flocculation →.	Five step screening (6mm) →grit removal →Primary sedimentation → polymer aided flocculation →	20mm screens on inlet
Secondary treatment ASP train eg. anoxic zone→ aerobic zone	conventional activated sludge process → secondary clarifier	Aerobic nitrifying activated sludge: anoxic zone → aerated zone→ flocculation→ secondary clarification (3:1 recycle used. Additional carbon added in winter to aid denitrification)	conventional activated sludge process with biological nutrient removal → Secondary Clarifier	Trickling filter then Solids contact re-aeration.
Hydraulic Retention time (HRT)	55 hrs	10.5 hrs	10-15 hrs	2 hrs
Sludge Retention time (SRT)	18-19 days	10-20 days	13-14 days	8 days
Mixed liquor suspended solids (MLSS)	3700-3900 mg/L	2990-5750 mg/L	2900-3900 mg/L	-

3.3 Results

All four of the selected treatment plants were able to provide at least 12 month data of regularly recorded physicochemical parameters. This data was then analysed to determine how these monitored physicochemical parameters changed in response to the seasonal and plant operational conditions. A summary of the data from up to 500 observations at each plant is presented as mean values ± SD in the Table 3-3. In addition, detailed descriptive statistical analysis of all available data for all four WWTPs is presented in the Appendix B.

The observed physicochemical parameters and frequency of data collection for influent and effluent varied considerably between the treatment plants. On size alone the Beenyup treatment plant is the biggest (131 ML/day) followed by Oxley Creek WWTP (58 ML/day), whereas Boneo and Rosny are much smaller with 9 and 6 ML/Day capacity respectively (Table 3-3). The highest hydraulic retention time (HRT) was observed in Oxley Creek treatment plant at 55 hours and lowest at Rosny treatment plant (2 hours) (which employs a trickle filter process). Mixed Liquor Suspended Solids (MLSS) target range for all three of the ASP plants was between 3400 to 4240 mg/L (as a trickling filter plant Rosny had no MLSS data available). Sludge Retention Time (SRT) was highest at Boneo (16.4 days) followed by Beenyup (12.42 days) and Oxley Creek (8-9 days). It was observed that SRT and MLSS vary within each treatment plant in response to the seasonal variation in wastewater inflows. In addition, the higher MLSS and SRT at the Boneo and Rosny plants are most likely due to comparatively colder climatic conditions than at the Oxley Creek and Beenyup treatment plants. Despite these differences, a significant reduction in the suspended solids and BOD/COD between the influent and effluent suggests that there was stable operation for each of the treatment plants (Table 3-3).

The data obtained showed that a direct comparison across all WWTPs was not possible due to differences in the size and design of the plants, variations in operational variability and in the frequency of data collection. A number of observations could be made, however, from the statistical analysis of the collected data.

- While the Beenyup WWTP had a mean inflow of 131.2 ML/day (± 7.15), there were observed variations in the inflow. This variation was identified as being primarily in response to rainfall events, as higher inflow coincided with the winter months (July-Aug 2014). This was also evident through variations in the mixed liquor suspended solids (MLSS) at this plant with higher ~3900 mg/L MLSS in winter (winter months) which is required to compensate for the reduced biological activity during the winter months.
- All of the treatment plants had stable influent pH over the studied 12 month period.
- The higher standard deviation for electrical conductivity at the Rosny WWTP is due to known seawater intrusion issues.
- BOD and COD were also observed to have significant decreases between the influent and the effluent in all four plants.
- Similarly, total phosphorus, total nitrogen, TKN and NH_3 concentrations in the influent were consistent over the 12 month period with very little standard deviation. In addition, the continuing ability to reduce the concentration of these chemical parameters was similar for all four treatment plants.

- In contrast to the other chemical parameters, only Oxley Creek demonstrated an ability to significantly reduce the concentration of phosphorus.

Table 3-3. Physicochemical parameters (mean ± SD) for influent and effluent from four wastewater treatment plants in Australia (January 2014 – April 2015).

Parameter	Rosny		Oxley		Beenyup		Boneo	
	Influent	effluent	influent	effluent	Influent	effluent	Influent	effluent
Flow volume (ML/day)	5.89 (±1.54)	-	58.13 (±39)	57.81 (±39)	131.22 (±7.15)	-	9.17 (±1.73)	-
Temp (°C)	18.3 (±1.37)	18.8 (±3.64)	25.8 (±1.62)	26.5 (±2.59)	23.1 (±2.46)	26.0 (±2.12)	21.3 (±1.34)	20.4 (±1.22)
pH	8.65 (±0.22)	7.18 (±0.26)	7.26 (±0.23)	7.81 (±0.14)	7.466 (±0.75)	7.16 (±0.77)	7.56 (±0.17)	6.80 (±0.12)
Turbidity (NTU)			185.6 (±53.48)	2.39 (±1.70)	117.7 (±19.29)	2.89 (±1.13)	176 (±50.09)	1.93 (±1.48)
SS (mg/L)	405.8 (±183.20)	9.03 (±5.19)	396 (±92.08)	5.8 (±1.48)	135.9 (±26.98)	17.9 (±19.20)	500(±176)	
Electrical Conductivity (mS/cm)	1567 (±1603.9)	1189 (±612.61)	1421 (±245.80)	1077 (±127.02)		115.3 (±5.13)	818.3 (±323.60)	897.9 (±41.77)
DO (mg/L)	0.20 (±0.15)	81.7% (±9.74)	1.55 (±2.67)	6.87 (±1.05)	3.68 (±1.11)	7.96 (±1.09)	1.70 (±0.93)	5.37 (±2.04)
$ALKCACO_3$ (mg/L)	-	-	308.23 (±58.10)	157.74 (±27.16)	335.84 (±17.80)	119.33 (±15.96)	-	0.18 (±0.54)
NH_3 (mg/L)	38.5 (±5.20)	20.1 (±5.96)	-	-	52.1(±3.52)	0.47 (±0.51)	-	0.18 (±0.29)
NH_4 (mg/L)	38.5 (±5.20)	19.7 (±5.30)	45.40 (±12.10)	1.86 (±0.22)	0.142 (±0.14)	0.14 (±0.14)	-	-
TKN (mg/L)	60.8 (±14.5)	-	-	0.61 (±0.22)	65.9 (±3.9)	3.42 (±1.63)	75.8 (±19.9)	-
NO_3 (mg/L)	10.04 (±0.82)	6.74 (±3.91)	-	-	-	12.3 (±2.35)	-	3.92 (±2.21)
TN (mg/L)	63.2(±10.96)	33.5 (±9.58)	59.56 (±15.88)	3.95 (±3.71)	65.8 (±4.38)	15.3 (±2.32)	-	-
TP (mg/L)	10.9 (±1.53)	6.20 (±1.14)	13.24 (±3.93)	1.70 (±1.71)	11.8 (±1.02)	11.4 (±0.86)	13.9 (±6.52)	-
BOD (mg/L)	357 (±166.89)	14.1(±26.31)	309(±94.57)		210 (±29.52)	7.79 (±6.15)	406(±86.30)	
COD (mg/L)	-	-	793 (±242)	40.90 (±12.94)	415 (±93)	51(±23)	906 (±207)	
MLSS (mg/L)	-		3841 (±411)		3495 (±292)		4427 (±806)	
SRT (days)			8-9		12.24 (±0.9)		16.4 (±3)	
SRT range (days)	-		18-19		10.59-13.46		10-20	

3.4 Discussion and Conclusions

The amount of physicochemical parameters recorded and the frequency of data collection for influent and effluent varied across the selected activated sludge treatment plants. Despite these observed variations, relatively small standard deviations were detected at all four plants for commonly recorded parameters such as pH, DO, temperature and electrical conductivity, indicating that there is relatively stable range for these parameters. Temperature range was much larger at Rosny and Boneo WWTPs as compared to Oxley Creek WWTP in response to colder winter conditions. Despite this, all of the treatment plants effectively removed BOD/COD from the wastewater and were generally efficient in reducing the concentrations of the other chemical parameters.

The overall conclusion that can be made is that, despite the differences in input and operation of the four treatment plants, the limited variation in the majority of the measured parameters at all of the studied wastewater treatment plants (excluding any seasonal variations) indicated that they were all operating under stable conditions over the time period of this study.

One major issue that was determined through this assessment of the operation of the four WWTPs was the fact that the Rosny WWTP is a very old plant that primarily uses a trickling filter system (followed by a solids contact re-aeration) with a HRT of only 2 hours. This fact combined with the results from the assessment of treatment performance means that it is very difficult to compare its operation with the other three newer activated sludge plants (Oxley Creek, Beenyup and Boneo). During the selection process the Rosny plant was chosen with the aim to compare the treatment capability of older plants that may or may not have optimal treatment capacity. The second initial aim was that if the performance of the Rosny plant was able to be compared to the other three WWTPs, then the influence of a cool climate on the ASP process could be assessed and compared to plants in other climatic regions of Australia. Through the results gained on the operating conditions given above (and the subsequent determined LRV rates for the studied microorganisms) it was concluded that, while the plant is operating under optimal conditions for its design, there is limited use in comparing the outcomes for the Rosny WWTP with the other three ASP treatment plants. It is therefore recommended that the determined treatment outcomes for the Rosny plant be considered on their own as representative for older trickling filter WWTPs. All information on the impact of a cooler climate on an activated sludge system within this study therefore needs to be drawn from the outcomes of the Boneo plant. It is also recommended that further studies be undertaken to further the information on seasonal and climatic impacts from the initial results established by this study.

4 General Materials and Methods

The collection and processing of samples for detection and quantification of the target microorganisms was undertaken using common sampling and analysis methods for all four WWTPs. Summaries on the sampling and analytical methods are provided below while the full detailed methodologies are provided in Appendix C.

4.1 Microorganisms targeted in selected activated sludge treatment plants

All four activated treatment plants (Oxley Creek, Beenyup, Boneo and Rosny) were sampled and tested for targeted microorganisms between August 2014 and May 2015. Each of the wastewater treatment plants was sampled at least bi-monthly and tested for the presence and number of viruses (adenoviruses, polyomaviruses and *Microviridae*), *Cryptosporidium* and *E. coli*.

An additional 20 samples of influent and effluent were also collected from the Oxley WWTP to determine if sampling of activated sludge plants for validation purposes should be carried out on a time based sampling relating to the calculated HRT, or if a more simplified simultaneous collection of samples at the influent and effluent of the activated sludge plant could give similar results.

A break-down of the total samples numbers collected and analysed for each target microorganism from all four sites is provided in Table 4-1. These samples were used for all subsequent analysis to provide information on input and output concentrations and the corresponding LRVs.

Table 4-1. Number of samples collected from ASP from all four sites (influent, effluent and LRVs).

Count/Values	Beenyup	Boneo	Oxley	Rosny	Grand Total
Influent *E.coli*	22	20	40	24	106
Effluent *E.coli*	20	20	40	24	104
LRVs *E. coli*	20	20	40	24	104
Influent Adenovirus	23	19	40	25	107
Effluent Adenovirus	22	19	39	25	105
LRVs Adenovirus	22	19	39	25	105
Influent Polyomavirus	23	19	40	25	107
Effluent Polyomavirus	22	17	38	25	102
LRVs Polyomavirus	22	17	38	25	102
Influent *Microviridae*	23	19	40	25	107
Effluent *Microviridae*	22	18	39	25	104
LRVs *Microviridae*	22	18	39	25	104

4.2 Sample collection and processing

General sampling was undertaken by collection of grab samples of influent and effluent wastewater were collected from the WWTPs based on the hydraulic retention time (HRT). In all cases local water utilities staff assisted in the sample collection.

On each sampling occasion, triplicate samples were collected for the influent (3 × 100 mL) in sterile labelled glass bottles after the grit removal screens. Triplicate samples of treated effluent (3 × 10 L) were also collected at the outlet of the clarifiers into sterile labelled carboy containers (Nalgene). As described above, on each sample occasion the collection time of the influent and effluent samples was separated by the calculated Hydraulic retention time provided by the partner Water Utility.

All samples were immediately transferred to the laboratory for further processing in cool boxes containing freezer blocks.

Figure 4-1. Sampling sites at the Oxley Creek Wastewater Treatment Plant.

4.3 Sample storage, shipment and processing

Influent and effluent samples collected from each site were processed at the CSIRO laboratories in Brisbane, Perth, Melbourne and Hobart.

The enumeration of *E. coli* was undertaken in each of the respective local CSIRO laboratories within 6 hours of sampling using the Colilert Quanti-tray 2000 (IDEXX Westbrook, Maine) technique as outlined in Appendix C. Samples collected for the detection of enteric virus and *Cryptosporidium* oocysts in the effluent were concentrated with Hemoflow FX80 dialysis filters (Fresenius Medical Care, Lexington, MA, USA) in the respective laboratories. These concentrated effluent and influent wastewater samples were then shipped to the Brisbane laboratory on ice via overnight courier. Further processing of samples was carried out at the Brisbane laboratory as described in Appendix C.

4.4 Sample quality assurance and quality control

All sampling and analysis steps were designed and undertaken using stringent quality assurance and controls.

Samples were collected and processed according to the best practices as outlined in the Standard Methods for Collection and Analysis of Water and Wastewater (Federation WE, 2005). This included samples being kept chilled during transport and processed within of collection 6 hours for *E. coli* analysis and within 24 hours for the concentration steps for virus and *Cryptosporidium* analysis. The shipping of concentrated samples back to the Brisbane laboratory was undertaken using dedicated transport chiller boxes containing sufficient ice blocks to maintain an appropriate temperature for the travel time. Samples were shipped using overnight couriers and for some shipping runs, temperature loggers placed inside the package were used to assess any fluctuations in temperature that could be expected during shipment. The temperature loggers were used consistently in the early sampling stages to determine if adjustments were needed to the number of ice packs required to maintain an appropriate low temperature during shipping. The temperature loggers were then used on a more random basis during the rest of the sampling period to ensure that consistent transport conditions were continued to be maintained.

Additional QA/QC procedures specific for the analysis and enumeration of the specific target microorganisms are listed in detail in the particular methodology sections in sections 5.1 and 6.1. These include details on:

- Standardised methods for the further concentration of samples used for detection of the viruses and *Cryptosporidium.*
- Appropriate storage of processed samples, particularly for storage of extracted DNA at -80°C to minimise sample degradation.
- Reduction of sample to sample variation by batch processing of DNA extractions and qPCR runs.
- Routine QA/QC methods for the PCR based detection methods as well as controls used to account for the presence of any PCR inhibition.

4.5 Calculation of Log Removal Value (LRV)

A log removal value (LRV) was set as a measure of the ability of the activated sludge processes to remove pathogenic microorganisms. The LRV results were determined according to the following equation:

$LRV = \log_{10}(A) - \log_{10}(B)$

Where: A=is the number of viable microorganisms in the influent prior to treatment

B=is the number of viable microorganisms in the effluent after treatment

A LRV of 1 is equivalent to 90% removal of a target microorganisms, a LRV of 2 is equivalent to 99% removal and an LRV of 3 is equivalent to 99.9% removal and so on.

4.6 Statistical analysis

Descriptive analysis of Log_{10} transformed data of determined microbial numbers in the influent and effluent was carried out using the Real Statistic add-on in Microsoft Excel. Relationships between different microorganisms in the influent and effluent samples were assessed using the non-parametric Spearman rank correlation with a two-tailed *P* value. Relationships between LRVs within sites were also calculated using Spearman rank correlation.

Statistical significances of the LRVs were determined by applying a Student's *t*-test to the T_{90} values. The critical P-value for the tests was set at 0.05. The null hypothesis was accepted if the *P* value was greater than (0.05) and in these cases differences between the compared data was considered to be non-significant.

5 Removal of Bacteria

Faecal coliforms, particularly *E. coli* have been commonly used in assessing the performance of wastewater treatment plants. Much of this has involved examining the number of cells detected in both the effluent and influent (Carducci and Verani, 2013; Haramoto *et al*., 2006), however, there has been some effort to determine log reduction values for *E. coli* to estimate (Chauret *et al*. 1999, Rose *et al*. 2004).

While the hypothesis of this study is that selected enteric viruses and potentially *Cryptosporidium* are going to be the most suitable microorganisms for use in validating activated sludge plants (based on their recognised higher resistance to environmental pressures and treatment methods), it was considered appropriate to also include the analysis of *E. coli* LRVs as a comparison with the determined viral and protozoan results. In addition, while the results of this study are focused on generating information for use in validation processes, *E. coli* has a significant role in the ongoing verification protocols for activated sludge plants (NRMMC, 2006). The results gathered in this study therefore, will have use for improving the understanding of requirements for these verification protocols.

5.1 Methodology

5.1.1 Total coliform and *E. coli* quantification

Total coliform and *E. coli* numbers were enumerated in the influent and effluent wastewater samples using the Colilert Quanti-tray 2000 (IDEXX Westbrook, Maine). Full details on volumes sampled, incubation details and quality control measures are provided in Appendix C.

The MPN number obtained from the Colilert Quanti-tray method for both influent and effluent samples were Log_{10} transformed for determining LRVs as outlined in Section 4.5.

5.2 Results

5.2.1 *E. coli* numbers in influent and effluent samples

Oxley Creek WWTP

E. coli numbers in the influent and effluent samples (n = 40) collected at the Oxley Creek WWTP from 23/10/2014 to 9/04/2015 (covering a spring, winter and autumn period) along with the calculated LRVs are presented in Figure 5.1. A detailed statistical analysis of the collected data including mean, median, range and geometric mean is also presented in Table 5.1.

No significant changes in the *E. coli* numbers in the influent were observed with a calculated mean of 8.3 $\log_{10} L^{-1}$ ± 0.21. The *E. coli* numbers in the effluent numbers were more variable (5.33 $\log_{10} L^{-1}$ ± 0.41). The calculated LRVs were relatively consistent over the sampling period with a mean of 3.04 $\log_{10}$ ± 0.37.

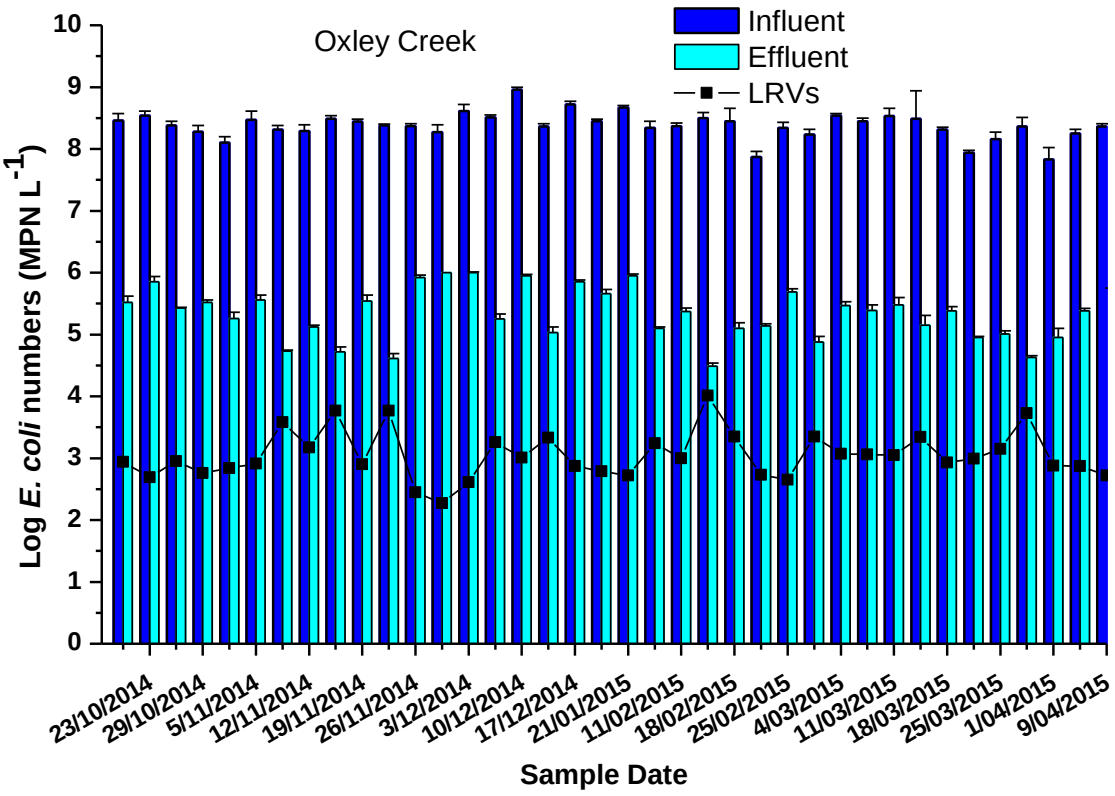

Figure 5-1. Comparative *Escherichia coli* numbers in the influent and effluent samples (n=40) at Oxley Creek wastewater treatment plant.

Table 5-1. Descriptive Statistical analysis on *E. coli* numbers in influent and effluent samples detected in the four WWTPs.

	Oxley		Beenyup		Boneo		Rosny	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Mean	8.4	5.3	8.2	5.6	9.5	5.9	7.9	6.5
Median	8.4	5.4	8.2	5.5	9.4	5.9	7.8	6.6
Standard Deviation	0.21	0.41	0.20	0.32	0.43	0.31	0.27	0.55
Range	1.1	1.5	0.8	1.2	2.1	1.2	1.0	1.8
Maximum	9.0	6.0	8.4	6.1	10.8	6.5	8.5	7.2
Minimum	7.8	4.5	7.5	5.0	8.7	5.4	7.6	5.4
Count	40	40	20	20	22	20	13	13
Geometric Mean	8.4	5.3	8.2	5.6	9.5	5.9	7.9	6.4

Numbers reported as Log_{10} L^{-1}

Beenyup WWTP

E. coli numbers in the influent and effluent samples (*n* = 22) collected at the Beenyup WWTP over a 12-month period from 17/06/2014 to 02/06/2015 are presented in Figure 5-2 and Table 5-1. The sampling period covered an entire Western Australian seasonal year (winter, spring, summer and autumn).

The mean *E. coli* numbers (9.48 $\log_{10}$ L^{-1} ±0.43) in the influent were higher than all other sites tested. The calculated mean numbers of *E. coli* in the effluent (5.66 $\log_{10}$ L^{-1} ±1.33) were similar to Oxley Creek but were more variable across the sampling period. Slightly higher LRVs (with a mean of 3.75 $\log_{10}$ ±1.38) were observed at the Beenyup site compared to the other WWTPs.

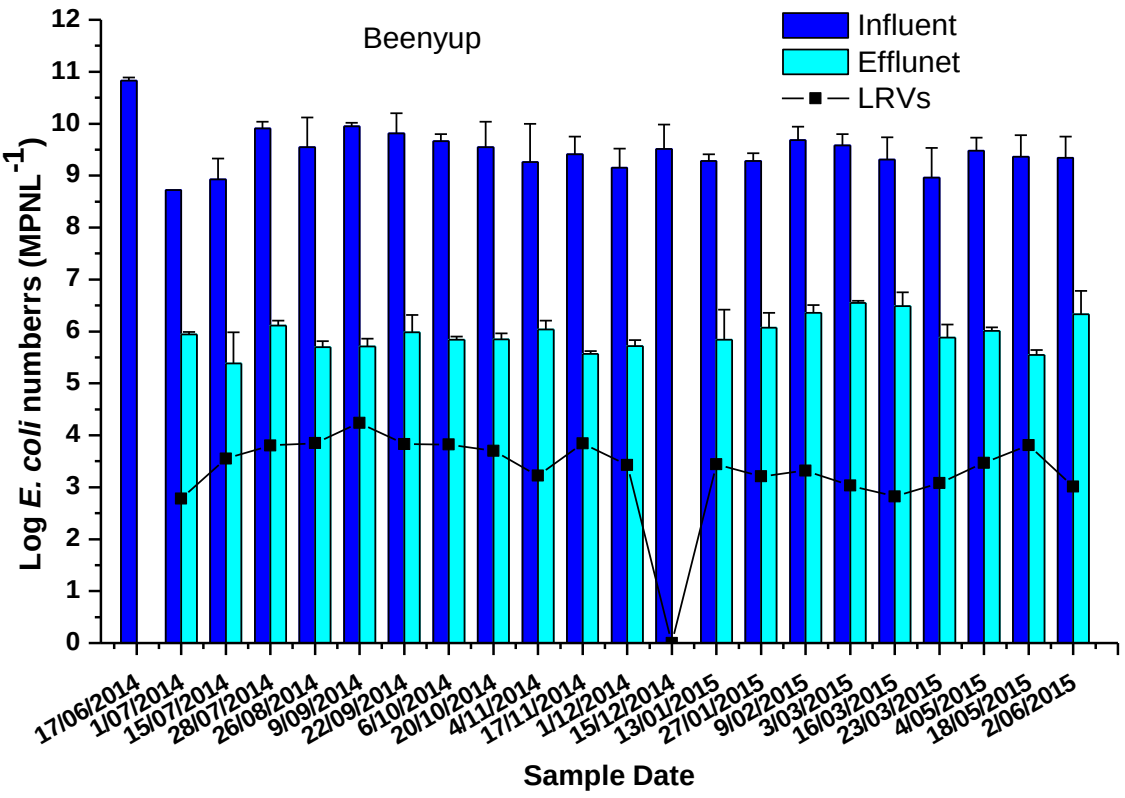

Figure 5-2. Comparative *E. coli* numbers in the influent and effluent samples at Beenyup wastewater treatment plant.

Boneo WWTP

Samples collected from the Boneo WWTP covered all four seasons from 19/05/2014 to 16/06/2015. *E. coli* numbers in the collected influent and effluent samples (*n* = 20) are presented in Figure 5-3 and Table 5-1.

The mean *E. coli* numbers in the influent (8.17 $\log_{10}$ L^{-1} ±0.20) varied little throughout the sampling period as can be seen by the small calculated standard deviation. The mean *E. coli* numbers in the effluent (5.58 $\log_{10}$ L^{-1} ± 0.32) were similar to Oxley creek and Beenyup WWTPs. The mean Log removal of 2.59 $\log_{10}$ ± 0.37 was approximately 0.5 – 1 $\log_{10}$ lower compared to Oxley creek and Beenyup WWTPs respectively.

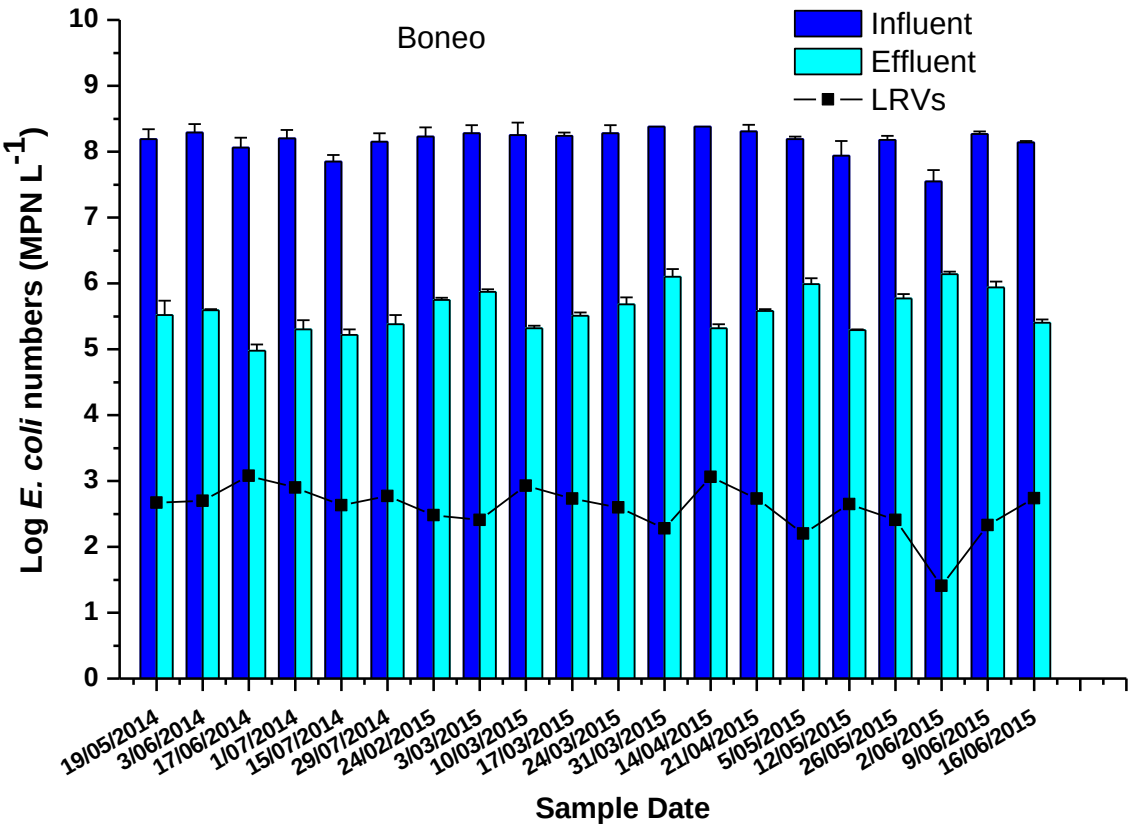

Figure 5-3. Comparative *E. coli* numbers in the influent and effluent samples at Boneo wastewater treatment plant.

Rosny WWTP

Samples were collected from the Rosny WWTP during the period from 19/05/2014 to 16/06/2015. This covered all four seasons. A total of 22 samples were collected from the Rosny site but *E. coli* data from 9 samples was found to be inconsistent and was not included in the final data analysis.

E. coli numbers in the influent and effluent samples (n = 13) collected at Rosny WWTP are presented in Figure 5-4 and Table 5-1. The mean *E. coli* numbers in the influent (7.88 $\log_{10}$ L^{-1} ± 0.27) were lower compared to the other sites tested, however, the mean *E. coli* numbers in the effluent (6.47 $\log_{10}$ L^{-1}± 0.5) were also higher than all the other sites. The resulting mean LRV of 1.41 $\log_{10}$ L^{-1}± 0.55 was therefore much lower than those determined at the three ASP WWTPs.

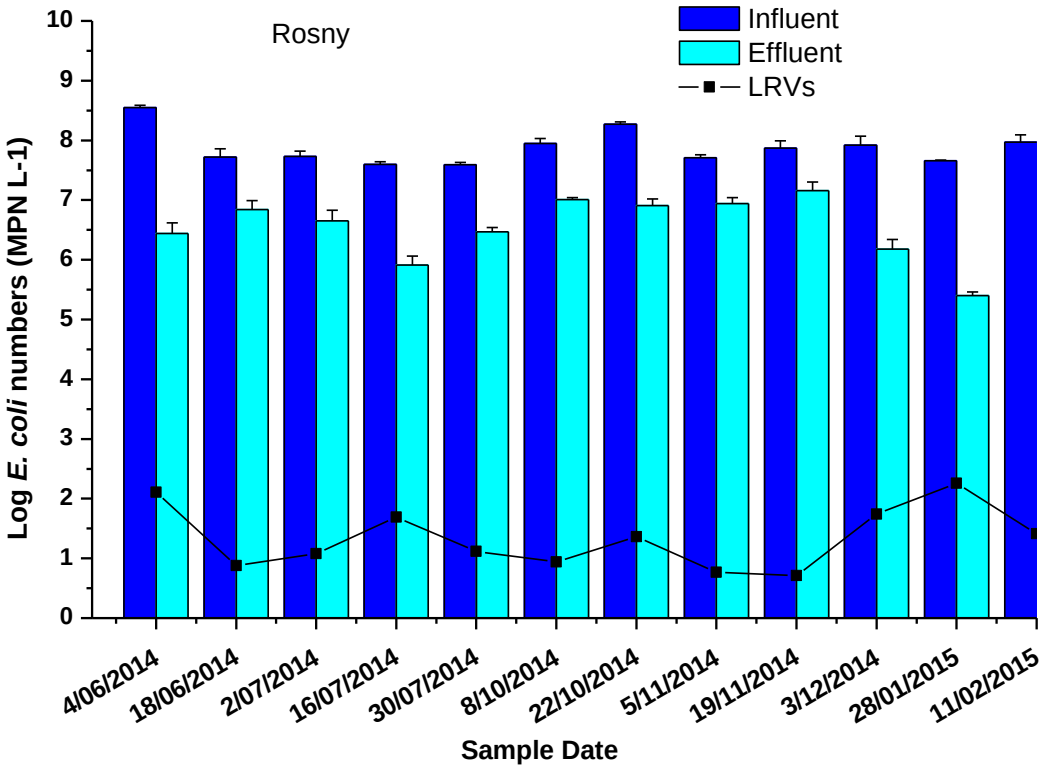

Figure 5-4. Comparative *E. coli* numbers in the influent and effluent samples at Rosny wastewater treatment plant.

5.2.2 Comparative distribution and removal of *E. coli* across sites

A comparative distribution of *E. coli* numbers within and across the WWTPs is presented in a Box plot (Figure 5-5).

The box plots show that the variations in the mean *E. coli* numbers in the influent varied as much between the WWTPs (from a low of 0.2 to a high of 1.6 $\log_{10} L^{-1}$) than within an individual plant (ranging from 0.8 to 2.11 $\log_{10} L^{-1}$). Similarly, *E. coli* numbers in the effluent also varied between WWTPS (from as low as 0.25 to as high as 1.14 $\log_{10} L^{-1}$) and within individual WWTPs with Boneo having the smallest range of 1.16 $\log_{10} L^{-1}$and Rosny the highest range of 1.76 $\log_{10} L^{-1}$ (Table 5-1).

Overall, *E. coli* was consistently reduced during the ASP in all systems although the removal rates varied from site to site. The median *E. coli* LRV at Beenyup (3.46 $\log_{10}$) was better than Oxley Creek (2.96 $\log_{10}$) and Boneo (2.66 $\log_{10}$). In contrast, the Rosny WWTP had a median LRV of only 1.36 $\log_{10}$ (Table 5.2). Analysis of the 10^{th} percentile LRVs shows that 10^{th} percentile values for LRVs were ~ 2 $\log_{10}$ across all the WWTPs with the two best performing treatment plants, Beenyup and Oxley, being 2.84 and 2.65 $\log_{10}$ respectively.

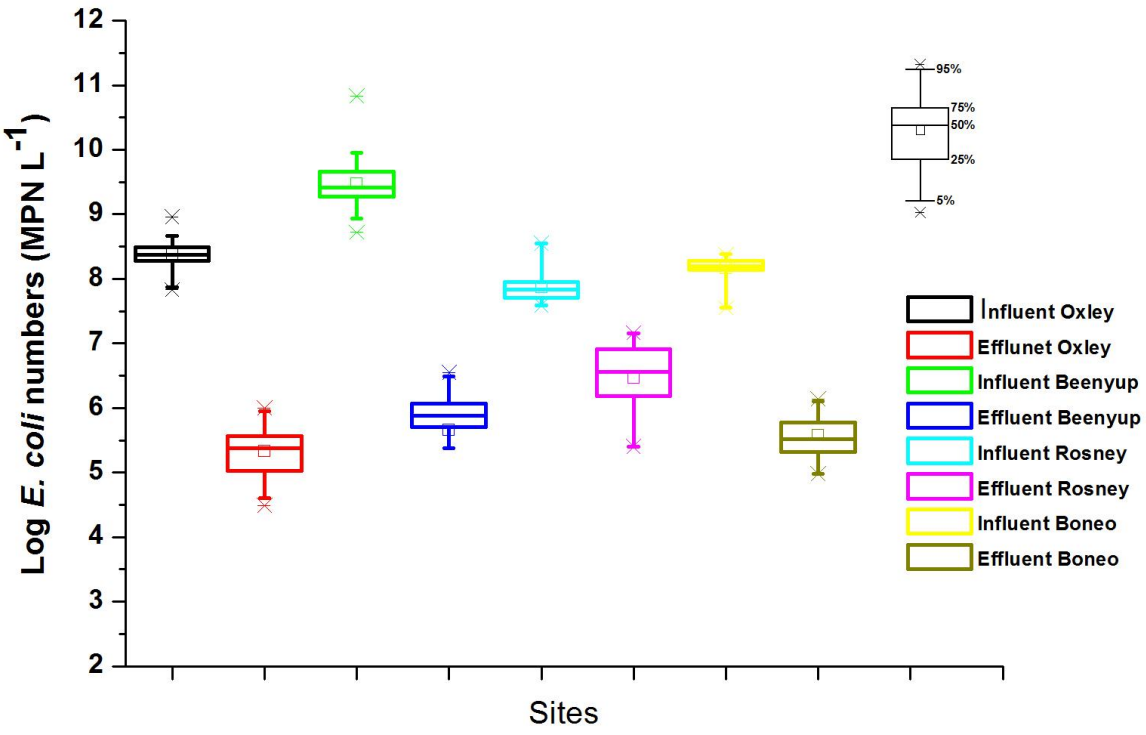

Figure 5-5. Comparative distribution of *E.coli* in influent and Effluent across all four sites.

The vertical line represents the range of data, the squares represent the mean. The small horizontal lines (whiskers) at the end of vertical lines represent the minimum and maximum values observed (range), and the crosses extending from the vertical line represent the 99% confidence limits.

Table 5-2. Descriptive statistical analysis of *Escherichia coli* log removal values (LRVs) across the four WWTPs.

	Oxley	Beenyup	Boneo	Rosny
Mean	3.0	3.5	2.7	1.4
Median	3.0	3.45	2.8	1.4
Standard Deviation	0.37	0.40	0.37	0.55
Range	1.7	1.5	1.7	1.5
Maximum	4.0	4.2	3.1	2.3
Minimum	2.3	2.8	1.4	0.7
Count	40	20	20	13
Geometric Mean	3.0	3.4	2.5	1.3
5^{th} percentile	2.4	2.8	1.4	ND
10^{th} percentile	2.6	2.8	2.2	ND
95^{th} percentile	3.8	4.2	3.1	ND

ND= no data as samples numbers was <20.

5.3 Discussion and conclusions

The results of this study indicate that *E. coli* numbers can vary in the influent entering the different WWTPs, however, this is primarily an influence of the source wastewater. Despite this, the three activated sludge plants (Oxley Creek, Beenyup, Boneo) consistently achieved similar removal efficiencies for *E. coli* with mean LRVs of 3 to 3.5 $\log_{10}$ and 10^{th} percentile LRVs of 2.2 to 2.8 $\log_{10}$. The LRVs obtained varied little between sample events with relatively consistent removal rates across the entire sampling period for each treatment plant. The limited variation in LRVs in the Boneo WWTP (with only one sampling occasion having an LRV less than 2 $\log_{10}$, Figure 5-3) suggests that the larger seasonal temperature variations have little influence on *E. coli* removal in activated sludge plants in cooler regions located in southern Australia. This should be confirmed, however, by a more comprehensive assessment of WWTP located in regions that have larger temperature variations between summer and winter.

The variations in the means and ranges of *E. coli* numbers were slightly greater in the influent water than in the effluent. This is reflective of the variable numbers in the different sources waters (influent) (ranging from a mean of 9.48 to 7.88 $\log_{10}$) while the number of *E. coli* cells detected in the effluents of each of the plants where more commonly around 5.38 to 6.56 $\log_{10}$). The cross site variation in *E. coli* numbers in influent wastewater observed in this study is comparable with the *E. coli* numbers (7 to >9 $\log_{10}$) reported from Japan and Canada (Carducci and Verani, 2013; Haramoto *et al*., 2006; Payment *et al*., 2001). The *E. coli* numbers in the effluent across the activated sludge WWTPs were similar with mean values between 5.33 and 5.95 $\log_{10}$ L^{-1} which are again comparable to the reported numbers in treated effluent in Japan and Canada (Carducci and Verani, 2013; Haramoto *et al*., 2006; Payment *et al*., 2001).

When the removal of *E. coli* by the three ASP treatment plants is considered as an LRV, the results showed that all three plants can consistently achieve removal rates with geometric mean of LRVs of 2.5 to 3.44 $\log_{10}$. These results are comparable to the LRV of 2.8±0.52 $\log_{10}$ reported in an earlier Australian study by Flapper *et al*. (2010). Other international studies have also determined high LRVs for *E. coli* and other related bacterial with Chauret *et al*. (1999) reporting $\geq$ 3.50 $\log_{10}$ reduction in the numbers of total coliforms, faecal coliforms, *Enterococcus spp.* and *Aeromonas spp.* during ASP treatment and Rose *et al*. (2004) reporting LRVs for *E. coli* of 1.62 to 3.11 $\log_{10}$ in a study of ASP plants from Michigan, USA.

As discussed in Section 4, there is limited ability to directly compare the results from the Rosny plant with the other three ASP plants. The Rosny plant consistently had lower LRVs than the ASP plants most likely because of the significantly shorter hydraulic retention time (2 hours). The treatment mechanisms involved in the trickling filter process could also be a reason for the lower removal rates, however, other factors that could also cause a lower HRT (for example the possibility that dislodged pieces of biofilm from the trickling filter) should not be excluded as reasons for the higher numbers of *E. coli* in the effluent. Regardless, the Rosny still achieved a relatively consistent removal of *E. coli* with a geometric mean LRV of 1.3 $\log_{10}$.

6 Removal of Viruses

Enteric viruses are the biggest concern for Australian water recycling schemes due to their high infectivity and low dose. In addition, viruses can be difficult to detect in wastewater due to their relative low numbers in large wastewater volumes (compared to the traditional microbial indicators such as faecal coliforms), traditionally difficulties with detection methodologies, and issues relating to the potential presence of a broad range of virus types with a wide spectrum of sources and environmental resistances. These factors have made it very difficult to determine how well activated sludge plants can remove the enteric viruses, whether this removal is consistent, and if an ASP can be validated. A major aim of this study was to determine how (or if) the removal rates of selected viruses, with their recognised resistance to environmental pressures and treatment processes, could be used in the validation of activated sludge plants.

A number of international studies have reported the presence of enteric viruses in high numbers in wastewater and effluent (Bofill-Mas *et al*., 2006; Albinana-Gimenez *et al*., 2009; Katayama *et al*., 2008; Nordgren *et al*., 2009; Aw and Gin 2010). However, there is limited information available on the fate of enteric viruses during wastewater treatment from Australian activated sludge treatment plants. As a result, developing an appropriate validation process requires an improved understanding of enteric virus numbers and removal (and resultant LRVs) during the ASP. Due to the impracticality of studying all of the wide range of enteric viruses, it is essential to select one or two surrogate viruses suitable to represent the broader range of enteric viruses in any validation protocols.

Two enteric viruses, adenovirus and polyomavirus have been suggested as potentially suitable as representative indicator pathogens. Human adenoviruses have been reported in wastewater in high numbers worldwide (Sidhu *et al*., 2012; Albinana-Gimenez *et al*., 2006) and are important candidates for process indicators because they are thermally stable and are resistant to ultraviolet light (Gerba *et al*., 2002; Meng and Gerba, 1996). Human polyomaviruses are also ubiquitous pathogens with a worldwide distribution (Albinana-Gimenez *et al*., 2006) and have also recently been proposed as indicators for the presence of human viral pathogens in contaminated water (Bofil-Mas *et al*., 2006; Fong *et al*., 2010).

Until recent advances in molecular technologies improved the detection capabilities for enteric viruses, somatic coliphages were commonly used to represent enteric viruses. Somatic coliphages are non-enveloped viruses structurally similar to enteric viruses but are much easier to culture due to the ease of culturing the host bacteria. Somatic coliphages have been shown to be present in relatively large numbers in wastewater and their removal during wastewater treatment is considered to be similar to that of enteric virus (Gantzer *et al*., 1998; Ottoson *et al*., 2006). However, somatic coliphage is a diverse group with four distinct families (*Myoviridae*, *Microviridae*, *Siphoviridae*, and *Podoviridae*) with each family containing several genera. A recent study has reported somatic coliphage belonging to the *Microviridae* are widely prevalent in sewage contaminated surface waters (Lee 2009). Therefore, somatic coliphage from the *Microviridae* may also be useful wastewater process indicators.

The research in this study therefore was designed to determine the applicability of using either adenovirus, polyomavirus or the *Microviridae* somatic coliphage as tools for validating activated sludge processes.

6.1 Methodology

The presence and number of the enteric virus and *Microviradae* were determined using quantitative real-time PCR. Details on the PCR reactions detection and quantification methods are given in Appendix C.

As viruses are present in lower number in larger sample volumes than enteric bacteria, concentrated of the samples was necessary to a volume containing sufficient viruses for an accurate detection. The concentration methods, DNA extraction procedure and quality control measures are also given in detail in Appendix C.

The numbers of each virus detected in each PCR reaction were converted to the original collected sample volumes and Log10 transformed. These transformed values were then used to determine the LVR for each sample occasion at each treatment plant.

6.2 Results

6.2.1 Enteric virus recovery efficiency with FX80 filters as part of the concentration method

The mean recovery efficiency for adenovirus in treated wastewater effluent when concentrated using the FX 80 hemaflow filter units was 36.66% with a standard deviation of ± 5.50%. There was confidence, therefore, that the concentration method used for concentrating viruses in the influent and effluent samples would be consistent enough to provide accurate data.

6.2.2 Virus numbers in the influent and effluent of the Oxley Creek WWTP

Comparative numbers of adenovirus, polyomavirus and *Microviridae* in influent and effluent samples collected at the Oxley Creek WWTP from 19/11/2014 to 22/04/2015 are presented in Figure 6-1. Detailed statistical analysis of the collected data (mean, median, range and geometric mean) is presented in Table 6-1.

All three viruses tested were present in the influent of the Oxley Creek WWTP with means of 6.5 to 7.4 Log_{10} L^{-1}. Adenovirus had the smallest and *Microviridae* the largest average numbers across the sampling period. *Microviridae* was observed to be the most stable of the three viruses with the smallest standard deviation and range. Polyomavirus and adenovirus numbers in the influent were a little more variable with adenovirus having a bigger standard deviation and range than polyomavirus. There was no discernible seasonal impacts for any of the three viruses over the sampling period (which covered spring, winter and autumn periods).

In the effluent of the ASP the Oxley Creek WWTP the average number of each virus detected ranged from 3.7 to 4.5 log_{10} L^{-1} (Table 6-1). *Microviridae* again had the smallest standard deviation and range of the three viruses. In contrast to the results observed in the influent, adenovirus had a

standard deviation and range very similar to *Microviridae* while polyomavirus had the largest range and standard deviation. Similar to what was observed in the numbers detected in the influent, there was no discernible impact of seasons on the numbers of viruses detected in the influent.

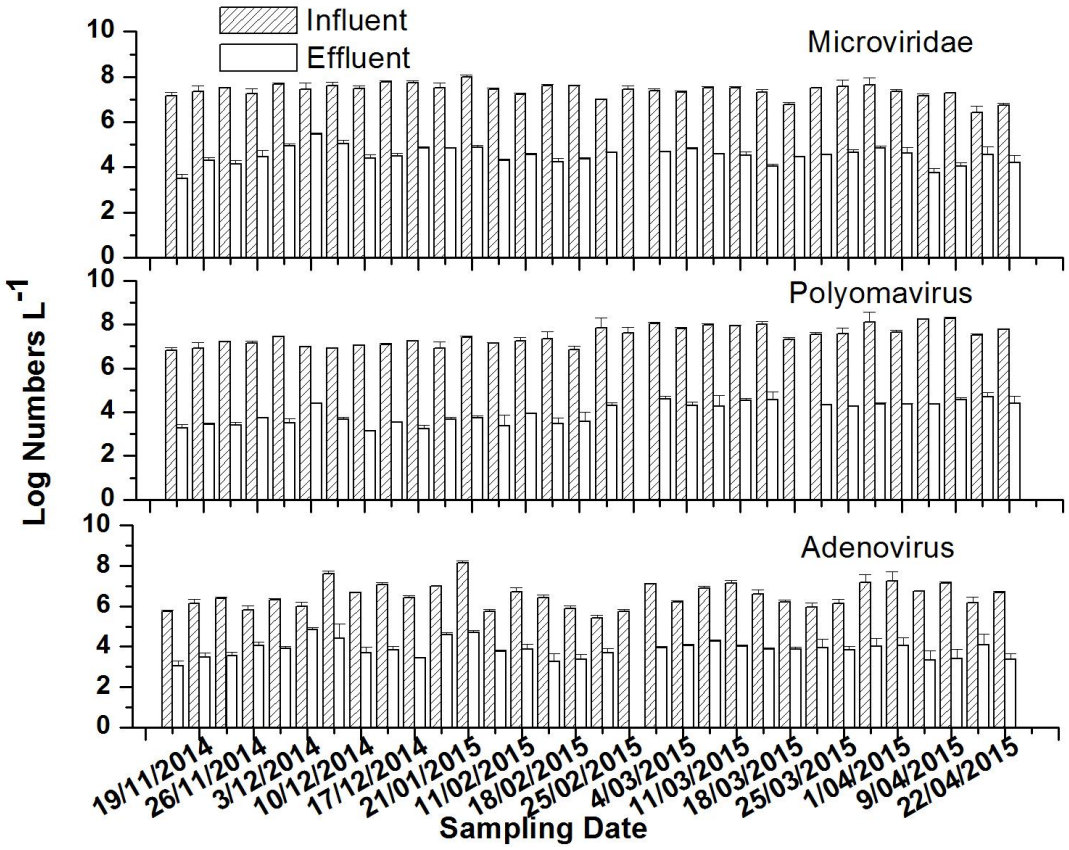

Figure 6-1. Comparative adenovirus, polyomavirus and *Microviridae* numbers in influent and effluent samples collected at the Oxley Creek WWTP.

Table 6-1. Statistical analysis of data from the Oxley Creek WWTP.

	Adenovirus		Polyomavirus		*Microviradae*	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
Mean	6.5	3.8	7.3	3.7	7.4	4.5
Median	6.5	3.8	7.2	3.7	7.5	4.6
Standard Deviation	0.61	0.44	0.54	0.66	0.33	0.38
Range	2.7	1.9	2.2	2.3	1.6	1.9
Maximum	8.2	4.8	8.3	4.7	8.1	5.4
Minimum	5.4	2.9	6.0	2.4	6.4	3.5
Count	40	39	40	38	40	39
Geometric Mean	6.5	3.7	7.3	3.67	7.4	4.5
5^{th} percentile	5.7	3.0	6.3	2.4	6.7	3.8
10^{th} percentile	5.7	3.2	6.7	2.8	7.0	4.0
50^{th} percentile	6.5	3.8	7.2	3.7	7.5	4.6
95^{th} percentile	7.6	4.7	8.2	4.6	8.0	5.2

Numbers reported as Log_{10} L^{-1}

As can be observed in Figure 6-2 under all the means and percentiles, polyomavirus had the biggest difference between the numbers in the influent and effluent (>3.5 $\log_{10}$). Adenovirus had the smallest difference between the numbers in influent and effluent (~ 2.7 $\log_{10}$) with *Microviridae* having marginally higher LRVs than adenovirus (~ 2.9 $\log_{10}$).

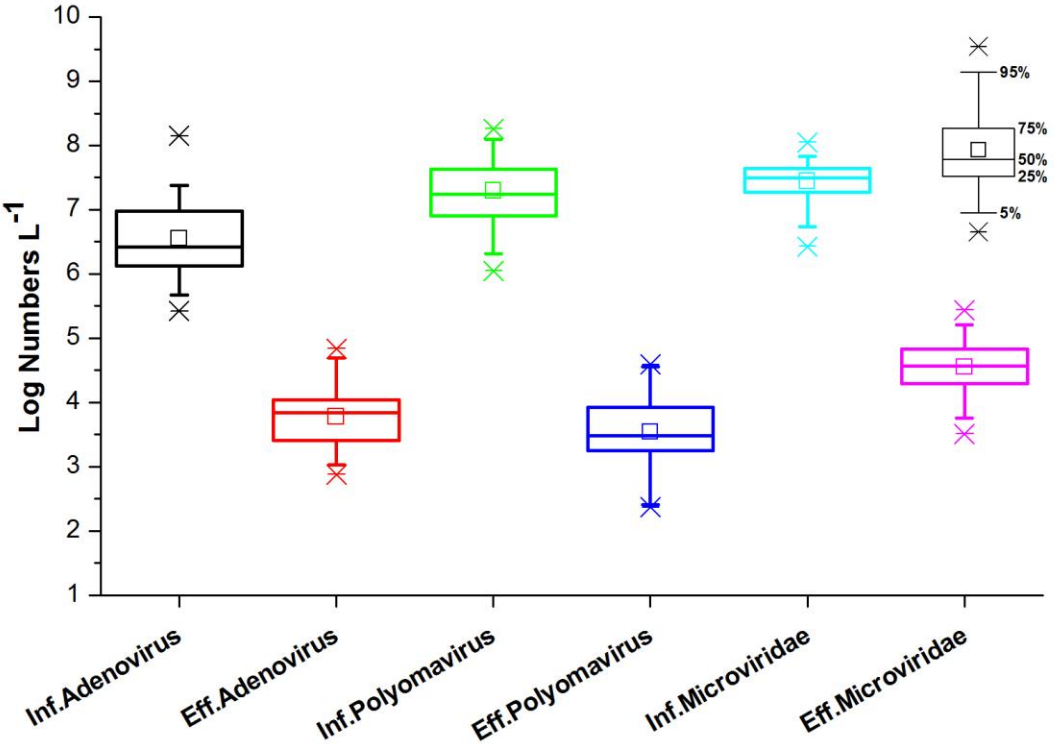

Figure 6-2. Comparative distribution of adenovirus, polyomavirus and *Microviridae* numbers in influent (Inf.) and effluent (Eff.) samples collected at the Oxley Creek WWTP.

The vertical line represents range of data, squares represent mean, small horizontal lines (whisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

6.2.3 Virus numbers in the influent and effluent of the Beenyup WWTP

Comparative numbers of adenovirus, polyomavirus and *Microviridae* in the influent and effluent samples (*n* = 23) collected at the Beenyup WWTP from 18/06/2014 to 19/05/2015 are presented in Figure 6-3. Detailed statistical analysis of the collected data (mean, median, range and geometric mean) is presented in the Table 6-2.

The numbers of adenovirus, polyomavirus and *Microviridae* in the influent were very similar over the sampling period with means ranging from 7.2 to 7.7 $\log_{10}$ L^{-1}. Unlike the Oxley Creek WWTP, adenovirus had the smallest range and Standard Deviation while polyomavirus and *Microviridae* had higher ranges and Standard Deviations that were almost identical to each other. Once again the seasons had no observable impact on virus numbers over the sampling period.

The average numbers of the three viral types detected in the effluent of the ASP at the Beenyup WWTP ranged from 5 to 5.4 $\log_{10}$ L^{-1}. Similar to the numbers detected in the influent, adenovirus again had the smallest standard deviation and range. *Microviridae* were the next with a range of

1.6 $\log_{10}$ $L^{-1}$and a standard deviation of 0.46, while polyomavirus had the largest range (2.2 $\log_{10}$ L^{-1}) and standard deviation (0.76). Again there were no observable impacts of the different seasons on the viral numbers detected.

The removal rates for the three viruses are provided in Figure 6-4. Regardless of the mean or percentile calculation, polyomavirus had the highest LRV (apart from the 95th percentile). Adenovirus and *Microviridae* were both lower than polyomavirus but which of these viruses had the better LRV varied depended on the calculation used.

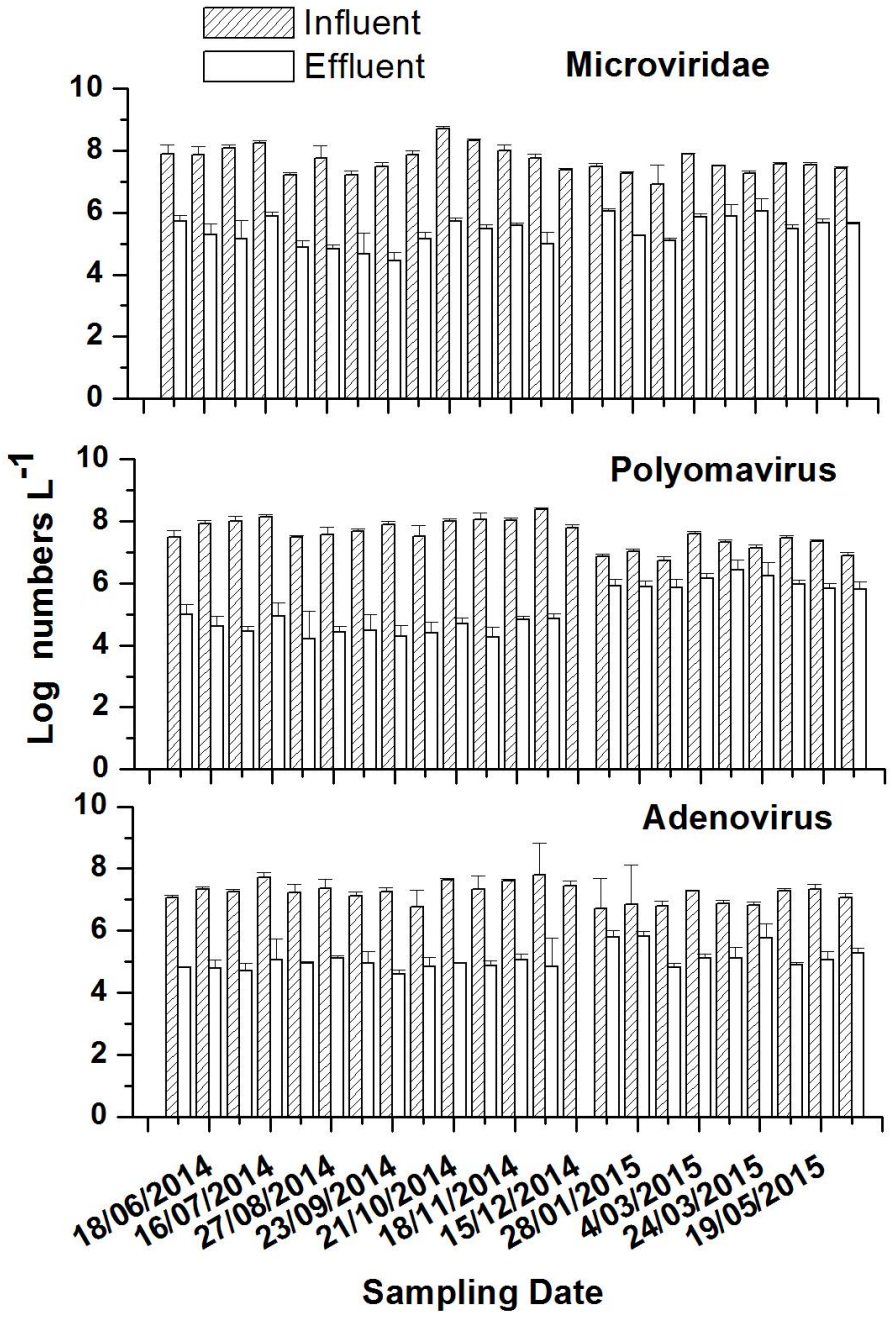

Figure 6-3. Comparative adenovirus, polyomavirus and *Microviridae* numbers in influent and effluent samples collected from the Beenyup WWTP.

Table 6-2. Statistical analysis of data from the Beenyup WWTP.

	Adenovirus		Polyomavirus		*Microviridae*	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
Mean	7.2	5.0	7.6	5.2	7.7	5.4
Median	7.3	4.9	7.6	4.9	7.6	5.5
Standard Deviation	0.31	0.34	0.45	0.76	0.42	0.46
Range	1.1	1.2	1.6	2.2	1.8	1.6
Maximum	7.8	5.8	8.4	6.4	8.7	6.1
Minimum	6.7	4.6	6.7	4.2	6.9	4.5
Count	23	22	23	22	23	22
Geometric Mean	7.2	5.0	7.6	5.1	7.7	5.4
5^{th} percentile	6.7	4.6	6.8	4.2	7.0	4.5
10^{th} percentile	6.8	4.7	6.9	4.3	7.2	4.7
50^{th} percentile	7.3	4.9	7.7	4.9	7.6	5.5
95^{th} percentile	7.8	5.8	8.3	6.4	8.6	6.1

Numbers reported as Log_{10} L^{-1}

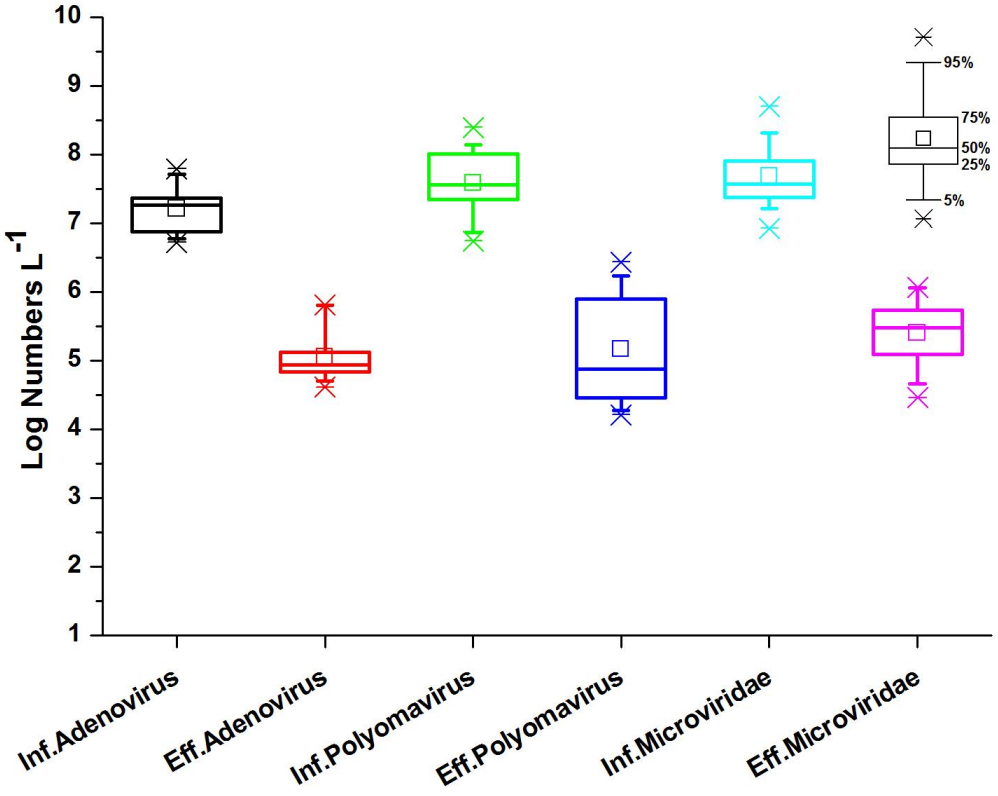

Figure 6-4. Comparative adenovirus, polyomavirus and *Microviridae* numbers in influent (Inf.) and effluent (Eff.) samples collected from the Beenyup WWTP.

The vertical line represents range of data, squares represent mean, small horizontal lines (whisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

6.2.4 Virus numbers in the influent and effluent of the Boneo WWTP

Comparative numbers of adenovirus, polyomavirus and *Microviridae* in influent and effluent samples collected at the Boneo WWTP from 19/05/2014 to 9/06/2015 are presented in the Figure 6-5. Detailed statistical analysis of the collected data (mean, median, range and geometric mean) is presented in the Table 6-3.

As observed at the Oxley Creek and Beenyup WWTP sites, adenovirus, polyomavirus and *Microviridae* numbers in the influent showed very little seasonal variation. In the influent, the mean number of viruses detected ranged from 5.9 $\log_{10}$ L^{-1} (adenovirus) to 7.9 $\log_{10}$ L^{-1} (*Microviridae*). The range in numbers of each virus was an inverse of the order of the mean numbers with a low of 1.8 $\log_{10}$ L^{-1} for *Microviridae* to a high of 3.2 $\log_{10}$ L^{-1} for adenovirus.

The mean numbers of the viruses detected in the effluent from the ASP at the Boneo WWTP differed from the results in the influent was that Polyomavirus had the smallest range and standard deviation closely followed by adenovirus. In contrast to the results from the effluent *Microviridae* had the largest range and standard deviation.

The removal rates for the three viruses are provided in Figure 6-6. Similar to the Beenyup WWTP, irrespective of the mean or percentile calculation, polyomavirus had the highest LRV being approximately 1 $\log_{10}$ or more higher than for the other two viruses. Adenovirus had LRVs that were a little higher than *Microviridae*.

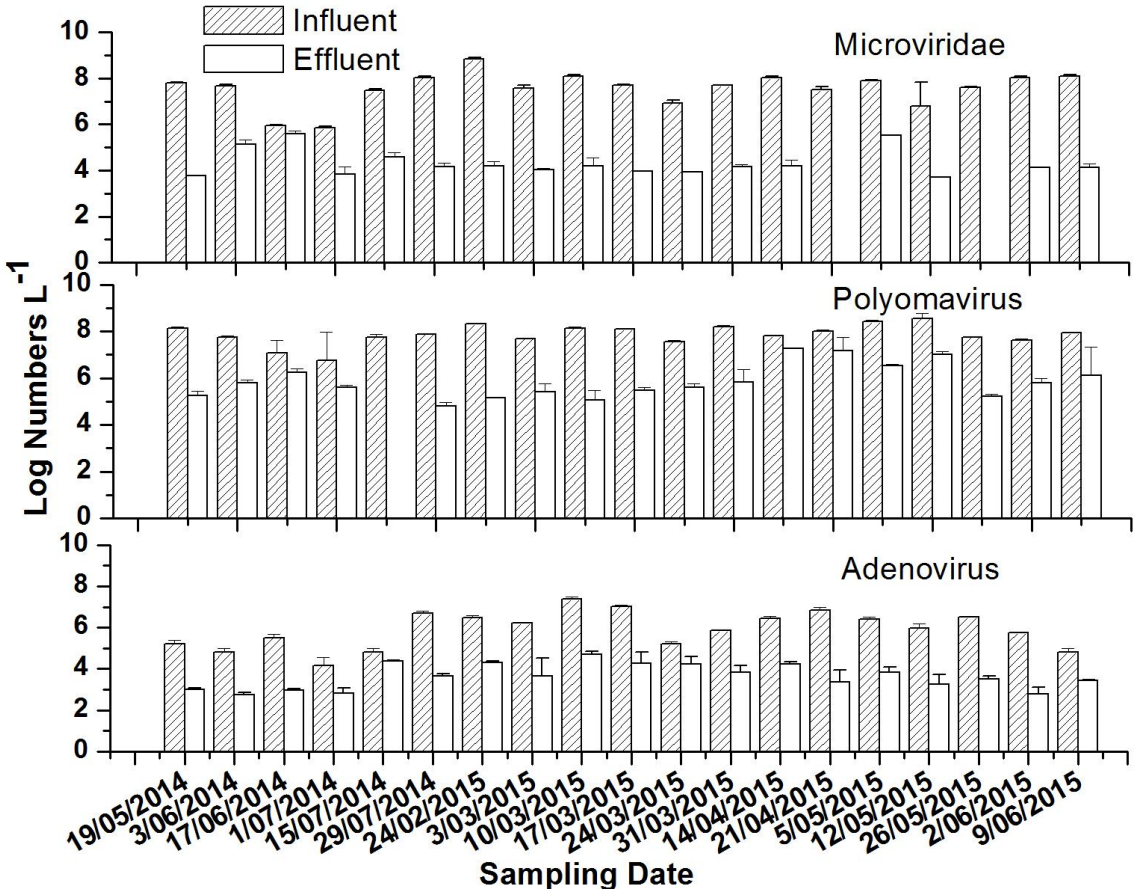

Figure 6-5. Comparative adenovirus, polyomavirus and *Microviridae* numbers in influent and effluent samples collected at the Boneo WWTP.

Table 6-3. Statistical analysis of data from Boneo WWTP.

	Adenovirus		Polyomavirus		Microviridae	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
Mean	5.9	3.6	7.6	4.3	7.9	5.8
Median	6.0	3.6	7.7	4.2	7.9	5.7
Standard Deviation	0.88	0.61	0.73	0.57	0.44	0.74
Range	3.2	2.0	3.0	1.9	1.8	2.5
Maximum	7.4	4.7	8.8	5.6	8.6	7.3
Minimum	4.2	2.7	5.8	3.7	6.8	4.8
Count	19	19	19	17	19	18
Geometric Mean	5.8	3.6	7.5	4.3	7.8	5.8
5^{th} percentile	4.2	2.7	5.8	ND	6.8	ND
10^{th} percentile	4.8	2.8	5.9	3.8	7.1	5.0
50^{th} percentile	6.0	3.6	7.7	4.2	7.9	5.7
95^{th} percentile	7.4	4.7	8.8	ND	8.6	ND

Numbers reported as Log_{10} L^{-1} ND= no data due to small sample number

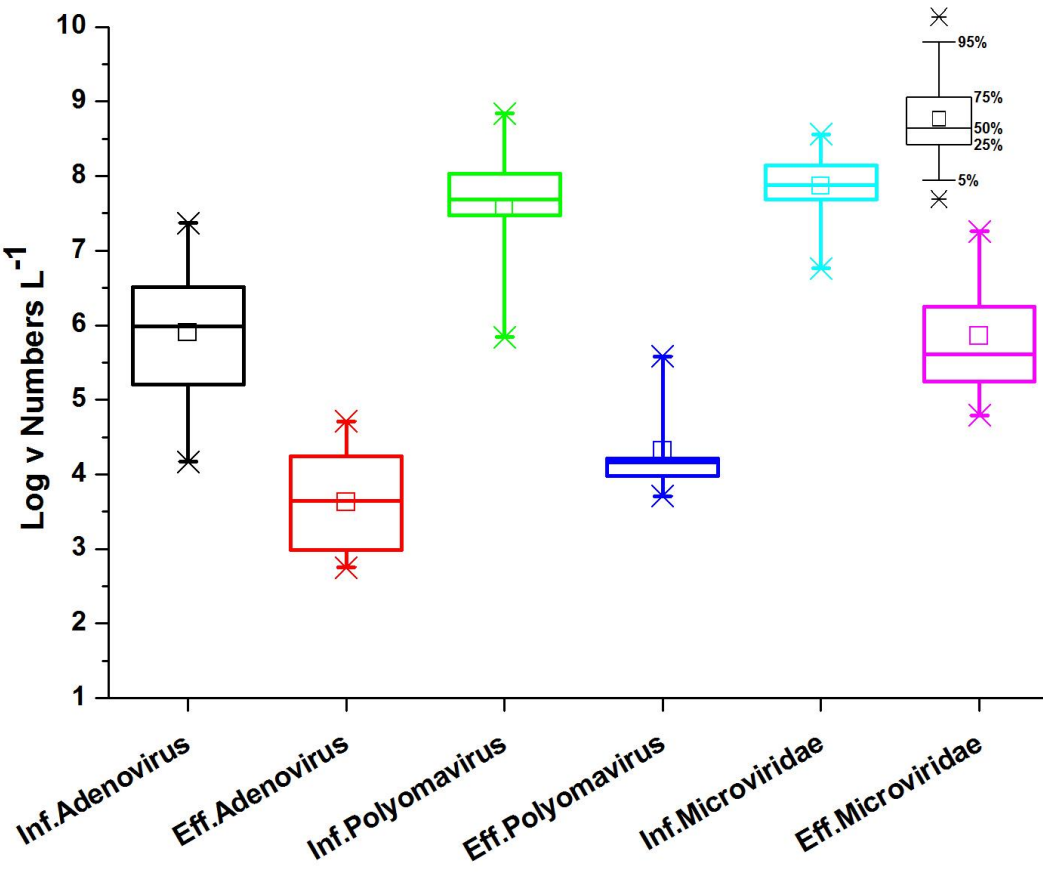

Figure 6-6. Comparative adenovirus, polyomavirus and *Microviridae* numbers in influent (Inf.) and effluent (Eff.) samples collected from Boneo WWTP.

The vertical line represents range of data, squares represent mean, small horizontal lines (whisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

6.2.5 Virus numbers in the influent and effluent at the Rosny WWTP

Comparative numbers of adenovirus, polyomavirus and *Microviridae* in the influent and effluent samples collected at the Rosny WWTP from 18/06/2014 to 11/05/2015 are presented in the Figure 6-7. Detailed statistical analysis of the collected data (mean, median, range and geometric mean) is presented in the Table 6-4.

As was observed in the Oxley Creek, Beenyup and Boneo WWTPs, *Microviridae* had the highest mean number (7.7 $\log_{10}$ L^{-1}) followed by polyomavirus (7.16 $\log_{10}$ L^{-1}) and then adenovirus (6.5 $\log_{10}$ L^{-1}). *Microviridae* and polyomavirus were very similar in range and standard deviation which were both lower than for adenovirus. Again as observed for the ASP WWTPs, adenovirus, polyomavirus and *Microviridae* numbers in the influent showed very little seasonal variation.

The results obtained for the virus numbers in the effluent at the Rosny WWTP showed that *Microviridae* also had the highest mean numbers and corresponding lowest range and standard deviation followed by polyomavirus and then adenovirus. Again as for the number in the influent, there were no evident seasonal impacts

When these influent and effluent numbers were converted into LRVs (Figure 6-8) it was determined that polyomavirus had the highest removal rates with *Microviridae* LRVs being slightly higher than adenovirus which had the lowest LRVs.

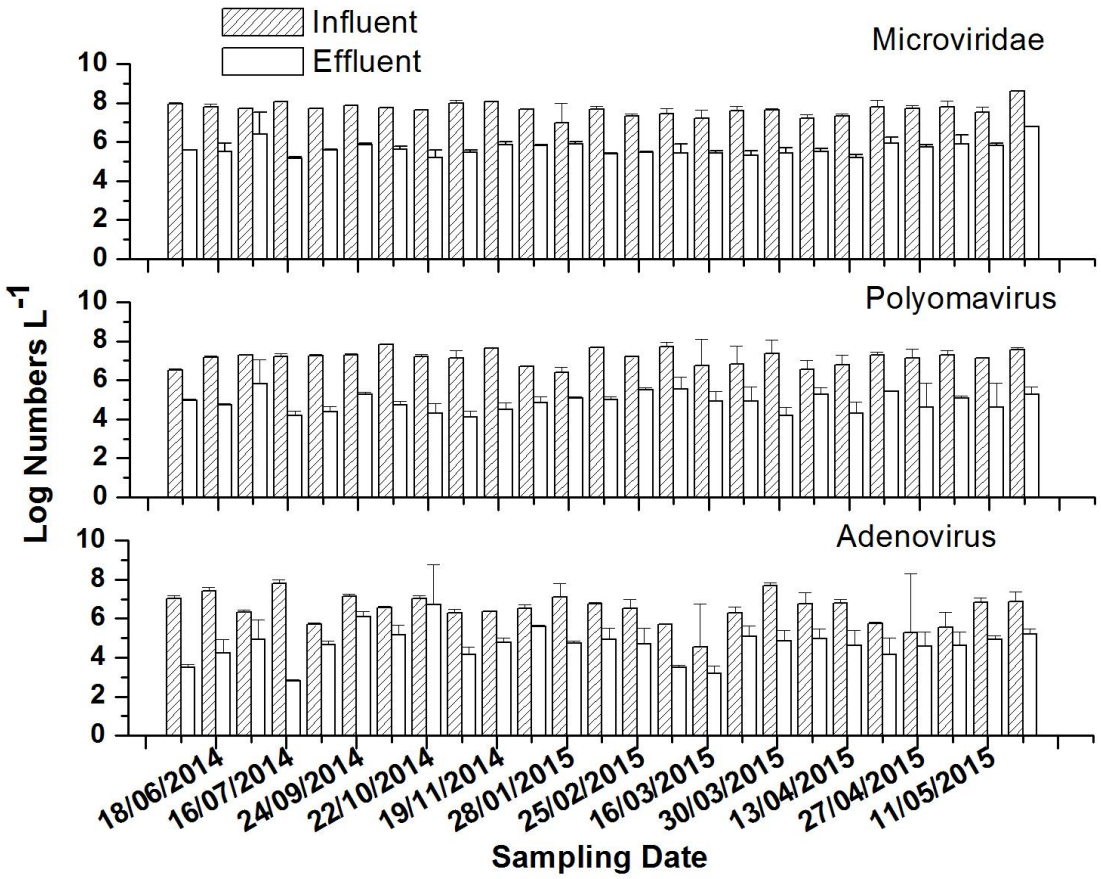

Figure 6-7. Comparative adenovirus, polyomavirus and *Microviridae* numbers in influent and effluent samples collected from Rosny WWTP.

Table 6-4. Statistical analysis of data from Rosny WWTP.

	Adenovirus		Polyomavirus		*Microviradae*	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
Mean	6.5	4.7	7.2	4.9	7.7	5.6
Median	6.6	4.7	7.2	4.9	7.7	5.6
Standard Deviation	0.76	0.85	0.38	0.47	0.33	0.37
Range	3.3	3.9	1.4	1.7	1.6	1.6
Maximum	7.8	6.7	7.8	5.8	8.6	6.8
Minimum	4.5	2.8	6.4	4.1	7.0	5.1
Count	25	25	25	25	25	25
Geometric Mean	6.5	4.6	7.1	4.8	7.7	5.6
5^{th} percentile	4.8	2.9	6.4	4.1	7.0	5.2
10^{th} percentile	5.4	3.4	6.5	4.2	7.2	5.2
50^{th} percentile	6.5	4.7	7.2	4.88	7.7	5.5
95^{th} percentile	7.8	6.5	7.8	5.73	8.4	6.7

Numbers reported as $Log_{10}\ L^{-1}$

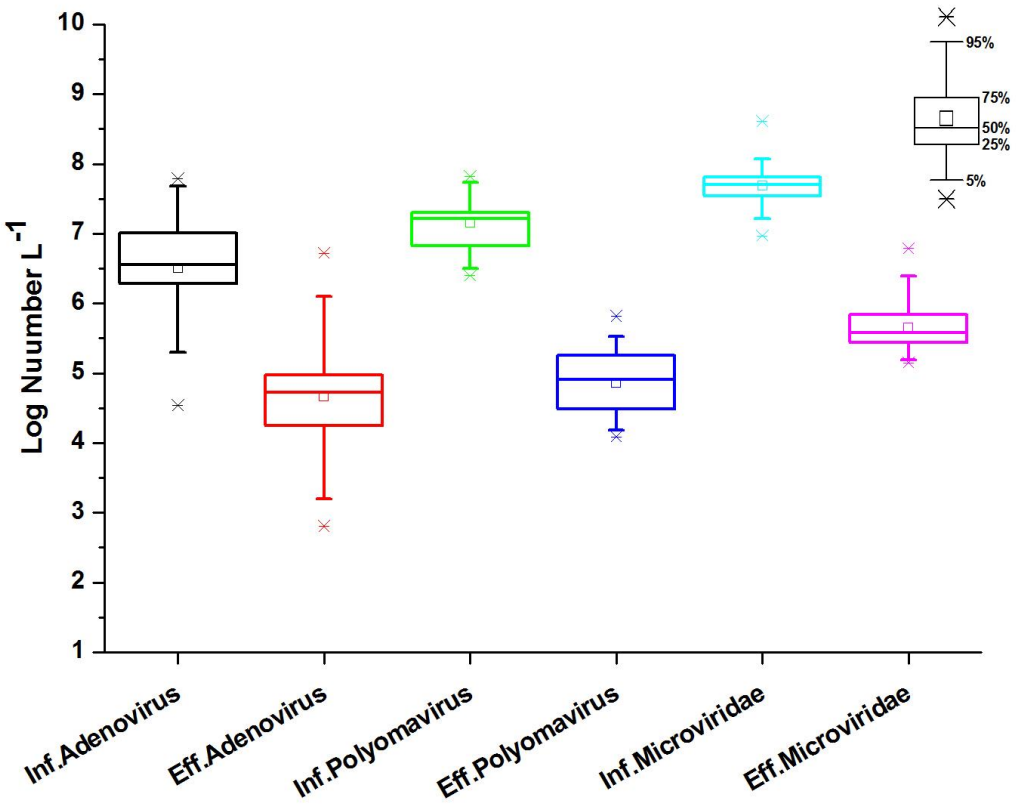

Figure 6-8. Comparative adenovirus, polyomavirus and *Microviridae* numbers in influent (Inf.) and effluent (Eff.) samples collected from Rosny WWTP.

The vertical line represents range of data, squares represent mean, small horizontal lines (whisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

6.3 Discussion and Conclusions

The results of this study indicate that, as was observed for *E. coli*, the numbers of viruses in the influent entering the different WWTPs varied across the sampling periods and between the wastewater treatment plants, however, this was observed to be primarily an influence of the source wastewater. All three of the ASP plants (Oxley Creek, Beenyup and Boneo) and the trickling filter plant (Rosny) were able to reduce the numbers of all three tested viruses, although the level of reduction varied between the plants. Despite this, there was determined significance for all of the viruses at two of the ASP plants (Oxley Creek and Beenyup). This could be a temperature related impact as, although there were no observed seasonal impacts, the average wastewater temperature at these two plants were $\geq$ 23˚C whereas the Boneo and Rosny plants had average wastewater temperatures of 21˚C and lower. The influence of geographical location is an issue that could not be fully resolved in this study.

6.3.1 Virus numbers in influent and effluent

Regardless of geographical location of the wastewater treatment plants in Australia, the median virus numbers in the influent at each plant were consistently 6 $\log_{10}$ or higher. Adenovirus numbers were always the lowest observed in the influent but still ranged between 10^6 to > 10^7 genomic units L^{-1} across the WWTPs. The numbers observed in Australian WWTPs in this study are comparable to the reported numbers (10^5 to 10^{-9} genomic units L^{-1}) from around the world (Carducci and Verani, 2013; Fong *et al*., 2010; Hewitt *et al*., 2011; Katayama *et al*., 2008; Kitajima *et al*., 2014b; Kuo *et al*., 2010; Laverick *et al*., 2004; Sidhu *et al*., 2013b). The wide variation in the reported virus numbers could be mostly attributed to variation in the source from issues such as community wellbeing and disease prevalence. Study related issues, however such as sample size, and concentration and detection methodologies can also have an influence on the comparative numbers detected. Regardless of these factors, adenovirus numbers (10^3 to 10^5 genomic units L^{-1}) observed in the effluent of this study were in agreement with reported numbers (10^3 to 10^6 genomic units L^{-1}) in the literature (He and Jiang, 2005; Kitajima *et al*., 2014b). The fact that adenovirus is detected worldwide and numbers in wastewater effluent and is similar to what has been determined in this study suggests that this virus could be suitable for use in an activated sludge plant validation protocol.

Human polyomavirus was also detected in high numbers (10^7 to 10^9 genomic units L^{-1}) in the influent from all WWTPs tested in this study although the numbers were always higher and had a larger range than adenovirus. The numbers reported here are similar to previously reported numbers in sewage (> 10^6 genomic units L^{-1}) in New Zealand (Hewitt *et al*., 2011). Kitajima *et al*. (Kitajima *et al*., 2014b), also reported high numbers of human polyomavirus (JCV strain) in primary wastewater from Arizona, USA (10^5 to 10^6 genomic units L^{-1}). The large observed range in the polyomavirus LRVs, particularly in the Boneo and Beenyup WWTPs (Figure 6-8) suggests that it may not be as suitable as adenovirus for use in the validation of activated sludge plants.

There is very limited information available on *Microviridae* numbers in sewage but coliphage belonging to families *Myoviridae* and *Siphoviridae* have been reported to most abundant in sewage (Muniesa *et al*., 1999). In this study, coliphage of the *Microviridae* family were found to be

high in number in both the influent (>10^7 L^{-1}) and effluent (10^5 L^{-1}) of all the wastewater treatment plants and were comparable in numbers to adenovirus and polyomavirus. Similar to adenovirus and polyomavirus, site specific removal (2 to 2.94 log_{10}) was observed for *Microviridae,* with the highest removal at the Oxley Creek WWTP. Bacteriophage belonging to *Microviridae* family have been reported to be stable in environment and the most resistant to UV and heat of the coliphage group (Lee and Sobsey, 2011). *Microviridae* removal rates were comparable to adenovirus (1.68 to 2.84 log_{10}) which suggest that it may be able to be used to indicate the removal of adenovirus in activated sludge plants, particularly if the loss of infectivity is considered to be an important factor to monitor.

6.3.2 Log removal of enteric virus across sites

All three WWTPs using ASP systems were able to remove viruses better that the trickling filter plant at the Rosny WWTP. Oxley Creek had the best removal rate followed by the Beenyup WWTP. Adenovirus and *Microviridae* LRVs were lower at the Boneo WWTP than at either Oxley Creek or Beenyup WWTPs. Conversely, variability in the LRVs within treatment plants was relatively low suggesting stable operation of WWTPs during the sampling period. In addition, the site specificity in the removal rates of the viruses indicated that operational variables are likely to be the major cause of pathogen removal variability. The operational variables considered to be most important include plant design parameters, operational conditions and environmental variables. Oxley Creek WWTP had the highest LRVs and the highest effluent temperature. Temperature has been previously considered an important factor in the performance of WWTP and removal of pathogens (Stevik *et al*., 2004). In addition, the Oxley Creek WWTP is also located in a subtropical environment which, having a more stable seasonal temperature range, appears to have a positive role in the removal of the viruses. The virus removal rates at the Oxley Creek WWTP in this study are similar to rates previously reported for Luggage Point, Bundamba and Oxley Creek treatment plants (Toze *et al*., 2012). If the influence of temperature is as important as suggested then higher enteric pathogen removal should be expected in WWTPs located in the warmer regions of Australia is expected. It is therefore possible that these treatment plants could be assigned higher LRVs for the ASP process.

Despite having the lower LRV of the three ASP WWTPs, the virus removal in the Boneo plants in this study is higher than have been previously determined in WWTPs in the southern states of Victoria and South Australia (McAuliffe and Gregory, 2010; Wen *et al*., 2009). This indicates that more studies are needed to assess the predictable removal capabilities of WWTPs in the southern regions of Australia, particularly taking climatic variables and operational impacts into detailed consideration.

Removal of RNA virus such as norovirus was not studied in this work as DNA viruses are reported to be more persistent during wastewater treatment (Albinana-Gimenez *et al*., 2006; He and Jiang, 2005). Therefore, we have focused on the removal of adenovirus during the ASP. However, it is expected that removal of RNA virus would be at least similar, and most likely higher than adenovirus. In a study on comparative RNA virus removal during wastewater treatment, a similar removal for norovirus (0.2-2.1 log_{10}), reovirus (0.9-1.4 log_{10}), and enterovirus (0.7-1.8 log_{10}) was

reported, however rotavirus removal (0.003-1.1 $\log_{10}$) was lower (Lodder and de Roda Husman, 2005).

In relation to variations in detection methodologies used, in this study, PCR analysis for adenovirus was carried out as adenovirus can detect both infectious and non-infectious viruses. PCR measures total removal of virus particles (in particular the genome), however this approach would not adversely affect the calculation of the LRVs because the ASP involves total removal of pathogens, which include physical removal and inactivation.

7 Removal of Protozoan Pathogens

In Australia, *Cryptosporidium* and *Giardia* oo(cysts) are as much a problem as viruses as they have similar infectious doses and are also resistant to a range of environmental conditions. While this overall research study had a working hypothesis that the viruses, in particular adenovirus, and *E. coli* will have the greatest application for validation of activated sludge processes, understanding the removal of protozoa (in particular *Cryptosporidium*) was considered to be important. Better data on determining *Cryptosporidium* LRVs may provide important information on either how these microorganisms could also be used in validation procedures, or at least show what linkages there are between viruses and *Cryptosporidium* LRVs.

Previously reported numbers of oocysts in wastewater matrices have been highly variable with a wide range of *Cryptosporidium* numbers and removals (e.g. 0-100% removal) reported at individual treatment plants (Graczyk *et al*., 2007; Ottoson *et al*., 2006b; Payment *et al*., 2001; Rose *et al*., 1996). The methods used for the detect *Cryptosporidium* in these studies is likely to be a major reason for this variation as the detection method commonly reported was initially developed for use in cleaner matrix (McCuin and Clancy, 2005) such as drinking water. As such these methods are not as applicable or reproducible in wastewater matrices. The issues with the detection methods would likely result in higher oocyst numbers being detected in the treated effluent compared to primary influent which is most likely reason of variable removal rates of oocysts with in a treatment and across treatment plants reported in the literature (Keegan *et al*., 2013; Montemayor *et al*., 2005; Quintero-Betancourt *et al*., 2003). More recently, immunomagnetic capture of oocysts followed by PCR have been reported to provide improved recovery rates of >50% for both influent and effluent samples (Kitajima *et al*., 2014a).

In this study, we have used a simpler method involving centrifugation for the concentration of oocysts from influent and effluent samples followed by the PCR detection with genus specific and species specific primers for the detection of *Cryptosporidium spp.* and *C. parvum*.

7.1 Methodology

7.1.1 Sample concentration

The presence and numbers of *Cryptosporidium* oocysts in the influent and effluent samples was determined using quantitative real-time PCR. Similar to the method used for detection of the viruses, as *Cryptosporidium* oocyst numbers are low in large volumes of wastewater, concentration of the samples was required prior to DNA extraction and PCR analysis. Details on the concentration methods, quality control measures and PCR reactions are given in Appendix C.

7.1.2 Detection of *Cryptosporidium* oocysts

The initial detection method focused on the detection of *Cryptosporidium parvum/hominis* by qPCR using species specific primers. However, due to the low detection rates attained, the method was converted to a *Cryptosporidium* spp. genus specific primer set. This new primer set provided greater sensitivity due to the detection of all *Cryptosporidium* oocysts and through the higher number of gene copies detected per oocyst. This enabling a detection method with an improved lower limit of quantification (LLOQ) compared to the original species specific primers.

The detected numbers of *Cryptosporidium* sp. oocysts in the concentrated samples were converted to the original starting sample volume and then Log_{10} transformed. These transformed numbers were then used to assess LRVs for *Cryptosporidium* in each of the treatment plants.

7.2 Results

7.2.1 Detection in raw and treated wastewater

Results for the analysis of *Cryptosporidium* oocysts is only available for the Beenyup and Boneo WWTPs. The Rosny WWTP was not tested as it is a trickling filter plant and therefore not able to be compared with the three ASP plants and the earlier analysis of *E. coli* and virus removal had shown that the Rosny plant had lower LRVs. Despite multiple trials, *Cryptosporidium* oocysts could not be detected with any consistency in samples collected from the Oxley Creek WWTP.

The initial testing was undertaken using *C. parvum/hominis* specific primers as these are the *Cryptosporidium* species causing the risks to human health. The numbers of *C. parvum/hominis* in raw wastewater samples were not quantifiable (< LLOQ of 30 gene copies) from a sample volume of 30 mL tested from Oxley Creek, Boneo or Beenyup WWTPs. In an attempt to overcome this problem, the raw wastewater sample volumes were increased to 50 mL and 100 mL, however, the number of *Cryptosporidium parvum* oocysts in the samples were not still quantifiable. As part of the QA/QC testing a known concentration (10^4) of *C. parvum* was spiked into in replicate 30 mL of raw sewage samples. In each case, the numbers were not quantifiable and inconsistencies were observed within and amongst replicate PCR runs. The results were highly variable and not reproducible most likely due to the low number of *C. parvum* oocysts in the wastewater and matrix difficulty. Another recognised reason for the detection issues is that the detection limit for *C. parvum* using qPCR is the fact that each oocyst has only 4 copies of the target gene per oocyst. It was determine that these issues were the cause of the inability to accurately detect *C. parvum* oocyts.

In an attempt to obtain results for *Cryptosporidium* in wastewater an alternative method was developed that used a lower sample volume (15 mL primary and 1 L secondary treated effluent) and the resulting concentrate was tested with the genus specific *Cryptosporidium* spp. primers. The genus specific primers will detect the oocysts of any *Cryptosporidium* species, not just *C. parvum,* and there are 20 copies of the target gene per oocyst (as opposed to the 4 copies for the *C. parvum* target site). Both of these detection characteristics were determined to be able to improve the detection limit to 1 oocyst L^{-1}.

This revised method was tested on three initial primary and three treated effluent samples from the Beenyup WWTP. This resulted in *Cryptosporidium* spp. oocysts being detected in the primary influent at numbers that ranged from 3.8 to 4.4 $\log_{10}$ L^{-1} and between 1.8 to 1.9 $\log_{10}$ L^{-1} in the treated effluent. As a result of these preliminary findings the testing of additional samples was undertaken at the three ASP plants (Oxley Creek, Beenyup and Boneo WWTPs).

As described above, results remained unattainable from the Oxley Creek WWTP despite the changes to the sampling and detection methodologies. Results were therefore only available for the Beenyup and Boneo WWTPs.

Cryptosporidium spp. numbers detected in the influent and effluent samples and $\log_{10}$ removal at the Beenyup and Boneo WWTPs are presented in Figures 7-1 and 7-2 and the statistical analysis of the numbers detected is given in Table 7-1. The Beenyup WWTP had mean *Cryptosporidium* spp. numbers of 4.1 $\log_{10}$ L^{-1} in the influent and 1.4 $\log_{10}$ L^{-1} in the effluent. The Boneo WWTP had similar mean numbers in the influent (4.5 $\log_{10}$ L^{-1}) but even lower numbers in the effluent (0.7 $\log_{10}$ L^{-1}). The low standard deviations of the numbers detected in the effluent at both WWTPs and in the influent at the Beenyup WWTP indicate that there was a relatively consistent number in the influent and a consistent removal at the Beenyup WWTP. There was a larger variability in the *Cryptosporidium* numbers in the Boneo WWTP, primarily because the numbers were below the PCR detection limit in the first seven samples. It is unclear whether this lack of detection was method related or actual high reductions in the Boneo WWTP. Despite this, the reduction in *Cryptosporidium* spp. numbers at both plants were found to be statistically significant (p<0.05). As with *E. coli* and viruses there were no observable influences from seasonal variations.

The resulting LRVs are shown in Figure 7-3 and the subsequent statistical analysis is given in Table 7-2. The analysis shows that the *Cryptosporidium* sp. had LRVs with a geometric mean of 2.8 $\log_{10}$ and 3.7 $\log_{10}$ at the Beenyup and Boneo WWPTs respectively. The low standard deviations for the LRVs at both treatment plants showed that the reductions in *Cryptosporidium* oocysts was consistent over the time period of the sample period.

7.3 Discussion and conclusions

The numbers of *C. parvum* in raw wastewater have previously reported to be low (10^1-10^2 L^{-1}) (Harwood *et al*., 2005; Montemayor *et al*., 2005). In this study, only *Cryptosporidium* sp. could be consistently detected in the wastewater. The number of *Cryptosporidium* sp. oocysts in the influent of the two WWTPs tested in this study, however, were determined to be higher than 4 $\log_{10}$ L^{-1} with the average numbers in the effluent being less than 1.5 $\log_{10}$ L^{-1}. This provided calculated mean LRVs of 2.8 and 3.8 $\log_{10}$. This is higher than the removal rates reported in other studies (Keegan *et al*., 2013; 2005; Montemayor *et al*., 2005).

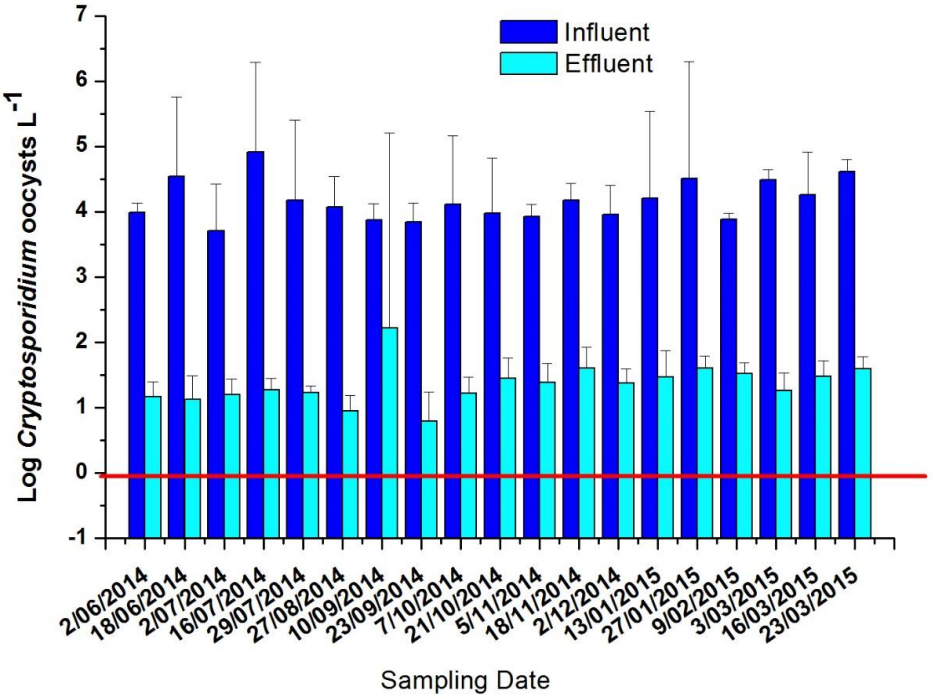

Figure 7-1. Comparative numbers and log_{10} removal of *Cryptosporidium* oocysts in influent and effluent samples from Beenyup WWTP.

Redline represents PCR detection limit of one oocyst L^{-1}

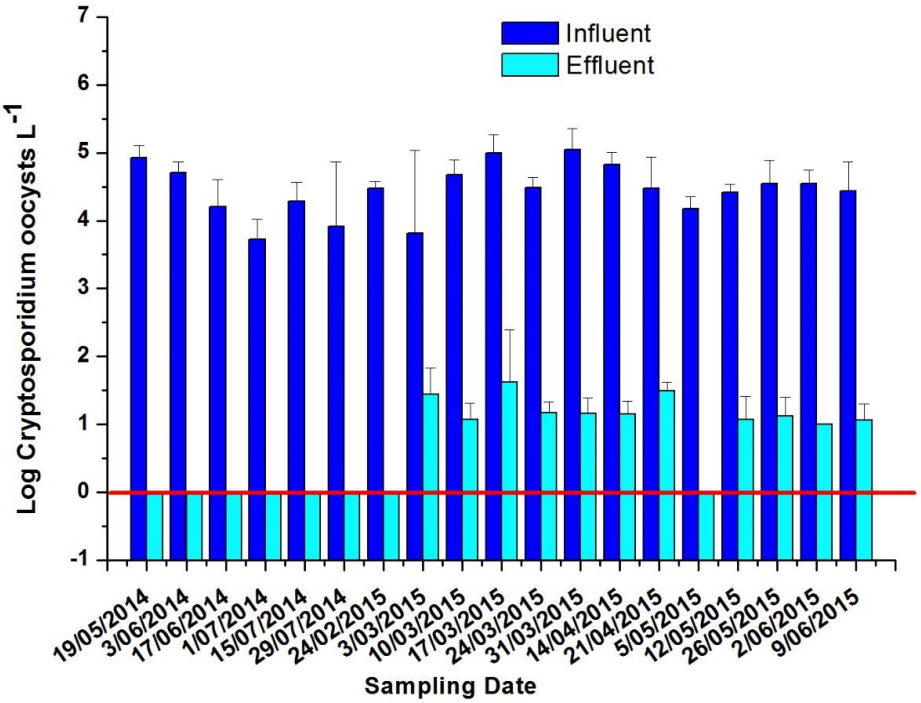

Figure 7-2. Comparative numbers and log_{10} removal of *Cryptosporidium* oocysts in influent and effluent samples at Boneo WWTP.

Redline represents PCR detection limit of one oocyst L^{-1}

Table 7-1. Statistical analysis of data on *Cryptosporidium* oocyst numbers the influent and effluent of the Beenyup and Boneo WWTPs.

	Beenyup		Boneo	
	Influent	Effluent	Influent	Effluent
Mean	4.1	1.4	4.5	0.7
Median	4.1	1.4	4.5	1.0
Standard Deviation	0.32	0.3	0.38	0.63
Range	1.2	1.4	1.3	1.6
Maximum	4.9	2.2	5.0	1.6
Minimum	3.7	0.8	3.7	0.0
Count	19	19	19	19
Geometric Mean	4.2	1.3	3.5	0.0
5^{th} percentile	3.7	0.8	3.7	0.0
10^{th} percentile	3.8	0.9	3.8	0.0
50^{th} percentile	4.1	1.4	4.5	1.1
95^{th} percentile	4.9	2.2	5.0	1.6

Numbers reported as $Log_{10}\ L^{-1}$

Table 7-2. Statistical analysis of data on average LRVs for *Cryptosporidium* oocyst numbers at the Beenyup and Boneo WWTPs.

	Beenyup	Boneo
Mean	2.8	3.8
Median	2.8	3.7
Standard Deviation	0.43	0.62
Range	2.0	2.6
Maximum	3.7	4.9
Minimum	1.7	2.4
Count	19	19
Geometric Mean	2.8	3.7
5^{th} percentile	1.7	2.4
10^{th} percentile	2.4	3.0
50^{th} percentile	2.8	3.7
95^{th} percentile	3.7	4.9

One issue with the revised method is that oocysts of all *Cryptosporidium* species present in a wastewater were detected, not just the two species (*C. parvum* and *C. hominis*) that cause the vast majority of disease in humans. This was done to increase the detection sensitivity to ensure a consistent detection of *Cryptosporidium* could be achieved. It could therefore be argued that there is an over estimation of the number of problematic *Cryptosporidium* in wastewater (because species that are unlikely to cause disease in human will be detected as well). We have made the assumption, however, that it could be expected that the vast majority of *Cryptosporidium* oocysts

in wastewater are of human origin (and therefore either *C. parvum* or *C. hominins*) and that all species of *Cryptosporidium* have a similar environmental resistance and that removal mechanisms in activated sludge treatment plants that influence the removal of *Cryptosporidium* would have the same effect on all species. Improvements in detection methods which allows the improved detection of *C. parvum/hominis* individually, or studies determining the relative proportion of *Cryptosporidium* species in wastewater may provide improved data on *C. parvum* removal by ASP at a later point in time.

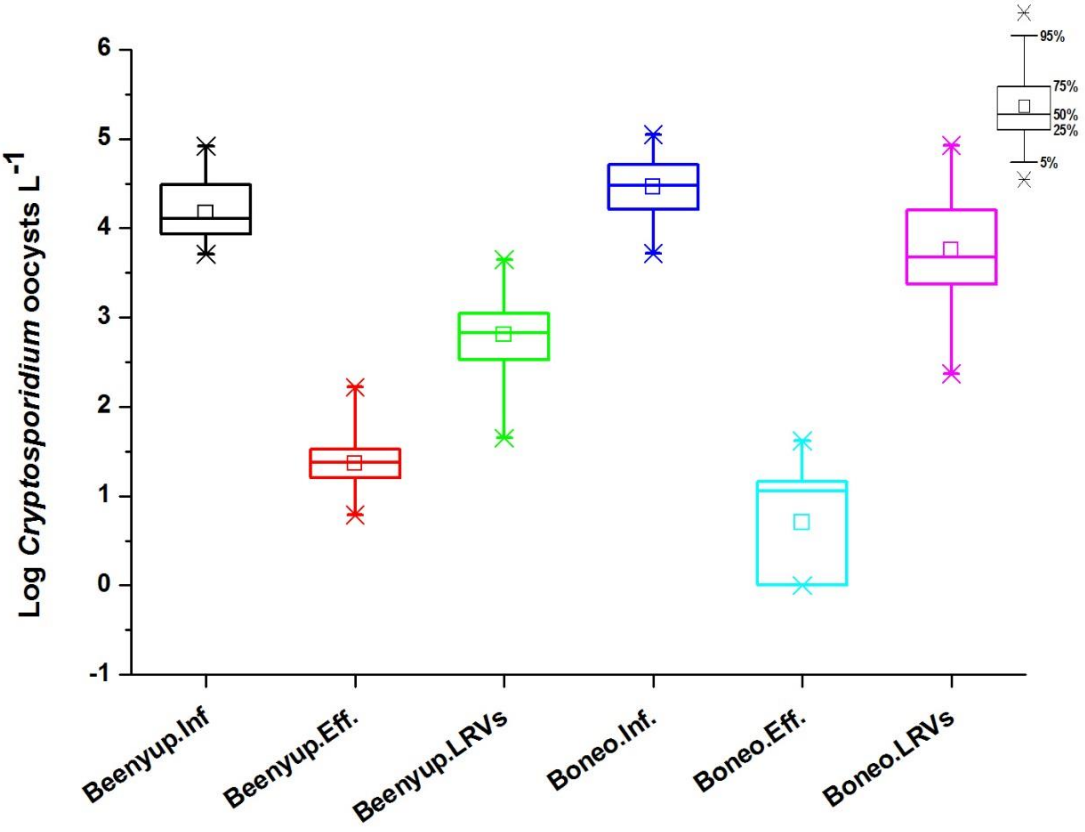

Figure 7-3. Comparative numbers and log_{10} removal of *Cryptosporidium* oocysts in influent and effluent samples at Beenyup and Boneo WWTPs.

The vertical line represents range of data, squares represent mean, small horizontal lines (whisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

As the research ultimately only allowed the testing 19 samples from each of two WWTPs, these findings should be considered as preliminary only. Therefore only limited conclusions that can be made at this stage, however, these initial removal rates are similar to those for the viruses. More samples need to be taken from these and other wastewater treatment plants before further conclusions can be accurately made. In addition, despite the observed (initial) conclusions that the LRVs for *Cryptosporidium* oocysts were similar to those for the viruses, an internal observation was that the laboratory methodology and effort required to detect the *Cryptosporidium* oocysts was much more involved and time consuming than for the viruses. This suggests that the determination of LRVs for *Cryptosporidium* is less useful for a validation protocol than the viruses tested such as adenovirus.

8 Comparison of Microbial Log Reduction Value Between the Wastewater Treatment Plants

A comparison of virus log removal values was made between the three ASP WWTPs (Oxley Creek, Beenyup and Boneo) to assess if the calculated LRVs varied across and within the treatment plants. Commonalities of LRVs between the plants could help assess their potential use in a validation protocol. Because *E. coli* has been so commonly used to-date for assessing the performance of WWTPs and can be used for on-going verification, it has been included in this section to determine how virus removal compared to the LRVs calculated for *E. coli.* The LRVs for *Cryptosporidium* spp. oocysts was not done as usable results were only available from two treatment plants (Beenyup and Boneo) from a reduced number of samples.

As explained in Section 3, the Rosny WWTP has not been included in this comparison due to the observed differences in design and physicochemical treatment outcomes. Instead, the Rosny WWTP is viewed on its own as an example of the treatment capability of a trickling filter plant to remove viruses.

All of the LRV calculations from Sections 4 and 5 on the reductions of enteric viruses and *E. coli* were combined and a descriptive statistical analysis (including 5^{th}, 10^{th} and 95^{th} percentile LRVs) was performed on the data prior to Pearson's correlation analysis (Figure 8-1 and Table 8-1). As shown in Figure 8-1, all three ASP WWTPs varied in their ability to removal the targeted enteric viruses, but as detailed in Table 8-1, Oxley Creek WWTP consistently had higher LRVs for all three viruses than Beenyup and Boneo WWTPs. Oxley Creek WWTP had LRV geometric means of 2.7, 3.5 and 2.9 $\log_{10}$ for adenovirus, polyomavirus and *Microviridae* respectively, which were between 0.5 to 1.4 $\log_{10}$ higher than was calculated for the other two WWTPs. Beenyup WWTP had higher LRVs for adenovirus and *Microviridae* than Boneo WWTP, but Boneo had higher LRVs for polyomavirus. Figure 8-1 also demonstrates that the Oxley Creek WWTP also had the small range of LRVs for all three viruses while the Boneo WWTP had the largest range.

In contrast to these observations for the enteric viruses, the Beenyup WWTP was the best for removing *E. coli* having LRVs for *E. coli* which were approximately 0.5 $\log_{10}$ higher than in Oxley Creek WWTP and 1 $\log_{10}$ higher than the Boneo WWTP. As shown in Table 8-1, the Beenyup WWTP had higher LRVs for *E. coli* than for any of the three viruses, whereas both Oxley Creek and Boneo WWTPs had higher LRVs for polyomavirus than for *E. coli*.

As explained above, due to the design differences and very short HRT, the Rosny WWTP has been examined individually as an example of a trickling filter plant. The results in Table 8-1 show that while the Rosny WWTP was able to reduce the numbers of all three viruses and *E. coli* (achieving a geometric mean LRV of 2.2 $\log_{10}$ for polyomavirus), the calculated LRVs were consistently lower than for the three ASP plants. This is most likely due to a combination of the trickling filter process and the very short HRT.

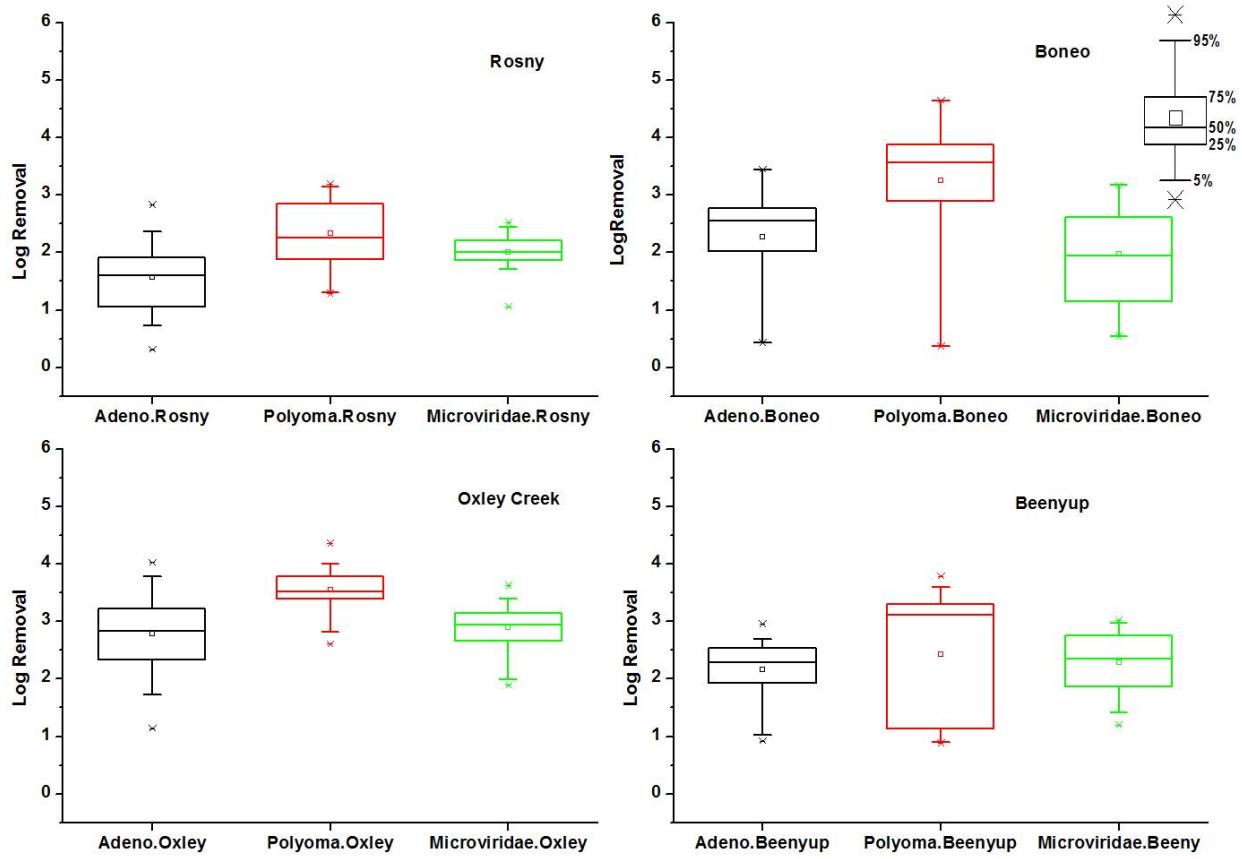

Figure 8-1. Comparative Log Reduction Values (LRVs) for adenovirus, polyomavirus and *Microviridae* in Box and whisker plot at all four WWTPs.

The vertical line represents range of data, squares represent mean, small horizontal lines (whisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

The estimated LRVs were analysed for the presence of correlations between the LRVs of enteric virus and *E. coli*. In general, there was extremely poor correlation between the removal of adenovirus, polyomavirus, *Microviridae* and *E. coli* for all the WWTPs (Table 8-2). The correlation data indicates that that there were no consistent, strong correlation between the LRVs for adenovirus, polyomavirus and *Microviridae* removal. However, statistical significance was determined for several of the correlations between the viruses at the Oxley Creek and Beenyup WWTPs. *E. coli* LRVs also had a significant correlation with the *Microviridae* LRVs at the Oxley Creek WWTP, however there were no other significant correlations between *E. coli* removal and any of the other viruses at Oxley Creek WWTP or either of the other two ASP WWTP. Only the correlation between polyomavirus and *Microviridae* was determined to be significant at the Boneo WWTP.

The Rosny WWTP had no significant correlations between the removal of any of the viruses or *E. coli* and the calculated correlations tended to be lower than those for any of the ASP WWTPs.

Table 8-1. Summary of the estimated Log Reduction Values (LRVs) for adenovirus, polyomavirus, *Microviridae* and *E. coli* in the different WWTPs.

	Adenovirus	Polyomavirus	Microviridae	*E. coli*
Oxley Creek WWTP				
Mean	2.8	3.6	2.9	3.0
Median	2.8	3.5	2.9	3.0
Geometric Mean	2.7	3.5	2.9	3.0
5th percentile	1.7	2.8	2.0	2.4
10th percentile	1.9	3.2	2.4	2.6
95th percentile	3.8	4.0	3.4	3.8
Beenyup WWTP				
Mean	2.4	2.6	2.5	3.5
Median	2.3	3.1	2.3	3.4
Geometric Mean	2.1	2.1	2.3	3.4
5th percentile	0.9	0.9	1.2	2.8
10th percentile	1.0	0.9	1.5	2.8
95th percentile	3.5	3.7	3.0	4.2
Boneo WWTP				
Mean	2.3	3.3	2.0	2.6
Median	2.6	3.6	1.9	2.6
Geometric Mean	2.1	3.0	1.8	2.5
5th percentile	0.4	0.4	0.5	1.4
10th percentile	1.0	2.0	0.8	2.2
95th percentile	3.4	4.6	3.2	3.1
Rosny WWTP				
Mean	1.8	2.30	2.0	1.2
Median	1.6	2.25	2.0	1.0
Geometric Mean	1.6	2.21	2.0	1.0
5th percentile	0.4	1.29	1.1	
10th percentile	0.8	1.40	1.5	0.7
95th percentile	4.5	3.18	2.8	

Table 8-2. Determined correlations between LRVs for adenovirus, polyomavirus, *E. coli* and *Microviridae* at each WWTP.

Treatment Plant	Adenovirus	Polyomavirus	*E. coli*
Oxley			
Adenovirus			0.19, P=0.22
Polyomavirus	**0.52**, P=0.0002		0.04, P=0.79
Microviridae	**0.4**, P=0.004	**0.58**, P=0.0000	**0.42**, P=0.006
Beenyup			
Adenovirus			0.11, P=0.62
Polyomavirus	**0.60**, P=0.002		0.26, P=0.22
Microviridae	**0.76**, P=0.0002	**0.65**, P=0.0002	0.35, P=0.23
Boneo			
Adenovirus			-0.10, P=0.66
Polyomavirus	0.12, P=0.59		-0.32, P=0.16
Microviridae	-0.08, p=0.71	**0.55**, P=0.01	-0.18, P=0.45
Rosny			
Adenovirus			0.20, P=0.35
Polyomavirus	-0.09, p=0.71		-0.19, P=0.35
Microviridae	0.37, p=0.09	0.67, p=0.09	0.04, P=0.3

P= Pearson's correlations; Significant correlation in bold

8.1 Discussion and Conclusions

Adenovirus had a consistently lower LRVs than polyomavirus and Microviridae in all the WWTPs (apart from *Microviridae* at the Boneo plant) which suggests that adenovirus has a higher stability than polyomavirus *and Microviridae*. DNA viruses (adenovirus and polyomavirus) are reported to be more stable and persist longer than RNA viruses (norovirus, polyomavirus and coxsackievirus) during wastewater treatment and in the environment (Love *et al*., 2010; Mena and Gerba, 2009). The data on the virus removal during the ASP process in this study also suggests that adenovirus is more conservative compared to the *Microviridae* coliphage. The removal of adenovirus is therefore most likely to be lower than other viruses of concern. Due to the conservative nature of adenovirus this means that it could be used as a suitable surrogate of virus removal for validation of the activated sludge process. While human polyomavirus were present in high numbers in the influent and effluent, their removal was much higher and more variable than adenovirus and *Microviridae*. Therefore polyomavirus is not as suitable a surrogate for validating the removal of more resistant viruses such as adenovirus.

The scientific consensus is that there is poor to no correlation between the levels of bacterial indicators and enteric viruses in treated wastewater (Carducci *et al*., 2009; Harwood *et al*., 2005). The results of this study support this view with very poor correlation between *E. coli* and the viruses tested. The use of *E. coli* for any validation process as a surrogate for other microorganisms such as viruses has been explored due to the relative ease of detection and low cost of the quantitative assays. However, due to higher LRVs for *E. coli* and very poor correlation with the enteric viruses, it is not recommended that *E. coli* be used as a surrogate for enteric virus removal or for validating ASP.

In contrast, the significance observed in cross-correlations between adenovirus and *Microviridae* numbers in the influent and effluent samples at the Oxley Creek and Beenyup WWTPs suggests that *Microviridae* could be a reasonable predictor of adenovirus removal in the ASP processes. As site specific variations were observed, however, more research would be needed to confirm that this is consistent across all Australian WWTPs.

In conclusion, the results have suggested that adenovirus could be good candidate for use in ASP validation process as it has high numbers in both influent and effluent and is more resistant to removal as compared to polyomavirus and *Microviridae*. *E. coli* is not recommended for validation of the ASP process due to higher removal rates and a lack of correlation with virus removal. In this study, enteric virus genomes were found to be a conservative measure of virus reduction, as this does not take into account infectivity of the virus. The built-in conservatisms of using the qPCR based approach for enteric virus quantitation provided adequate assurances to consistently determine and apply actual LRVs to the ASP plants at WWTPs.

The finding of the attempted correlation is that the lack of any strong correlations between the plants reinforces the hypothesis that all ASP plants need to be validated on an individual basis. The influences of geographical location, plant design and operation, and differences in the wastewater composition due to the input from the local population are the most likely causes for the individual characteristics of each WWTP.

9 Validation Protocol Development

As in the Aims and Objectives, the principal aim of this study was to generate information on the ability to use observations on microbial log reduction values to assist in developing a validation protocol for activated sludge plants used for treated wastewater for water recycling purposes. The determination of LRVs and their comparison across different wastewater treatment plants, however, are only one part of what is required for the development of a validation protocol. Other important issues include the standardisation of sampling protocols, linking of LRVs with plant performance, and determining if there are plant operational parameters that can be linked with the validation protocol for use in the ongoing verification of validated plants.

To assist with these important issues, this section covers information generated on the sampling strategies and determining if any links could be made to determine if there were any relationships between measured physicochemical parameters and the presence of the studied microorganisms.

9.1 Comparison of sampling on hydraulic retention time or simultaneous samples

Standardisation of the timing of sampling of influent and effluent at WWTPs is important for obtaining informative and comparable data for a validation process. Two approaches have been commonly used when comparing microbial reduction across an activated sludge plant:

(i) sampling based on HRT of the treatment plant; and

(ii) collection of simultaneous samples at the inlet and outlet.

Both approaches have advantages and disadvantages. Samples collected using a hydraulic retention time can be considered to be a better representation of the actual condition of the activated sludge treatment process at a treatment plant. However, calculation of the HRT is difficult and depends on the plant operation at any one time. In addition, the calculations may not be very precise and can be influenced by eternal impacts such as stormwater inflows. Another problem is that when the HRT is longer than only a few hours, the necessity for storage of one portion of the samples after collection (usually the influent) also presents a challenge due to the necessity of storage at 4°C to maintaining sample integrity. Even with appropriate storage, there is a high potential for changes to occur in the sample.

As activated sludge plants are large biological reactors, there is extensive mixing of the solids and liquor. This means that particles such as microorganisms entering the plant in the effluent may not even follow a HRT and could therefore be more reflective on other measures such as SRT or MLSS. Due to this mixing, it was therefore considered worthwhile testing the hypothesis that simultaneous sampling of the influent and effluent could produce results comparable to sampling undertaken using a calculated HRT.

In order to determine the influence of mode of sample collection on the LRVs, a series of 40 simultaneous samples of the influent and effluent at the Oxley Creek treatment plant were collected and compared to the LRV results from 20 routine samples that had been based on the calculated HRT (~55hours). The resulting LRVs for *E. coli*, adenovirus, polyomavirus and

Microviridae (using the methodologies outlined in Sections 5 and 6) were all tested and compared to ensure that results were obtained from a range of microorganisms.

9.1.1 Results of Sampling at HRT versus simultaneous samples

Data on 40 simultaneous samples and 20 samples collected using the calculated HRT from the Oxley Creek was compared. The outcomes of the LRV analysis from the simultaneous and HRT sampling is detailed in the Table 9-1 and shown as a statistical box plot in Figure 9-1.

As can be seen from the data in Table 9-1 there was little difference between the LRV results derived from the simultaneous sampling and sampling based on HRT for any of the three viruses. A Student t-test comparing the simultaneous and HRT sampling outcomes found no statistically significant difference (*t* test, $P > 0.05$) between the LRVs derived from either sampling method for adenovirus, polyomavirus and *Microviridae*. These results indicate that any validation protocols that involve the analysis of LRVs for viruses can employ a sampling methodology that uses simultaneous sampling of the influent and effluent, rather than trying to sampling over a theoretical HRT residence time.

In contrast, the LRV data for *E. coli* was more variable between the two sampling methods (as seen in Figure 9-1) and there was a statistically significant difference found (*t* test, $P = 0.01$). If bacteria are therefore to be part of validation protocols, then the *E. coli* results indicate that more thought needs to be given to how sampling is undertaken to obtain valid bacterial LRVs. It should be noted, however, that it has not been established that sampling based on a theoretical HRT provides a more accurate result for *E. coli* LRVs than the simultaneous sampling method. The standard deviations and range obtained for the *E. coli* LRVs were similar under both sampling methods and, therefore, using the simultaneous sampling method for *E. coli* could be as appropriate to use as the HRT sampling method.

Table 9-1. Descriptive statistical analysis of the LRV data for simultaneous samples and samples collected on HRT at Oxley Creek WWTP.

	E. coli		Adenovirus		Polyomavirus		Microviridae	
	Simultaneous	*HRT*	*Simultaneous*	*HRT*	*Simultaneous*	*HRT*	*Simultaneous*	*HRT*
Mean	3.0	2.8	2.8	2.8	3.6	3.5	2.9	2.9
Median	3.0	2.8	2.8	2.0	3.5	3.6	2.9	2.9
Standard Deviation	0.37	0.34	0.63	0.70	0.34	0.34	0.38	0.34
Range	1.7	1.2	2.9	2.9	1.8	1.2	1.7	1.3
Maximum	4.0	3.4	4.0	4.4	4.4	4.0	3.6	3.5
Minimum	2.3	2.2	1.1	1.5	2.6	2.8	1.9	2.2
Count	40	20	39	19	38	19	39	19
Geometric Mean	3.0	2.8	2.7	2.8	3.5	3.5	2.9	2.9
5^{th} Percentile	2.5	2.2	1.7	1.5	2.8	2.8	2.0	2.2
10^{th} Percentile	2.6	2.2	1.8	1.5	3.2	2.8	2.3	2.2
50^{th} Percentile	3.0	2.8	2.8	2.9	3.5	3.6	2.9	2.9
95^{th} Percentile	3.8	3.4	3.8	4.4	4.0	4.0	3.4	3.5

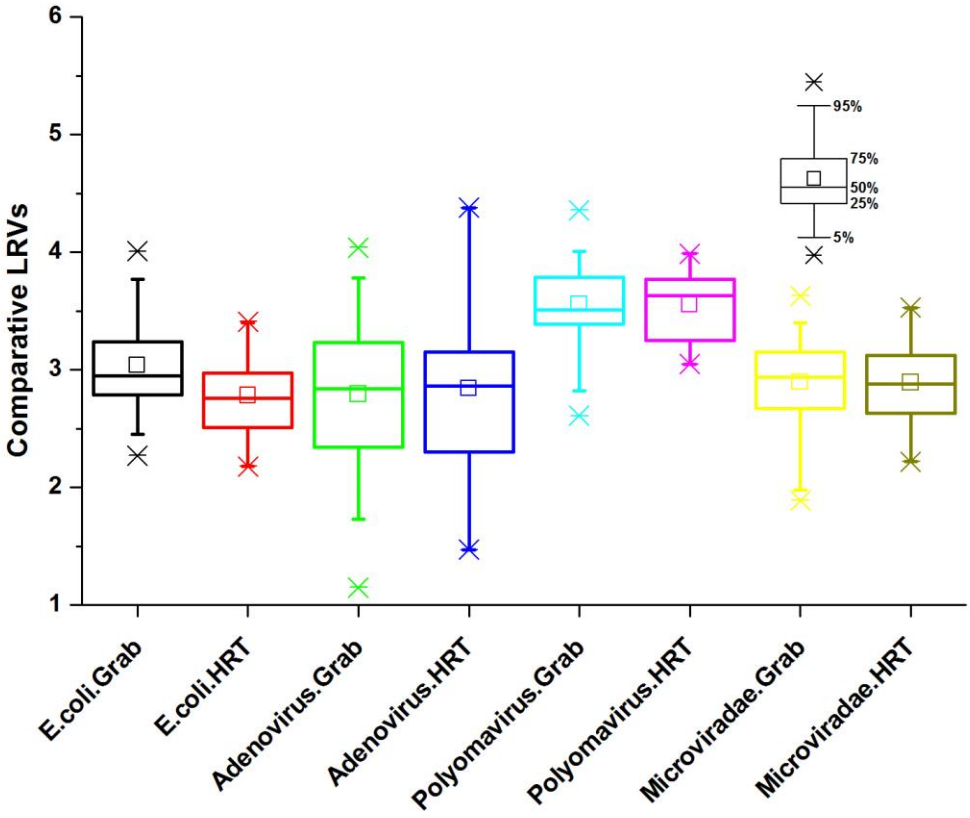

Figure 9-1. Comparative Log Reduction Values (LRVs) observed from the simultaneous samples (n=40) and paired samples (n=20) on Hydraulic Retention Time (HRT) from Oxley Wastewater Treatment Plant.

The vertical line represents range of data, squares represent mean, small horizontal lines (whisker) at the end of vertical lines represent min and maximum, the crosses extending from the vertical line represent the 99% confidence limits.

While it had been determined that simultaneous sampling could produce comparable results to those obtained using sampling based on HRT, it was tested to determine the minimum sampling number needed to obtain a usable LRV. The working hypothesis has been that the results from 20 sample events will be sufficient to obtain an accurate, usable LRV, however, an assessment was undertaken to determine if 20 samples were sufficient compared to 10, 30 or even 40 sample events. The comparison was made using the data available from the simultaneous sampling for adenovirus and *Microviridae*. Descriptive statistical analysis was performed on a pool of 40, 30, 20 and 10 samples (Table 9-2).

The results obtained show that there is little different in calculating LRVs using 10, 20, 30 or 40 samples, although the 30 and 40 sample calculations have slightly smaller means and geometric means than the LRVS calculated from only 10 sample events. The similarity observed between the LRV means and geometric means indicates that using 20 or 30 samples captures sufficient data to calculate adequate LRVs and, therefore, 20 samples would be an acceptable sample number to use for producing the required LRVs within a validation protocol. The LRVs obtained when using the data from 40 samples is not sufficiently different to warrant the additional sampling. While the geometric means for LRVs calculated using 10 samples are only marginally higher than those calculated using 20+ samples, it is the likely that 10 samples would be less applicable for other statistical assessments and at greater risk of being impacted by lost or NO DATA values and therefore should not be the set sample number.

Table 9-2. Summary statistics on LRVs data derived from variable samples number for adenovirus and *Microviridae*.

	Adenovirus				*Microviridae*			
Sample numbers	**40**	**30**	**20**	**10**	**40**	**30**	**20**	**10**
Mean	2.8	2.8	2.9	3.0	2.9	2.9	2.9	3.0
Median	2.8	2.8	3.0	3.1	2.9	3.0	3.0	3.1
Standard Deviation	0.63	0.64	0.68	0.58	0.38	0.35	0.36	0.31
Range	2.9	2.9	2.9	1.7	1.7	1.6	1.6	1.1
Maximum	4.0	4.0	4.0	4.0	3.6	3.6	3.6	3.6
Minimum	1.1	1.1	1.1	2.3	1.9	2.0	2.0	2.5
Count	39	29	20	10	39	29	20	10
Geometric Mean	2.7	2.7	2.8	3.0	2.9	2.9	2.9	3.0

9.2 Assessment of Linkages Between Microbial LRVs and Physicochemical Results in Wastewater Treatment Plants

Validation of treatment systems requires an understanding of how well the system is able to remove a contaminant (in this case microbial pathogens) under normal operating conditions and be able to determine under what conditions the treatment system is not effectively removing the contaminant. One of the best ways to achieve this is to determine a link between the removal of the contaminant and a standard measurable operational condition or another easily measurable physicochemical parameter.

Many engineered wastewater treatment systems such as UV irradiation and ozonation are well understood in terms of physicochemical parameters which influence the removal of enteric pathogens. Keeping factors such as turbidity/ suspended solids in optimum range in these systems helps in consistent removal of enteric pathogens. Any change in turbidity is therefore an indication that UV systems will not be operating under optimal conditions and, therefore potentially letting pathogens pass through untreated. In case of ASP, however, the relationships between parameters are less understood due to variability in design, operational conditions and geographical conditions.

This study has determined the LRVs for three viruses and *E. coli* for three operating activated sludge plants as well as obtaining a series of measured physicochemical parameters for these plants. To aid the development of a validation protocol for activated sludge plants an assessment was undertaken to determine if there were linkages between the LRVs for these microorganisms and the measured physicochemical parameters.

It had been previous suggested that the use of Principal Component Analysis (PCA) may be a way to identify these linkages (Flapper *et al*. 2010), therefore the influent and effluent data obtained in this study was analysed using PCA to identify the factors affecting the correlation between physicochemical and microbiological parameters.

9.2.1 Rational for using Principal Component Analysis

Principal Component Analysis (PCA) is the most commonly used unsupervised multivariate regression analysis to explore the relationship between different physicochemical and microbiological parameters (Flapper *et al.,* 2010; Muela *et al.,* 2011). The reason PCA is so frequently used is that it can identify groups of variables which have complex relationships. PCA analysis assumes that observed variables are correlated with a small number of underlying variables or latent variables. The observed correlation matrix then can manifest variables aiming to highlight the underlying latent principal components. The first principal component identified (PC1) accounts for most of the variance in the data. The second principal component identified (PC2) relates to the second largest amount of variance in the data and is uncorrelated with the first principal component, and so on. The multiple correlations in PCA are explained by eigenvalues. Eigenvalues indicate the amount of variance explained by each component. Eigenvectors are the weights used to calculate components scores. PCA analysis involves three main steps:

1. Standardisation of measured variables to ensure that they have equal weights in the analysis by e.g., mean = 0, standard deviation = 1, diagonals of the matrix are equal to 1;
2. Calculation of the covariance matrix by identifying the eigenvalues and eigenvectors; and
3. Elimination of components that account for a very small proportion of the variation.

PCA as an unsupervised regression analysis is ineffective in certain case such as when within-group variation is greater than the between-group variation. In such situations, the use of supervised classification methods such as Partial Least Square Discriminant Analysis (PLS-DA) PLS-DA is considered.

Partial Least Square Discriminant Analysis (PLS-DA) is a supervised analysis performed to sharpen the separation between groups of observations. This is normally done by rotating PCA components so that a maximum separation among classes is obtained, and to understand which variables carry the class separating information - in the example of this study, if there are any obvious physicochemical components which are closely linked to influent or effluent microbial numbers.

In this study, data limitation, especially for the less frequently collected physicochemical parameters such as BOD, COD and suspended solids, resulted in poor fitting of the PCA models. As part of the model fitting, missing data was estimated using the data that was available. In these cases all the missing values were populated with a small value (the half of the minimum positive values in the original data which was assumed to be the detection limit). This approach is commonly used for missing data in PCA/PLS-DA analysis. The multivariate analysis of the data provided information on which parameters could be closely linked to the virus numbers in the treated effluent.

9.3 Analysis of correlations at the Oxley Creek WWTP

As the Oxley Creek WWTP was the plant with the highest LRVs for the viruses and had the largest data set for the physicochemical data, it was selected as the treatment plant to test the PCA analysis. Prior to undertaking the multivariate analysis on the influent and effluent data sets (n=40 for each), descriptive statistical analysis was performed to compare the measured physicochemical

and microbiological parameters from the influent and effluent samples. A brief summary of data analysis (mean, median, range, maximum and maximum) is presented in the Appendix D.

Figure 9-2 shows principal component analysis (PCA) results obtained after the analysis of the influent and effluent data set for the Oxley Creek wastewater treatment plant. The PC1 vs PC2 score plot does not show clear class separation between the two groups (influent and effluent), although it represents the main part of the data variance (68.0 and 13.6%, respectively). Despite the lack of clear separation, the plot does show that there is a clear distinction between the influent and effluent samples, which is to be expected due to the large respective differences in the microbial numbers. In the loading scatter plot, most of the physicochemical parameters cluster away from the microorganism suggesting no relationship between them. Total Conductivity (TC), Mixed Liquor Suspended Solids (MLSS), turbidity and suspended solids clustered with the microorganisms, however, the clustering was at distance from the origin (red circle) indicating that there was limited correlation between the values for the microorganisms and these four physicochemical parameters.

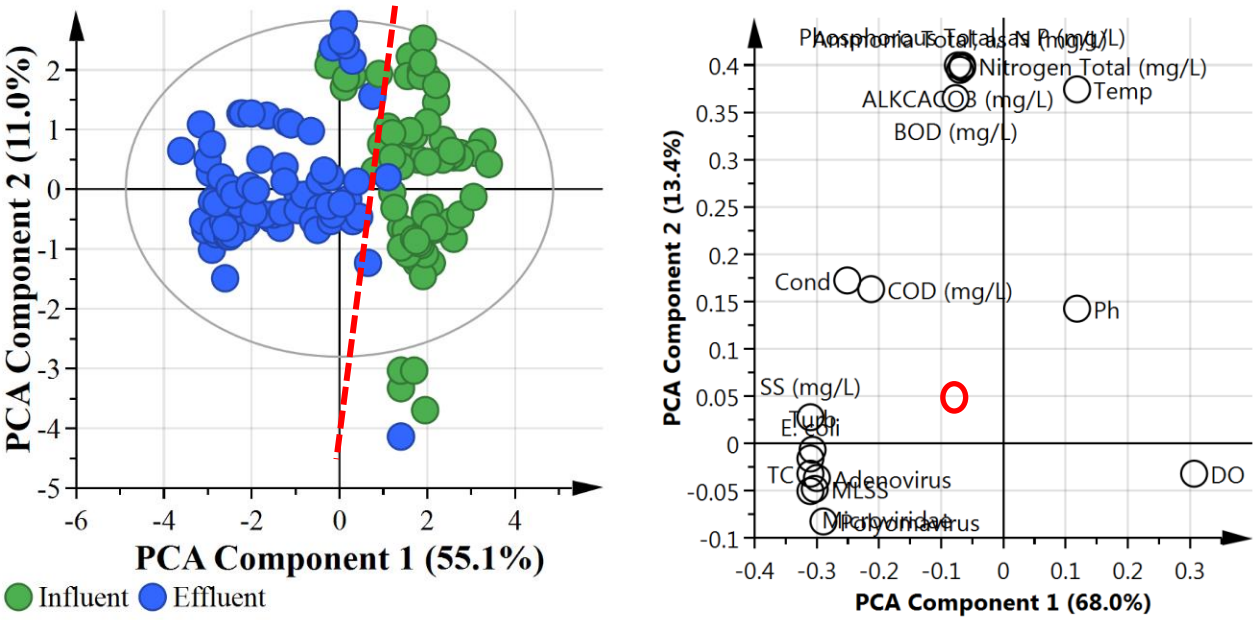

Figure 9-2. PCA Score Scatter Plot and Loading Scatter Plot comparing Oxley creek influent and effluent samples.

The red dotted line highlights the separation within the data. Note: the PCA eclipse (solid line) represents 95% confidence interval, with R^2X and Q^2 (cum) values of 0.814 and 0.695, respectively.

For within group analysis (influent or effluent), PLS-DA was applied after excluding variables with less than 50% data (50%cut off limit) (Figure 9-3). These PLS-DA modelling results showed that for the Oxley Creek WWTP, while the model is not a perfect fit there is some links between suspended solids and turbidity and adenovirus numbers in the influent and effluent. To further explore these links correlation analysis was performed.

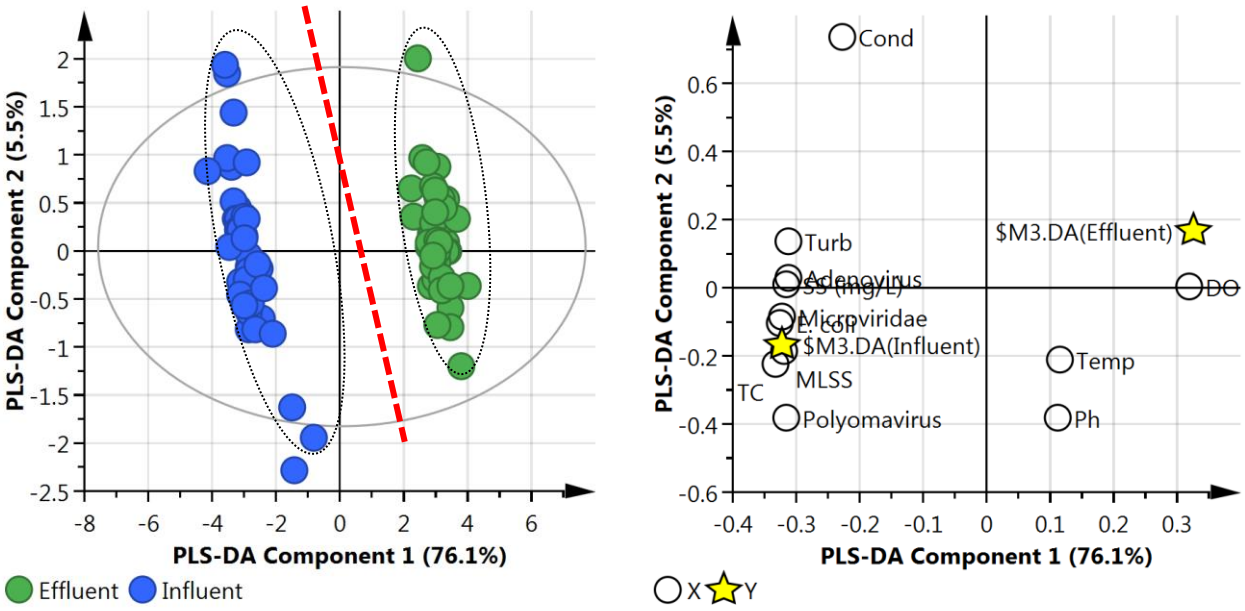

Figure 9-3. PLS-DA Score Scatter plot and PLS-DA Loading Scatter plot of Oxley Creek influent and effluent data.

Note: the PLS-DA eclipse (grey solid line) represents the 95% confidence interval. The red dotted line highlights the separation within the data. The black dotted highlights the influent and effluent groupings.

As the data on the SS, nitrogen, BOD and COD had significant number of observations missing (due to the fact that these parameters are recorded less frequently at the Oxley Creek WWTP (fortnightly to monthly)) these parameters were excluded from the correlation analysis. Despite this, analysis of the remaining physicochemical data against the microbial numbers in the influent and effluent for the Oxley Creek WWTP indicated that there were not significant correlations between the parameters tested.

An in depth individual PCA analysis of the other sites is not reported here as the other sites had smaller pools of data (ranging from n=20 to 24). Despite this some brief information on the data analysis from Beenyup, Boneo and Rosny WWTPs is provided in the Appendix D.

9.3.1 PCA analysis of the combined data from all WWTPs

While there was limited correlation or relationships noted for one individual WWTP, data from all sites were pooled under two classes (influent and effluent) to further explore the possible links between measured physicochemical and microbiological parameters using a much larger pool of data than was available for any one individual plant. The PCA analysis from all sites also indicates that there are not significant correlations between physicochemical and microbiological parameters (Figure 9-4).

The PCA analysis on the pooled data was not conclusive with a wide spread of data. The Loading Scatter Plot also found that there was no clustering of the physicochemical or microbiological parameters was observed. Like for the analysis on the single Oxley Creek WWTP, the analysis for the pooled data from all four sites indicated that there were no significant correlations between any of the physicochemical and microbiological parameters tested.

The subsequent PLS-DA model for the pooled data results are presented in Figure 9-5. The PLS-DA Scatter plot indicated that there was still a poor fit amongst all the parameters, however, the associated Loading Scatter Plot indicated that there was some inverse relationships between Microviridae, polyomavirus and influent water temperature, although the correlations between these relationships were low.

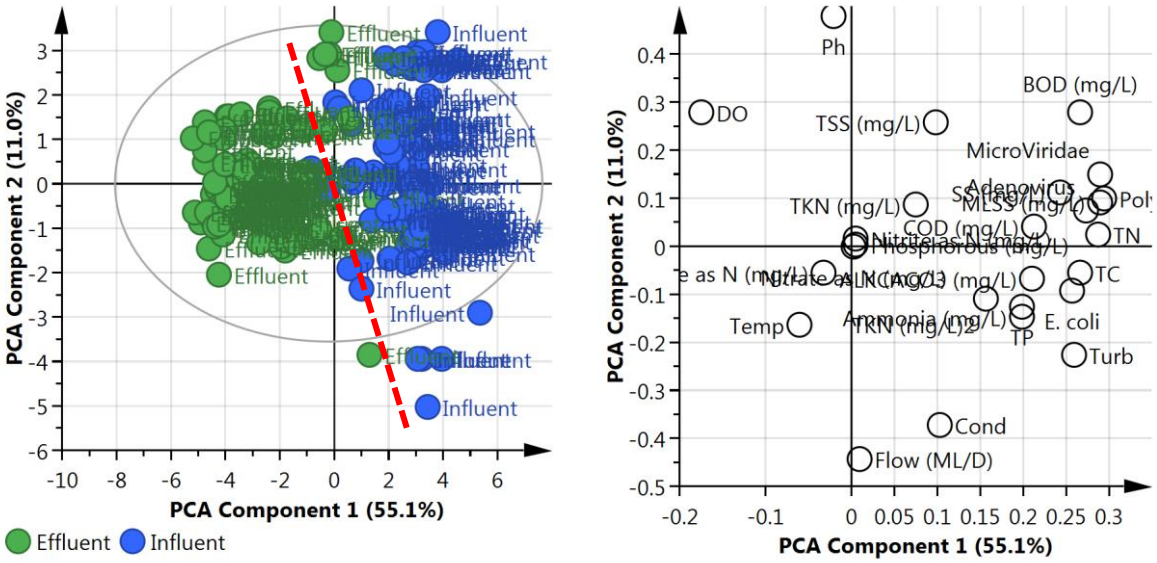

Figure 9-4. PCA Score Scatter Plot and Loading Scatter Plot comparing all influent and effluent samples.

The red dotted line highlights the separation within the data. Note: the PCA eclipse (solid line) represents the 95% confidence interval, with R^2X and Q^2(cum) values of 0.947 and 0.355, respectively.

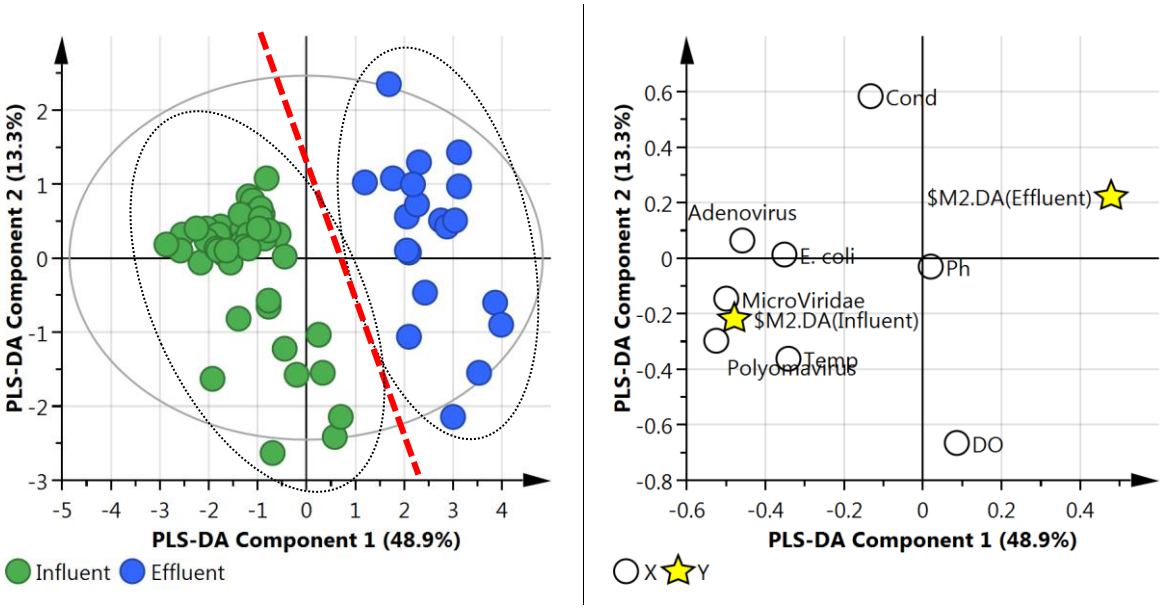

Figure 9-5. PLS-DA Score Scatter plot and PLS-DA Loading Scatter plot of all influent and effluent data.

Note: the PLS-DA eclipse (grey solid line) represents the 95% confidence interval. The red dotted line highlights the separation within the data. The black dotted highlights the influent and effluent groupings. PLS-DA R^2X, R^2Y and Q^2 values are 0.622, 0.882 and 0.848 respectively.

9.4 Discussion and conclusions

9.4.1 Sampling at HRT versus simultaneous sampling

In this study, samples were collected from the Oxley Creek wastewater treatment plant using either simultaneous samples or samples based on the calculated HRT to determine if there was any statistically significant difference between the LRVs calculated from the two sampling methods. Simultaneous sampling of the influent and effluent is more convenient and resolves issues of sample collection, storage and shipping, however, there would be expected to be difference caused by the fact that the influent sample does not match the effluent sample. In contrast, sampling using the calculated HRT should theoretically resolve this issue as the effluent sample should be a similar batch of liquor to the influent sample. In reality, however, activated sludge plants could be considered large batch reactors with considerable mixing, therefore, there is no guarantee that a collected effluent sample is, in fact, closely related to the HRT matched influent sample. Determining the HRT at a treatment plant requires calculation of correct HRT at the time of sampling. HRT can often vary depending upon the volume of inflow which can be influenced by factors such as rainfall and seasonal variations such as surge in population during holiday seasons for smaller treatment plants. In addition, as already discussed, there are also practical reasons that a HRT based sampling can cause problems associated with shipping and storage. For a practical, accurate validation protocol, it is therefore important to ensure that there is statistical veracity in the finalized sampling methodology.

Comparison of the data analysis of the simultaneous and HRT samples showed there was no statistically significant difference in the LRVs (*t* test, $P > 0.05$) from either method for *E. coli*, adenovirus, polyomavirus or *Microviridae*. A similar investigation of an activated sludge plant in France found that virus removals estimated from grab samples were closely aligned with the removals from automatic composite samples (Rolland *et al*., 1983). The findings of the current study suggest that using either sampling method would produce similar results for the calculation of LRVs for activated sludge plant.

Therefore, it is recommended that, due to the practicalities associated with simultaneous sampling, this method is used in activated sludge validation protocols to determine the LRVs for enteric viruses and other microorganisms.

To ensure that optimal sampling frequency is also set in a validation protocol, it was deemed important to also determine the most practically accurate number of samples required to capture the variation in the LRVs for the WWTP. The results demonstrated that 10 samples are too few to capture variations in the LRVs and to cover any lost samples. The mean and geometric means of 20, 30, and 40 samples for adenovirus and *Microviridae* were statistically similar, suggesting no additional benefit could be obtained from the collection of more than 20 samples. In accordance with the available data we propose that the collection of a minimum of 20 samples is set as the requirement for validation purposes but it is noted that sample numbers above 20 will not provide any further significant benefit.

9.4.2 Correlation between physicochemical factors and microbiological parameters

Like for any treatment system, to validate an activated sludge plant, it is important to determine that the plant is operating efficiently, then determine the treatment capability of the ASP (in this case for removing pathogens), and finally determine at what point the plant is no longer able to achieved those measured removals. In most cases a treatment system or plant can be assessed to be achieving optimal performance and maintaining that level of performance through the monitoring of one or more physicochemical parameters.

For activated sludge systems the most easily determined physicochemical parameters are one such as temperature, pH, dissolved oxygen, suspended solids, BOD and COD. The existence of a correlation between these physicochemical parameters and the microbiological parameters would make it possible to demonstrate real-time removal of pathogens is being maintained by measuring these physicochemical parameters.

In this study, principal component analysis (PCA) was used to analyse influent and effluent data sets from all three ASP wastewater treatment plants to identify the correlation between physicochemical and microbiological parameters. The Oxley Creek WWTP was studied in greatest detail as it was the plant with the highest microbial LRVs and the most available data on the physicochemical parameters. The results of the PCA analysis of the Oxley Creek WWTP plant data did not highlight any specific relationships with little to very poor correlation between on-line measured parameters (DO, temp, EC, turbidity) and microbiological data (virus and *E. coli*). This observation suggests that these factors are not good predictors of pathogen presence in influent, effluent or removal during the ASP. In addition, a relatively large difference in the virus numbers between the influent and effluent with relatively small variations in physicochemical parameters was an additional reason for the poor correlation between the microbiological and physicochemical parameters.

These findings on the PCA on data for the Oxley Creek WWTP matched other international studies. In a French study on ASP processes, limited correlations were also found between virus numbers in the effluent and physicochemical parameters such as chemical oxygen demand, and suspended solids (Rolland *et al*., 1983). In addition, they reported that virus removals exceeded the removal of suspended solids, chemical oxygen demand and turbidity. A similar outcome was determined in a year-long study on an activated sludge treatment plant in Spain where a PCA of data indicated no significant correlations between physicochemical parameters (COD, BOD, SS, TKN, and NO_3) and microbiological parameters (Muela *et al*., 2011). In another study, on enteric pathogens and indicators removal during membrane bioreactor (MBR) and up-flow anaerobic sludge blankets (UASB), no physicochemical parameter (NH_4, PO_4, COD, BOD, TOC, and SS) was able to predict the presence of enterovirus genome in the effluent samples (Ottoson *et al*., 2006a). The results of our study are in agreement with these international findings, as analysis of the pooled data from all the studied treatment plants could not determine any significant correlations between physicochemical and microbiological parameters.

In a different finding, Flapper *et al*. (2010) found that using a PCA on data from in laboratory-scale experiments that there was a significant link between total alkalinity, organic matter, turbidity and

NO_3 with F-RNA coliphage, *E. coli*, enterococci, *Cryptosporidium* and *Giardia* removal. Furthermore, they found that suspended solids, turbidity, TKN and COD were negatively correlated factors linked to the calculated LRVs. It is unclear why Flapper *et al*. found these links although it may be possible that it is an impact of using a laboratory scale ASP. In a laboratory-scale setting, physicochemical parameters are likely to be less variable than in a large operational WWTP which, if so, would lead to less process variability and therefore better links between microbiological and physicochemical parameters. This type of process stability is difficult to obtain in large-scale wastewater treatment plants.

In the present study, virus removal values correlated better to each other than the most commonly used physicochemical parameters (Figures 9-4 and 9-5). This suggests that microbial parameters are more likely to predict the overall presence or removal of enteric pathogens than any of the physicochemical parameters. This is in accord with a previous finding where viruses were reported to be better indicators of other enteric virus than bacteria or protozoa (Muela *et al*., 2011). A lack of correlation and the higher sensitivity of the PCR detection method to detect changes in pathogen and indicator number (influent and effluent quality) also suggests that monitoring of microbiological parameters are currently the most appropriate means to determine accurate LRVs. Therefore, the use of an enteric virus model (recommended to be adenovirus) using PCR enumeration is more appropriate for demonstration of virus removal during the ASP.

With improvements in the technology in on-line sensors developing rapidly, it may be possible in the future to monitor additional physicochemical parameters during the wastewater treatment which may provide better correlations to pathogen removal.

Because of the nature that LRVs are derived data and therefore cannot be compared to the measured physicochemical data using PCA, an attempt was made to analyse linkages between the physicochemical parameters and the microbial LRVs using Bayesian Network modelling. The outcomes of this Bayesian Network modelling are reported in a paper by the UNSW researchers Roser and Khan (2015). They reported that the Bayesian Network analysis determined that the MLSS data was a poor predictor of *Microviradae* LRVs similar to the results of our PCA analysis which also showed poor link between MLSS and enteric virus or *E. coli*.

Roser and Khan (2015) also reported that the Bayesian Network analysis found potential links of low LRVs being closely associated with high reduced nitrogen (–20/33 mg/L), and higher LRVs (>2.3 $\log_{10}$) associated with much lower than average NH_4^+-N and TN of 2.2 and 7.4 mg/L respectively. It appears that, while these physicochemical parameters may not be directly correlated to pathogen removal, they may likely be associated with optimally performing ASP processes. This intimates that ensuring that better monitoring of optimal performance through appropriate operational monitoring of these parameters may be a strong assistance in verification and on-going operational monitoring. This does require further research on a wider number of WWTPs using ASP across a wide range of geographical location work to conclusively establish if these physicochemical parameters can be used for appropriate operational monitoring to indicate optimal pathogen removal in activated sludge plants.

In conclusion, the data provided by this study was not able to identify any usable direct links between the calculated microbial LRVs and any physicochemical parameters. A basic validation

protocol is still possible through an assessment of appropriate log reduction credits via the direct measurement of the reduction of agreed microbial surrogates (recommended to be adenovirus) in individual activated sludge plants operating under optimal conditions. Optimal performance for individual activated sludge plants can be made through the independent assessment of agreed performance on selected physicochemical parameters. What is still not possible, however, despite the attempts made in this study, is the direct linking any of physicochemical parameters with plant performance (as part of on-going operational monitoring and verification) to determine when the ASP is not achieving the required microbial reductions to ensure appropriate health risks levels are maintained.

This does not mean that such an aim may never be achieved, however, it will be necessary to study more activated sludge plants over longer time periods to obtain a greater pool of data that could enable a more in-depth statistical analysis than was possible in this study. In addition, the continual development and improvement in on-line sensor technologies and analysis methods may also identify linkages that were not possible in this study.

10 Summary and Recommendation

10.1 Validation of activated sludge plants

This study has provided rigorous scientific data that can underpin the development of validation protocols for activated sludge plants to demonstrate that they are capable of effectively achieving an agreed level (LRVs) of pathogens. A brief summary of the important findings from this report directly relevant to ASP validation protocols are summarised below:

- The activated sludge process evaluated in this study varied in their ability to remove pathogens and indicators, with the Oxley Creek WWTP located in Brisbane (in a subtropical environment) having the best removal rates, followed by the Beenyup WWTP located in Western Australia (mediterranean-style climate) and finally the Boneo WWTP located in Victoria (temperate climate) being an intermediate case. The trickling filter plant (Rosny WWTP) which was used as an example of these form of treatment consistently had LRVs lower than the ASP plants.
- None of the plants tested showed any influence of seasonal impacts for any of the microorganisms tested.
- Regardless of the WWTP or location, ASP was found to be consistently able to achieve average *E. coli* removals of 2.7 to 3.5 $logs_{10}$.
- The variation in *E. coli* removal with in a treatment plant was low with standard deviations of *ca* 0.5. However, across all three ASPs the performance was more variable with standard deviations *ca* 1.0.
- For optimally performing ASP processes such as Oxley Creek WWTP, an average of LRVs of approximately 3 log_{10} for viruses could be expected to be achieved. More details are needed, however, to determine if geographical location and climate have influences on the final LRVs achieved by an individual WWTP.
- Conversely, trickling filter plants comparable to that used by the Rosny WWTP may not attain pathogen removal rates as high as activated sludge plant.
- The presence of high numbers of adenovirus in both influent (10^6 to 10^8 L^{-1}) and effluent (10^3 to 10^5 L^{-1}) from all the tested wastewater treatment plants, all located in different regions of Australia suggests that they are a good candidate for use in validation processes.
- On an average adenovirus removal and *Microviridae* removal was comparable (`~ 2.5 log_{10}). The non-ASP plant at the Rosny WWTP had much lower removal rates with an average LRV for adenovirus only 1.8 log_{10}.
- The differences in adenovirus, polyomavirus and *Microviridae* at the individual WWTPs tested in this study demonstrates that any validation process must be undertaken at an individual WWTPs to determine the log removal credits possible and to determine the conditions to demonstrate the optimal performance of the activated sludge plant.
- The two WWTPs studied for the removal of *Cryptosporidium* spp. oocysts found LRVs between 2.8 and 3.7 log_{10}. Continuing improvements in the detection of *Cryptosporidium* in wastewater and testing of more WWTPs is required to provide more appropriate data to

demonstrate how well *Cryptosporidium* oocysts are removed by WWTP and its appropriateness for use in validation protocols of ASP plants.

- The variability in the observed LRVs of between the viruses and *E. coli*, and the absence of any correlation between *E. coli* and adenovirus numbers across the tested WWTPs suggest that a virus such as adenovirus would be the preferred microorganism for use in any validation protocols.
- The comparable observed removal rates for *Microviridae* in the ASP to adenovirus indicates that it could be used in conjunction with, or in place of adenovirus where needed, for the validation of activated sludge plants. The reported higher environmental stability and resistance to wastewater treatment of adenovirus, however, still makes it the preferred microbial surrogate for validation.
- Poor to no correlation between online measured parameters (DO, temp, pH, EC, turbidity) and the microbiological data suggests that there is still insufficient information to set one or more physicochemical parameters as good predictors of pathogen presence or removal during the ASP.
- Testing demonstrated that the results obtained from samples collected simultaneously from the influent and effluent and from samples collected using a calculated HRT are not significantly different. This means that simultaneous sampling can be used for validation of ASP as it is more practical and overcomes issues relating to time based sampling.
- For validation purposes, the collection of at least 20 uniformly spread simultaneous samples over at least an entire calendar year are required to cover for the potential spikes in disease and seasonal variation.

10.2 Issues requiring further investigation

This study has taken an in-depth analysis on the log removal potential of three activated sludge plants and one trickling filter plant located in different geographical and climatic regions. Despite the data and analysis on LRVs provided, there are issues which require further investigation for improving any future versions of an ASP validation protocol. These include:

- Generation of more data from more sites, preferably for longer time periods that can add to the analysis and comparative statistics.
- Increasing the number of WWTPs studied for *Cryptosporidium* LRVs to obtain appropriate data on removal by ASP plants.
- Any further studies on ASP plants should also examine the comparative removal of RNA viruses such as norovirus and rotavirus to further assess if adenovirus is also a good surrogate for the removal of RNA viruses.
- Although this study was unable to establish a clear link between a single or group of physicochemical factors and microbial LRVs, the potential link between suspended solids, turbidity, NO_3 and COD/BOD and the reductions in pathogen numbers should be examined further. The increased information will enable a more detailed assessment of any links between microbial reductions and physicochemical parameters which, hopefully, will

enable usable real time information on the WWTP performance and any potential impacts on pathogen removal.

- It had originally been planned to test the findings from the larger WWTPs on small and regional treatment plants, however, time and the initial findings eventually precluded this. These smaller plants are more at risk of operating out of the specifications assessed at the time of validation which has a corresponding potential reduction in pathogen removal and increased health risks. It is therefore important that a sufficient number of small and regional activated sludge plants be assessed using the methods and protocols developed in this study to determine if these small plants are able to be validated and routinely monitored for suitable microbial LRVs.

Appendix A. Pathogen removal during wastewater treatment

Table A 1. Wastewater treatment operational parameters and reported pathogen and indicators removal.

Location Reference	Pathogens studied	Sampling site	Treatment plant type	Actual pathogen numbers in influent and effluent	Log reduction	LRV calculation
Spain Montemayor *et al.,* (2005)	*Cryptosporidium*	Inlet and outlet	Plants A-E serve population from 35,000 to 170,000Activated sludge and sedimentation apart from largest Pant B which Physical–chemical precipitation	103 to 139 (oo)cysts/L maximum 340, min 40 (oo)cysts, effluent 1.8 to 5.96 (oo)cysts/L max. 16 and min 0.4 (oo)cysts	Geometric mean removal rate of $\log_{10}$ 1.66±0.27	Combined data from all plants
Swedish plants Ottoson, (2006)	Enteroviruses, noroviruses, *Cryptosporidium* and *Giardia*	Inlet and outlet	Four plants with activated sludge process (ASP) chemical precipitation as primary treatment, followed by a conventional activated sludge process.		*Cryptosporidium* and *Giardia* of 1.30±0.46 $\log_{10}$ and 3.32±0.46 $\log_{10,}$ while noroviruses and enteroviruses were removed by 0.9 and 1.3 log, respectively.	Combine averages
Ottawa, **Canada** **Chauret *et al.*, (1999).**	*Cryptosporidium*, *Giardia* and number of indicators	Inlet and outlet	Facility treating approximately 500 ML/d of sewage Activated sludge plant (ASP) with primary treatment, secondary treatment mesophilic anaerobic digestion (MAD)		*Cryptosporidium* and *Giardia* of 1.30±0.46 $\log_{10}$ and 3.32±0.46 $\log_{10,}$ while noroviruses and enteroviruses were removed by 0.9 and 1.3 log, respectively.	
Spain Reinoso, and Becares, (2008).	*Cryptosporidium*, *Giardia* and helminths		Two plants 250–300 (m3/day) of pig slurry conventional activated sludge treatment coagulant 40% ferric chloride plus Hyfloc FIC-100, nitrification–denitrification tanks (3500 m^3 in volume with seven	10^4–10^5 (oo)cysts/L for *Cryptosporidium*, 10^3 cysts/L for *Giardia* and 10^2–10^3 eggs/L for helminths	*Cryptosporidium* (oo)cysts 0.65-0.72 $\log_{10}$ helminth eggs >3 $\log_{10}$ removal	

Location Reference	Pathogens studied	Sampling site	Treatment plant type	Actual pathogen numbers in influent and effluent	Log reduction	LRV calculation
			days of HRT)			
Australia Keegan *et al* 2013	Adeno-v, crypto, *Giardia*, sulphite reducing clostridia (SRC), F-RNA bacteriophage	Influent, effluent	4 plants, activated sludge, and integrated fixed film activated sludge	Data not provided	Adenovirus 1.58 to 2.15 log_{10}, *Cryptosporidium* 0-2.1 log $_{10}$ *Giardia* 0.9-3.7 log_{10} All four plants had variable reduction for all pathogens	Results analysed to determine 5th and 10th percentile for LRV's for individual plants and combined data from all plants.
Petrinca *et al* 2009	HepA virus, Adenovirus, Rotavirus, Astrovirus, coliphage F-RNA phage	Influent, intermediat e treatment points, effluent, chlorinated effluent	one of two plants treating sewage for 1.2 million people in the Milwaukee metropolitan, treats 100 million Gallons/Day Activated sludge		F-RNA phage ~2log reduction	
Milwaukee Wisconsin, USA Sedmak *et al* 2005	Reovirus, enterovirus, adenovirus,	Influent, effluent (after final chlorination)	Activated sludge	Fluctuation in virus numbers reovirus, a maximum titer of 12,027 MPN/L and enterovirus 3,347 MPN/L	2-3 log (avg 2.41 log)	
Tenerife-Canary Islands, Spain Abreu-Acosta *et al*, 2011	Faecal coliforms, enterococci, *E coli*, *Clostridium perfringens*, somatic coliphages, *Salmonella*, *Campylobacter*, *Cryptosporidium*, *Giardia*, helminths	Inlet, outlet, storage pond effluent	Constructed wetlands	Influent with *Giarida* 205/L and *Cryptosporidium* 3.79/L	2 log	

Location Reference	Pathogens studied	Sampling site	Treatment plant type	Actual pathogen numbers in influent and effluent	Log reduction	LRV calculation
Michigan, **USA** **Simmons *et al* 2011**	Adenovirus, enterovirus, norovirus, HepA virus	Influent (raw sewage), pre-disinfection (after secondary biological treatment), post disinfection (final effluent)	4 plants-2 activated sludge, 1 oxidation ditch, 1 rotating biological contractor		1.9-5.0 log (avg 4.2 log) for infectious virus	
New Zealand Hewitt *et al* 2011	Adenovirus, enterovirus, norovirus,	Influent, effluent	WWTP served large (130,000-1,000,000), medium (10,000-64,000) and small (<1100-4000) community size moving bed biofilm reactors, activated sludge, waste stabilisation ponds 10 plants – 4 activated sludge, 4 waste stabilisation pond, 2 moving bed biofilm reactor	Typical influent culturable virus numbers 2-3 log10/L compared to 1-2 log10/L inthe effluent.		
Michigan, **USA** **Srinivasan *et al* 2011**	*E. coli*, Enterococci, *B. thetaiotaomicron*	Influent, primary effluent, sec effluent (pre and post chlorination), tertiary effluent	Activated sludge		5 log for *E. coli* and enterococci (by culture), 3 log for *B. thetaiotaomicron* (by qPCR)	
China	*Giardia*, *Cryptosporidium*,	Influent, primary and	3 plants – 1 activated sludge, 1 anaerobic-anoxic-oxic		For activated sludge plant: 3.34 log of	

Location Reference	Pathogens studied	Sampling site	Treatment plant type	Actual pathogen numbers in influent and effluent	Log reduction	LRV calculation
Fu *et al*, 2010	somatic coliphages, faecal indicators	secondary clarifier, flocculation clarifier, sand filter, membrane filter, reclaimed water (post chlorination)	process, and 1 oxidation ditch process		*Cryptosporidium,* 3.46 log of *Giardia*, 2.95 log of somatic coliphages, and 4.09 log of faecal coliforms	
North-western Ireland Cheng *et al* 2012	*Cryptosporidium*, *Giardia*, *E. coli*, enterococci, *Clostridium perfringens*	Influent, activated sludge, effluent, biosolids	4 plants – 2 activated sludge, 1 oxidation ditch, 1 percolating settlement		84% of *Cryptosporidium*, 98% of *Giardia*, and 99.5% of microsporidian spores	
Georgia, USA **Lui *et al* 2013**	Adenovirus	Influent, sec effluent, granular media effluent, membrane filtration effluent, final effluent	Activated sludge		5.1 log	
Hamburg, Germany Ajonia *et al* 2013	*Giardia*	Specific details not provided	Activated sludge	Influent 50 to 7548 cysts/L.	78% removal efficiency	
Sao Paulo, Brazil Hachich *et al*	Enterovirus, *Giardia*, *Cryptosporidium*, *Ascaris* sp eggs	Influent, effluent	4 plants – 2 activated sludge, 1 MBR, 1 anaerobic pond	Influent range *Giardia* 2.8	2-5 log of *Giardia* and, 2-3 log of Enterovirus	Geometric average

Location Reference	Pathogens studied	Sampling site	Treatment plant type	Actual pathogen numbers in influent and effluent	Log reduction	LRV calculation
2013				x 10^3/L Cryptosporidium 32/L Enterovirus 19/L Effluent *Giardia* 18L Cryptosporidium 0.1/L Enterovirus 0/L Data from one pant, variation between plants		
Traverse City, Michigan Kuo *et al* 2010	Adenovirus, Norovirus	Influent, primary sedimentation influent, MBR influent, MBR effluent	MBR		5 log removal of adenovirus and 0-3.6 log of Norovirus	
Galicia, Spain Castro-Hermida *et al* 2008	*Cryptosporidium*, *Giardia*	Influent, effluent	12 plants – 5 activated sludge, 4 oxidation, 2 oxidising beds, 1 biological aeration		Up to 83% of *Cryptosporidium* and up to 90% of *Giardia*	
Norway Myrmel *et al* 2006	Norovirus, Rotavirus, astrovirus, adenovirus, circovirus, HepA virus, *E. col*i	Influent, effluent	3 plants – 1 activated sludge, 1 biofilm process, 1 primary treatment screening		TBA[1]	
Italy Carducci *et al* 2008	HepA virus, adenovirus, somatic coliphages, TTV, *E. coli,* enterococci, *Salmonella*	Influent, effluent	Activated sludge	7.18 x 10^6 MPN/100mL *E. coli* (influent), 1.41x10^6 MPN/100mL *enterococci*	2 log adeno –v, 1.58 log TTV, 1.74 E. coli	Calculated using QPCR (displayed graphically in paper)

Location Reference	Pathogens studied	Sampling site	Treatment plant type	Actual pathogen numbers in influent and effluent	Log reduction	LRV calculation
				(influent)	1.99 enterococci, 2.2 log somatic coliphage	
Florida, Arizona and California Rose *et al.,* (2004)	Bacterial, vial and protozoan pathogens	Influent, effluent	Variable size from 0.9-25 mega gallons per day. Residence time for activated sludge varied from 3-8 days, nitrification 8-13 days, nutrient removal 8-16 days activated sludge (plants A-D), biological nitrification-denitrification (Facility E), enhanced biological phosphorus removal (Plant F)		Bacterial and virus removal through the activated sludge plants (1.39-3.0 log_{10}) with *Clostridium* removed (1.17-2.69 log_{10}), *Giardia* 2 log_{10} and *Cryptosporidium* 1.5 log_{10}.	

Appendix B. Summary statistics on historical data from four WWTPs

Table B 1. Historical data – Beenyup Influent January 2014 – April 2015.

	ALKCACO3 mg/L	*BOD mg/L*	*COD mg/L*	*pH*	*SS mg/L*	*TKN mg/L*	*TN mg/L*	*TP mg/L*	*MLSS AT16*	*Inflow mL/D*
Mean	340.05	222.18	441.82	7.35	148.83	67.39	67.60	11.74	3841.63	131.22
Median	340.00	220.00	431.50	7.50	150.00	67.10	67.10	11.50	3897.50	129.57
Standard Deviation	20.23	28.58	45.15	0.89	28.46	5.29	5.52	1.18	411.02	7.15
Range	105.00	180.00	172.00	7.68	170.00	26.70	26.70	6.41	3450.00	55.31
Maximum	395.00	340.00	542.00	7.68	250.00	83.70	83.70	15.70	5350.00	163.52
Minimum	290.00	160.00	370.00	0.00	80.00	57.00	57.00	9.29	1900.00	108.21
Count	43.00	38.00	22.00	72.00	264.00	45.00	43.00	45.00	365.00	456.00
Geometric Mean	339.46	220.53	439.70	#NUM!	146.19	67.19	67.39	11.68	3818.04	131.03

Table B 2. Historical data - Beenyup Effluent January 2014 – April 2015.

	ALKCACO3 mg/L	*BOD mg/L*	*COD mg/L*	*pH*	*SS mg/L*	*TKN mg/L*	*TN mg/L*	*TP mg/L*	*PH*
Mean	125.36	8.75	55.09	7.15	19.50	3.69	14.26	7.98	7.16
Median	130.00	5.00	48.00	7.30	10.00	2.89	14.00	8.12	7.27
Standard Deviation	15.57	6.59	24.60	1.11	20.48	1.82	2.29	1.45	0.78
Range	69.00	35.00	106.00	10.00	195.00	9.40	10.40	7.20	7.84
Maximum	159.00	40.00	127.00	10.00	200.00	11.40	20.60	12.00	7.84
Minimum	90.00	5.00	21.00	0.00	5.00	2.00	10.20	4.80	0.00
Count	67.00	41.00	22.00	93.00	259.00	60.00	60.00	60.00	91.00
Geometric Mean	124.37	7.43	50.78	#NUM!	14.73	3.37	14.09	7.85	#NUM!

Table B 3. Historical data - Boneo Influent January 2014 – June 2015

	Inflow kL/D	*pH*	*EC mS/cm*	*BOD 5 mg/L*	*COD mg/L*	*TKN mg/L*	*Ammonia mg/L*	*P mg/L*	*SS mg/L*	*Alk-Total mg CACO3/L*	*TDS mg/L*	*Total Nitrogen Calc (TCN) mg/L*
Mean	9169.15	7.56	1119.09	406.83	906.10	75.80	49.05	13.93	532.20	341.95	497.33	56.43
Median	8370.00	7.50	1100.00	380.00	940.00	75.00	47.00	14.00	530.00	340.00	510.00	54.00
Standard Deviation	1733.00	0.17	128.06	86.30	207.87	19.97	7.95	6.52	200.99	37.03	55.09	16.06
Range	5300.00	0.80	680.00	430.00	880.00	106.00	42.00	34.00	930.00	250.00	210.00	52.00
Maximum	12627.00	8.10	1600.00	690.00	1400.00	140.00	71.00	34.00	1100.00	500.00	580.00	86.00
Minimum	7327.00	7.30	920.00	260.00	520.00	34.00	29.00	0.00	170.00	250.00	370.00	34.00
Count	41.00	41.00	22.00	41.00	41.00	41.00	41.00	41.00	41.00	41.00	15.00	7.00
Geometric Mean	9022.06	7.56	1113.05	398.91	881.95	73.30	48.42	#NUM!	494.11	340.16	494.30	54.53

Table B 4. Historical data - Boneo Effluent June 2014 – April 2015

	pH	*Turbidity mg/L*	*Nitrate mg/L*	*Ammonia mg/L*	*Conductivity mS/cm*
Mean	6.80	1.93	3.92	0.18	897.89
Median	6.80	1.98	3.48	0.08	902.40
Standard Deviation	0.12	1.48	2.21	0.29	41.77
Range	2.18	9.93	12.54	4.22	384.38
Maximum	7.68	9.98	12.58	4.23	1140.42
Minimum	5.50	0.05	0.04	0.01	756.03
Sum	584362	155236	334896	15264	73162493
Count	85875	80545	85497	85253	81483

Table B 5. Historical data - Rosny Influent April 2014 - June 2015

	Ammonia as N mg/L	*BOD5 mg/L*	*Nitrate and Nitrite as N mg/L*	*Nitrogen mg/L*	*Phosphorus mg/L*	*TKN as Nitrogen mg/L*	*Total Suspended Solids mg/L*	*Influent kL/day*
Mean	35.62	507.91	1.04	64.13	36.58	60.82	352.64	5890.70
Median	37.00	500.00	0.90	63.30	29.35	59.00	354.00	5564.16
Standard Deviation	5.14	122.47	0.82	12.69	27.08	14.45	81.66	1586.20
Range	21.60	514.00	2.90	48.10	68.70	51.00	348.00	23978.84
Maximum	42.80	760.00	3.00	93.90	76.80	94.00	501.00	23984.64
Minimum	21.20	246.00	0.10	45.80	8.10	43.00	153.00	5.80
Count	22.00	11.00	11.00	11.00	22.00	11.00	11.00	527.00
Geometric Mean	35.18	492.63	0.70	63.07	25.82	59.38	341.37	5719.27

Table B 6. Historical data - Rosny Effluent April 2014 – June 2015

	Ammonia as N mg/L	*BOD5 mg/L*	*COD mg/L*	*Conductivity mS/cm*	*DO*	*Nitrate and Nitrite as N mg/L*	*Nitrate as N mg/L*	*Nitrogen mg/L*	*pH*	*Phosphorus mg/L*	*Temperature*	*TSS mg/L*
Mean	18.64	17.33	177.00	1129.23	81.67	7.66	3.18	31.94	6.88	6.31	17.08	11.45
Median	17.50	11.50	177.00	1059.00	83.50	7.40	1.40	31.85	7.29	6.57	17.70	9.15
Standard Deviation	4.29	28.17	NA	319.27	9.74	3.87	3.24	4.78	1.73	1.19	6.44	14.06
Range	16.70	142.00	0.00	1273.00	38.00	12.00	7.70	18.10	6.81	5.60	23.60	72.00
Maximum	28.40	147.00	177.00	2120.00	93.00	14.70	8.20	39.40	7.97	7.60	25.20	76.00
Minimum	11.70	5.00	177.00	847.00	55.00	2.70	0.50	21.30	1.16	2.00	1.60	4.00
Count	23.00	24.00	1.00	13.00	12.00	13.00	10.00	24.00	13.00	24.00	13.00	24.00
Geometric Mean	18.18	11.95	177.00	1099.03	81.04	6.77	1.89	31.58	6.38	6.14	14.73	8.91

NA = Not Available

Table B 7. Historical data - Oxley Creek Influent July 2014 – June 2015

	Suspended Solids mg/L	*Alkalinity (as CaCO3) mg/L*	*Ammonia (Total, as N) mg/L*	*Nitrogen (Total) mg/L*	*Phosphorus (Total, as P) mg/L*	*Biochemical Oxygen Demand mg/L*	*Chemical Oxygen Demand mg/L*	*pH*	*Conductivity mS/cm*
Mean	421.61	323.37	50.18	66.12	14.47	304.48	841.68	7.10	1417.79
Median	420.00	331.45	48.35	66.25	14.18	296.00	837.00	7.04	1410.00
Standard Deviation	115.51	53.04	12.69	16.32	4.00	81.06	213.74	0.30	172.62
Range	660.00	279.92	67.35	86.30	21.47	439.00	1091.00	1.27	764.00
Maximum	780.00	401.25	76.70	103.00	24.25	502.00	1311.00	7.85	1770.00
Minimum	120.00	121.33	9.35	16.70	2.79	63.00	220.00	6.58	1006.00
Count	50.00	50.00	50.00	50.00	50.00	50.00	50.00	29.00	29.00
Geometric Mean	403.83	318.08	48.13	63.71	13.81	291.47	808.55	7.10	1407.31

Table B 8. Historical data - Oxley Creek Effluent July 2014 – June 2015

	Suspended Solids mg/L	*Alkalinity (as CaCO3) mg/L*	*Ammonia (Total, as N) mg/L*	*Nitrate (as N) mg/L*	*Nitrogen (Total) mg/L*	*Phosphorus (Total, as P) mg/L*	*COD (soluble) mg/L*	*pH*	*Temperature*	*Conductivity mS/cm*	*Dissolved Oxygen mg/L*	*Turbidity mg/L*
Mean	7.15	165.67	4.42	0.61	5.64	1.52	35.51	7.80	26.58	1074.27	6.74	2.39
Median	6.00	163.65	1.62	0.60	3.23	0.92	36.00	7.81	27.95	1099.00	6.77	2.20
Standard Deviation	2.26	30.86	6.46	0.24	6.06	1.59	13.31	0.16	3.18	107.58	1.03	1.70
Range	9.00	178.11	31.71	0.99	33.76	7.50	43.00	0.60	12.55	447.00	5.84	8.01
Maximum	14.00	262.20	31.80	1.19	34.85	7.70	57.00	8.06	31.00	1234.00	9.84	8.70
Minimum	5.00	84.09	0.09	0.20	1.09	0.20	14.00	7.46	18.45	787.00	4.00	0.69
Count	41.00	64.00	54.00	64.00	69.00	68.00	11.00	45.00	44.00	45.00	45.00	29.00
Geometric Mean	6.87	162.78	1.52	0.56	3.96	0.92	33.03	7.80	26.38	1068.65	6.66	2.00

Table B 9. Summary of common influent water quality parameters measured during the sampling period (June 2014- April 2015) from the Boneo WWTP.

	pH	*Temp*	*DO mg/L*	*Cond mS/cm*	*Turb mg/L*	*COD (mg/L)*	*BOD (mg/L)*	*TKN (mg/L)*	*Ammonia (mg/L)*	*Phosphorous (mg/L)*	*SS (mg/L)*	*ALKCACO3 (mg/L)*
Mean	8.18	21.26	1.49	1048.00	183.29	822.22	420.00	59.33	44.00	12.32	500.00	330.00
Median	8.30	21.40	1.50	1100.00	168.00	730.00	410.00	61.00	44.00	13.00	555.00	330.00
Standard Deviation	0.45	1.34	0.77	293.37	49.32	188.73	85.59	5.85	2.00	3.46	176.18	8.94
Range	1.40	4.20	2.13	1026.00	150.00	480.00	210.00	16.00	6.00	7.80	440.00	20.00
Maximum	8.80	23.00	2.41	1600.00	290.00	1100.00	530.00	64.00	47.00	16.00	690.00	340.00
Minimum	7.40	18.80	0.28	574.00	140.00	620.00	320.00	48.00	41.00	8.20	250.00	320.00
Count	13.00	8.00	7.00	12.00	7.00	9.00	9.00	6.00	6.00	6.00	6.00	6.00
Geometric Mean	8.17	21.22	1.25	1006.71	178.69	803.87	412.25	59.07	43.96	11.89	469.35	329.90

Table B 10. Summary of common effluent water quality parameters measured during the sampling period (June 2014- April 2015) from the Boneo WWTP.

	pH	*Temp*	*DO mg/L*	*Cond mS/cm*	*Turb mg/L*	*Nitrate mg/L*	*Ammonia mg/L*
Mean	7.69	20.42	5.37	858.43	3.05	4.15	0.12
Median	7.81	20.53	5.66	871.00	2.95	4.01	0.10
Standard Deviation	0.38	1.22	2.04	51.49	0.84	1.30	0.05
Range	1.08	3.19	4.94	138.00	2.90	4.28	0.15
Maximum	7.93	21.78	7.34	923.00	4.40	6.85	0.23
Minimum	6.85	18.59	2.40	785.00	1.50	2.57	0.08
Count	7.00	6.00	6.00	14.00	14.00	10.00	10.00
Geometric Mean	7.68	20.39	4.99	856.98	2.93	3.98	0.11

Table B 11. Summary of common influent water quality parameters measured during the sampling period (June 2014- April 2015) from the Beenyup WWTP.

	pH	Temp	DO mg/L	Cond mS/cm	Turb mg/L	COD (mg/L)	BOD (mg/L)	MLSS (mg/L)	ALKCACO3 (mg/L)	SS (mg/L)	TKN (mg/L)	TN mg/L	TP mg/L	NH3 (mg/L)	Flow (ML/D)
Mean	7.43	23.10	3.68	1.98	117.74	463.10	220.27	3867.20	342.50	155.00	71.43	71.41	12.04	52.01	131.21
Median	7.50	22.00	3.45	1.30	113.00	451.00	225.00	3900.00	345.00	147.50	71.10	70.30	11.80	52.70	129.10
Standard Deviation	0.19	2.46	1.11	1.26	19.29	43.71	22.69	321.29	20.58	45.36	4.52	4.26	1.27	3.38	7.51
Range	0.71	6.00	2.40	3.10	40.30	141.00	85.00	1130.00	80.00	220.00	16.50	14.50	4.30	9.10	31.52
Maximum	7.80	27.00	5.10	4.00	139.00	542.00	245.00	4350.00	370.00	340.00	80.40	80.40	14.50	56.90	153.59
Minimum	7.09	21.00	2.70	0.90	98.70	401.00	160.00	3220.00	290.00	120.00	63.90	65.90	10.20	47.80	122.07
Count	22.00	5.00	4.00	5.00	5.00	10.00	11.00	23.00	10.00	20.00	19.00	10.00	10.00	7.00	20.00
Geometric Mean	7.43	23.00	3.56	1.71	116.49	461.28	219.06	3853.99	341.90	151.04	71.29	71.30	11.98	51.92	131.02

Table B 12. Summary of common effluent water quality parameters measured during the sampling period (June 2014- April 2015) from the Beenyup WWTP.

	pH	Temp	DO mg/L	Cond mS/cm	Turb mg/L	COD (mg/L)	BOD (mg/L)	ALKCACO3 (mg/L)	SS (mg/L)	TKN (mg/L)	TN (mg/L)	TP (mg/L)	NH3 (mg/L)	NO3 (mg/L)	NO32 (mg/L)	NH4 (mg/L)
Mean	7.22	26.00	7.96	33.84	2.89	56.56	10.45	126.67	19.31	4.41	14.11	7.76	0.69	9.37	9.60	0.18
Median	7.20	26.00	8.40	1.00	2.31	47.00	9.90	125.00	10.00	3.67	13.80	7.26	0.33	9.04	9.60	0.18
Standard Deviation	0.18	2.12	1.09	56.56	1.13	31.77	10.11	9.35	24.69	2.43	2.45	1.83	0.69	1.43	1.98	0.11
Range	0.74	5.00	2.60	122.00	2.53	106.00	35.00	30.00	94.00	9.09	10.26	6.80	1.92	3.61	2.80	0.16
Maximum	7.60	29.00	8.70	122.00	4.31	127.00	40.00	140.00	100.00	11.40	20.60	12.00	2.02	11.00	11.00	0.26
Minimum	6.86	24.00	6.10	0.00	1.78	21.00	5.00	110.00	6.00	2.31	10.34	5.20	0.10	7.39	8.20	0.10
Count	22.00	5.00	5.00	7.00	5.00	9.00	11.00	9.00	13.00	15.00	15.00	15.00	7.00	7.00	2.00	2.00
Geometric Mean	7.22	25.93	7.89	#NUM!	2.72	50.17	8.27	126.35	13.95	3.95	13.92	7.58	0.46	9.28	9.50	0.16

Table B 13. Summary of common influent water quality parameters measured during the sampling period (June 2014- April 2015) from the Rosny WWTP.

	pH	*Temp*	*DO mg/L*	*Cond mS/cm*	*BOD (mg/L)*	*Ammonia as N mg/L*	*Nitrate and Nitrite as N (mg/L)*	*Nitrogen (mg/L)*	*Phosphorous (mg/L)*	*TSS (mg/L)*	*TKN as Nitrogen (mg/L)*	*Flow (mL/D)*
Mean	7.33	18.28	4.02	1567.64	505.71	36.96	1.11	64.23	10.96	333.13	59.67	5.87
Median	7.10	18.20	5.00	1080.00	500.00	37.15	1.00	63.10	10.75	351.50	57.00	5.65
Standard Deviation	0.65	1.37	2.23	1603.89	153.02	1.95	0.96	12.09	1.69	75.77	15.90	0.88
Range	2.16	4.30	5.70	5515.00	514.00	7.00	2.90	48.60	5.30	240.00	51.00	4.65
Maximum	8.83	20.40	5.80	6390.00	760.00	40.00	3.00	93.90	13.40	393.00	94.00	9.88
Minimum	6.67	16.10	0.10	875.00	246.00	33.00	0.10	45.30	8.10	153.00	43.00	5.23
Count	13.00	9.00	13.00	11.00	7.00	14.00	8.00	15.00	8.00	8.00	9.00	25.00
Geometric Mean	7.30	18.23	2.37	1268.23	483.24	36.92	0.67	63.23	10.84	322.10	58.01	5.82

Table B 14. Summary of common effluent water quality parameters measured during the sampling period (June 2014- April 2015) from the Rosny WWTP.

	pH	*Temp*	*DO mg/L*	*Cond mS/cm*	*BOD (mg/L)*	*Ammonia as N mg/L*	*Nitrate and Nitrite as N (mg/L)*	*Nitrate as N (mg/L)*	*Nitrite as N (mg/L)*	*Nitrogen (mg/L)*	*Phosphorous (mg/L)*	*TSS (mg/L)*	*Flow (mL/D)*
Mean	7.12	18.48	0.38	1182.35	12.00	18.99	6.56	7.73	0.82	32.85	6.62	7.11	5.91
Median	7.08	18.60	0.40	978.00	12.50	17.50	4.00	8.10	0.80	32.10	6.54	6.50	5.72
Standard Deviation	0.28	2.32	0.28	727.48	3.44	4.96	4.61	0.72	0.05	3.97	0.78	2.60	0.96
Range	1.21	9.20	1.00	3202.00	13.00	16.70	13.60	1.30	0.12	13.00	3.20	7.40	5.00
Maximum	7.97	23.30	1.10	4030.00	18.00	28.40	14.70	8.20	0.90	39.30	8.50	11.40	10.30
Minimum	6.76	14.10	0.10	828.00	5.00	11.70	1.10	6.90	0.78	26.30	5.30	4.00	5.30
Count	20.00	18.00	11.00	20.00	16.00	19.00	11.00	3.00	4.00	17.00	19.00	18.00	25.00
Geometric Mean	7.12	18.34	0.31	1077.94	11.46	18.40	5.13	7.71	0.82	32.62	6.58	6.67	5.86

Table B 15. Summary of common influent water quality parameters measured during the sampling period (June 2014- April 2015) from the Oxley Creek WWTP.

	pH	*Temp*	*DO mg/L*	*Cond mS/cm*	*Turb mg/L*	*COD (mg/L)*	*BOD (mg/L)*	*MLSS mg/L*	*SS (mg/L)*	*ALKCACO3 (mg/L)*	*Ammonia Total, as N (mg/L)*	*Nitrogen Total (mg/L)*	*Phosphorous Total, as P (mg/L)*
Mean	6.92	26.25	0.43	1429.65	308.30	795.36	284.63	3642.75	396.98	308.23	45.40	59.56	13.42
Median	7.16	26.50	0.17	1474.50	299.00	790.50	287.00	3625.00	410.00	318.60	45.95	59.25	13.28
Standard Deviation	0.78	1.28	0.82	243.35	81.81	233.39	78.14	251.88	92.08	58.10	12.10	15.88	3.93
Range	3.43	7.40	4.70	1132.00	435.00	1091.00	363.00	925.00	405.60	279.50	56.75	75.80	18.96
Maximum	7.73	27.80	4.78	1692.00	565.00	1311.00	426.00	4175.00	525.60	400.80	66.10	92.50	21.75
Minimum	4.30	20.40	0.08	560.00	130.00	220.00	63.00	3250.00	120.00	121.30	9.35	16.70	2.79
Count	40.00	40.00	40.00	40.00	37.00	22.00	22.00	40.00	22.00	22.00	22.00	22.00	22.00
Geometric Mean	6.87	26.21	0.24	1401.42	297.01	754.23	269.24	3634.34	382.10	301.21	42.98	56.90	12.65

Table B 16. Summary of common effluent water quality parameters measured during the sampling period (June 2014- April 2015) from the Oxley Creek WWTP.

	pH	*Temp*	*DO mg/L*	*Cond mS/cm*	*Turb mg/L*	*COD (mg/L)*	*SS (mg/L)*	*ALKCACO3 (mg/L)*	*Ammonia Total, as N (mg/L)*	*Nitrate as N (mg/L)*	*Nitrogen Total (mg/L)*	*Phosphorous Total, as P (mg/L)*
Mean	7.39	27.45	4.56	1036.33	1.88	40.90	5.86	157.74	1.86	0.61	3.95	1.70
Median	7.36	28.05	4.55	1075.00	2.00	40.45	5.20	161.43	0.27	0.66	2.64	1.16
Standard Deviation	0.57	1.97	0.41	163.57	1.06	12.94	1.48	27.16	3.55	0.22	3.71	1.71
Range	2.32	10.40	2.03	738.00	5.30	31.30	5.00	135.70	14.84	0.78	16.46	5.78
Maximum	8.87	29.50	5.60	1297.00	6.30	57.00	10.00	219.79	14.90	1.00	17.80	6.00
Minimum	6.55	19.10	3.57	559.00	1.00	25.70	5.00	84.09	0.06	0.22	1.35	0.22
Count	40.00	40.00	40.00	39.00	39.00	4.00	23.00	23.00	23.00	23.00	23.00	23.00
Geometric Mean	7.37	27.37	4.54	1021.76	1.66	39.31	5.72	155.23	0.42	0.57	3.11	0.94

Appendix C. Detailed sampling and detection methodologies

Microorganisms targeted in selected activated sludge treatment plants

Four activated treatment plants (Oxley Creek, Beenyup, Boneo and Rosny) were sampled from August 2014 to May 2015. Each of the wastewater treatment plants (WWTPs) were sampled at least bi-monthly for enteric viruses (adenoviruses, polyomaviruses and *Microviridae*), protozoa and *E.coli*. Additional grab samples (*n* = 20) of influent and effluent were collected from the Oxley WWTP to determine if sampling for the WWTP validation should be carried out on the HRT or if simultaneous grab samples at inlet and outlet could be sufficient. A break-down of samples numbers and data obtained after sample analysis (input, output, LRVs) from all sites is provided in Table 4.1.

Table C 1. Number of samples collected from ASP from all four sites (influent, effluent and LRVs).

Influent *E.coli*	22	20	40	24	106
Effluent *E.coli*	20	20	40	24	104
LRVs *E. coli*	20	20	40	24	104
Influent Adenovirus	23	19	40	25	107
Effluent Adenovirus	22	19	39	25	105
LRVs Adenovirus	22	19	39	25	105
Influent Polyomavirus	23	19	40	25	107
Effluent Polyomavirus	22	17	38	25	102
LRVs Polyomavirus	22	17	38	25	102
Influent *Microviridae*	23	19	40	25	107
Effluent *Microviridae*	22	18	39	25	104
LRVs *Microviridae*	22	18	39	25	104

Sample collection and processing

Grab samples for influent and effluent wastewater samples were collected from the selected WWTPs based on the hydraulic retention time (HRT), sample collection was assisted by the water utilities staff. On each sampling occasion, time separated triplicate samples were collected for the influent (3 × 100 mL) in sterile labelled glass bottles after the grit removal screens. Triplicate samples of treated effluent (3 × 10 L) were also collected in sterile labelled carboy containers (Nalgene). All samples were immediately transferred to the laboratory for further processing in cool boxes containing freezer blocks.

Figure C-10-1. Sampling sites at the Oxley Creek Wastewater Treatment Plant.

Sample storage, shipment and processing

Influent and effluent samples collected from each site were processed at the CSIRO laboratories in Brisbane, Perth, Melbourne and Hobart for the enumeration of *E. coli* with Colilert Quanti-tray 2000 (IDEXX Westbrook, Maine) technique. Samples for the detection of enteric virus and *Cryptosporidium* oocysts in the effluent were concentrated with Hemoflow FX80 dialysis filters (Fresenius Medical Care, Lexington, MA, USA) in the respective laboratories. Concentrated effluent and influent wastewater samples were shipped to the Brisbane laboratory on ice via overnight courier. Further processing of samples was carried out at the Brisbane laboratory.

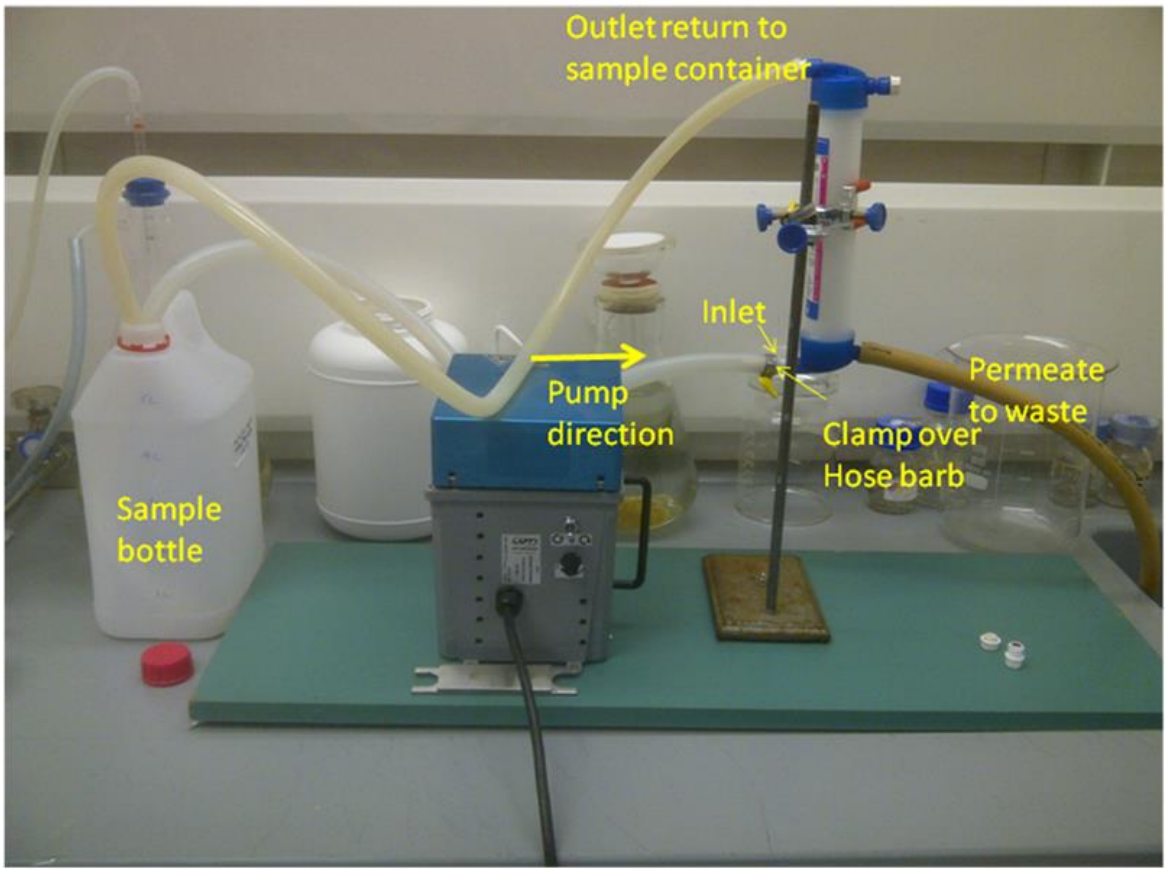

Figure C-10-2. Effluent sample concentration setup using Hemoflow FX80 dialysis filters.

Sample quality assurance and quality control

Samples were collected and processed according to the best practices as out lined in the Standard Methods for Collection and Analysis of Water and Wastewater (Federation WE, 2005). In brief, as outline in the previous section, samples were kept cold and processed within 24 hours of collection. Where, samples are shipped they were in boxes with ice block in overnight couriers and for some shipping runs temperature loggers inside the package were used to determine the range of temperature during shipment with an aim to make adjustment to the ice packs requirement as required. QA/QC procedures are listed in detail in the methodology section (section 5.1 and section 6.1). In particular, for the PCR based detection methods detailed information is provided in the methodology.

Standard methods for the concentration of sample were used referenced for each pathogen/indicator. Recovery rates of these methods are also provided in the subsequent sections. Processed samples involving DNA extraction were stored at -80°C to minimise sample degradation. To reduce sample to sample variation, batch processing for DNA extraction and qPCR runs were undertaken. Negative and positive controls were always added to the qPCR runs validated qPCR methods were used.

Bacterial Analysis

Methodology

In this section brief, information on the processing of influent and effluent samples collected is provided.

Total coliforms and *E. coli* quantification

Total coliforms and *E. coli* numbers in the influent and effluent wastewater samples were enumerated using the Colilert Quanti-tray 2000 (IDEXX Westbrook, Maine) technique. A 100-µl aliquot of primary wastewater was added to 99.9 ml of sterile water (1:1,000 dilution) in sterile phosphate buffered (PBS) to set up Quanti-trays for each of the replicate samples according to manufacturer's instructions. Further dilutions of primary wastewater were made as appropriate. Similarly, 100 or 1000 µL sample of treated effluent was used for enumeration of coliforms and *E. coli* numbers. Plates sealed with IDEXX Quanti-Tray Sealer were then incubated at 37 °C for 18 hours. The wells showing chromogenicity (yellow) were counted as total coliforms. The yellow wells fluorescing under UV (365 nm) illumination were counted positive for *E. coli*.

The MPN number obtained from the Colilert Quanti-tray method for both influent and effluent samples were then Log_{10} transformed for determining LRVs.

Quality control and quality assurance

Colilert Quanti-trays were set up for all three replicate samples as mentioned above and on each occasion negative controls were set up with sterile water. Plates were scored after 18 h of incubation at 37 °C.

Statistical analysis

All *E. coli* data from three sample replicates was $\log_{10}$ transformed prior to descriptive statistical analysis. Log removal values (LRVs) were derived as outlined in the section 4.6.

Viral Analysis

Wastewater concentration for detection of enteric viruses

Primary wastewater samples were concentrated and purified with Amicon® Ultra (Ultracel - MWCO 50 kDac) centrifugal filters (Millipore, Billerica, MA) in triplicate. The Ultracel centrifugation unit equipped with an ultrafiltration membrane, which can achieve high virus concentration and purification efficiency as demonstrated in our previous study (Sidhu *et al*., 2013b) . Briefly, 10 mL influent sample was centrifuged at 1,500 rpm for 5 minutes at 4 °C and the resulting supernatant was added to Amicon® column, and centrifuged at 4,500 rpm for 10 min to obtain a final volume of 1,000 µL and frozen at -80 °C for DNA extraction and downstream qPCR in a batch.

Concentration of enteric viruses in the effluent samples was carried out within ~24 h of collection by hollow-fibber ultrafilters, Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) as previously described (Hill *et al*., 2005). Briefly, the effluent 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, tubes were cleaned and disinfected by soaking in 1% bleach followed by washing with distilled water and then sterilized by autoclaving. At the end of the concentration process, the flow of water was reversed by running pump in an anti-clockwise direction to remove concentrated water sample in the filter and tubing (Sidhu *et al*., 2013a; Toze *et al*., 2012). Then pressurised air was passed through the filter cartridge from the top to recover as much water as possible. The sample (approximately 100 mL) was centrifuged at 4,500 rpm for 5 minutes at 4°C and pellets kept for protozoa pathogen analysis as outlined below. The resulting supernatant was further concentrated by JumboSep with 100 K MWCO filters (Pall, Australia) to a final concentration of approximately 5mL (Toze *et al*., 2012). The samples were then aliquoted and frozen at -80 °C for batch processing of enteric viruses.

DNA extraction

Concentrated primary and treated wastewater samples were subjected to DNA extraction for the quantification of enteric viruses. Enteric DNA was extracted from each of the concentrated primary and treated wastewater samples (200 µL) using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc.,

Valencia, CA) as per manufacturer instructions. The extracted DNA samples were stored at -80°C for qPCR analysis.

Enteric virus quantification

Enteric virus numbers in the influent and effluent samples were quantified using the previously published primer sequences and amplification conditions for qPCR assays used in this study, and are shown in Table 6-1. Human adenoviruses and *Microviridae* qPCR assays were performed in 20 µL reaction mixtures using SsoAdvanced™ Universal Probe Supermix (Bio-Rad Laboratories). The qPCR mixtures contained 10 µL of Supermix, 250 nM of each primer and 250 nM of probe (for human adenoviruses) and 10 µL of Supermix, 400 nM of each primer and 400 nM of probe (for *Microviridae*) and 3 µL of template DNA. The human polyomavirus qPCR mixtures contained 12.5 µL of Supermix, 250 nM of each primer, 250 nM of probe and 3 µL of template DNA. Quantitative PCR reactions were performed on Bio-Rad CFX96 (Bio-Rad, Hercules, CA, USA) using iQ Supermix (Bio-Rad, Hercules, CA). Each qPCR reaction mixture (25µL) contained 12.5 µL of SuperMix, 200-400 nM of each primer and corresponding probe, and 3 µL of template DNA. Bovine serum albumin (BSA) was added to each reaction mixture to a final concentration of 0.2 µg µL-1 to relieve PCR inhibition (Kreader, 1996). For each PCR experiment, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included.

Table C 2. Primers, probes and thermal cycling condition for qPCR assays.

Target	Primer sequence (5'-3')	Cycling parameters	Reference
Adenovirus (HAdv)	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	10 min at 95°C, 50 cycles of 20 s at 95°C and 20 s at 60°C and 20s at 72°C	(Heim *et al*., 2003)
Human Polyomavirus (HPyv)	F: AGT CTT TAG GGT CTT CTA CCT TT R: GGT GCC AAC CTA TGG AAC AG P: FAM-TCA TCA CTG GCA AAC AT- BHQ1	10 min at 95°C, 50 cycles of 20 s at 95°C and 20 s at 55°C and 60s at 60°C	(McQuaig *et al*., 2009)
Somatic coliphage *(Microviridae)*	F: TAC CCT CGC TTT CCT GC R: GCG CCT TCC ATG ATG AG P: FAM-CAT TGC TTA TTA TGT TCA TCC CG-TAMRA	10 min at 95°C, 50 cycles of 20 s at 95°C and 20 s at 61°C and 20s at 72°C	Lee, 2009

Abbreviations: F: Forward primer; R: Reverse primer; P: Probe, Single-letter code: D= G+A+T; R= A+G.

Cloning of target gene sequences and standard curves

DNA isolated from the human adenovirus strain 41 (ATCC VR-930), human polyomavirus strain Bkv (ATCC 45024) and *Microviridae* prototype coliphage *φX 174* (ATCC 13706-B1) was PCR amplified and purified using a QIAquick PCR Purification Kit (Qiagen) followed by cloning into a pGEM-T Easy Vector system II (Promega, Madison, Wis., USA). Recombinant plasmids with corresponding inserts were

purified using a Plasmid Mini Kit (Qiagen). Standards were prepared from the plasmid DNA. 10-fold dilution ranging from 1×10^6-1×10^0 copies per µL of plasmid DNA standards were prepared and stored at -20 °C. A 3-µL template from each dilution was used to prepare standard curve for each qPCR assay.

qPCR performance characteristics

qPCR standards were analysed in order to determine the amplification efficiencies (E) and the correlation coefficient (r^2). The repeatability (intra-assay agreement) and reproducibility (inter-assay agreement) of the each qPCR assay were assessed by determining the precent coefficient of variation (CV). The CV values were calculated from the Cq values of each standard ranging from 3×10^6- 3×10^0 gene copies. The intra-assay repeatability was calculated based on the quantification cycle (Cq) values by testing each dilution 10 times in the same qPCR run. The inter-assay reproducibility was calculated based on the Cq values by testing each standard on five different days. The qPCR lower limit of quantification (LLOQ) was also determined from the Cq values obtained for each standard. The lowest amount of diluted standards detected in 100% triplicate assays was considered qPCR LLOQ. All qPCR reactions were performed in triplicate. For each qPCR assay, a negative (sterile water) control was included.

Recovery efficiency of FX80 filters

In order to determine the recovery rate of the Hemoflow FX80 filters treated effluent samples were spiked with known number of adenovirus and numbers were compared with the un-seeded samples (background). Briefly, treated effluent samples (5x20L) were collected from Oxley Creek WWTPs in Brisbane, three samples were spiked with known numbers of adenovirus (10^3 mL^{-1}) and two non-spiked (background). After thoroughly mixing spiked containers samples were passed through the FX80 as previously described and the sample was concentrated to a final volume of 100 mL. This was followed by further concentration by JumboSep with 100 K MWCO filters (Pall, Australia) to a final concentration of approximately 5mL. Then DNA was extracted from a portion of sample (200 µL) using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) followed by qPCR.

The recovery efficiency of the HFUFS was calculated as follows:

% Recovery = (Total PDU of adenovirus in the retentate ⁄Initial PDU of adenovirus in the 20 L

inoculated) X 100.

Protozoan Analysis

Sample concentration

Primary wastewater (influent) samples (30mL) were centrifuged at 4,750 rpm for 20 minutes at 4 °C, the supernatant was then decanted and the pellet stored at -20 °C for protozoa qPCR analysis. Collected treated effluent (10L) samples were concentrated within 24 h of collection by a HFUF, using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) as previously described

by Hill *et al.,* (2005). Resulting 100-150 mL concentrated samples was centrifuged at 47500 rpm for 20 minutes to obtain a pellet which was suspended in 1 mL of MilliQ water. Please see above section 6.1.1. All samples were labelled and stored at -80°C for batch processing. Nucleic acid was concentrated from 150 µL of influent sample and 200 µL of effluent samples in a batch.

Nucleic acid extraction

For protozoa qPCR analysis, DNA was extracted from the pellets (influent and effluent) samples, obtained using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). Briefly, 150 µL of influent and 200 µL of effluent sample was mixed with 180 µL of buffer ATL and subjected to 10 one minute freeze thaw cycles in liquid nitrogen and water bath at 95°C. Proteinase K (20 µL) was then added to the sample and vortexed for 30 seconds, followed by incubation at 56°C for five minutes. The manufacturer-recommended protocol was then followed for the DNA extraction. The extracted DNA was stored at -80 °C until required.

Quantification of *Cryptosporidium*

qPCR assay was performed using previously published primer set, and a probes (Table 7-1). The *Cryptosporidium* spp. primer set used in this study targets 18S rRNA genes detects both human specific species (*C. hominis* and *C. parvum*) and animal specific species (*C. canis*, *C. muris*, *C. andersoni* and others) as described previously (Jothikumar *et al*., 2008). Whereas, the second primer set which target *Cryptosporidium* oocyst wall protein (COWP) gene is specific for *C. parvum*.

qPCR amplifications were performed in a 20-µL reaction mixture using Sso Fast™ Probes Supermix (Bio-Rad Laboratories, CA). The qPCR mixtures contained 10 µL of Supermix, 300 nM of each primer and 300 nM of probe and 3 µL of template DNA. Standards (positive controls) and sterile water (negative controls) were included in each qPCR run. All qPCR reactions were performed in triplicate. qPCR assays were performed using a Bio-Rad® CFX96 thermal cycler.

Table C 3. Primers, probes and thermal cycling condition for qPCR assays.

Target	Primer sequence (5'-3')	Cycling parameters	Reference
Cryptosporidium spp.	F: ATGACGGGTAACGGGGAAT R: CCAATTACAAAACCAAAAA GTCC P: FAM- CGCGCCTGCTGCCTTCCTTAGATG -BHQ1	10 min at 95°C, 50 cycles of 20 s at 95°C and 30 s at 55°C and 20s at 72°C	Jothikumar et a., 2008
Cryptosporidium parvum	F: CAAATTGATACCGTTTGTCCTTCTG R: GGCATGTCGATTCTAATTCAGCT P: HEX-TGCCATACATTGTTGTCCTGACAAATTGAAT-BHQ1	10 min at 95°C, 45 cycles of 20 s at 95°C and 30 s at 60°C and 30s at 60°C	(Guy *et al.*, 2003)

Abbreviations: F: Forward primer; R: Reverse primer; P: Probe, Single-letter code: D= G+A+T; R= A+G..

Preparation of qPCR standard curve

Amplicon for the detection and quantification of *Cryptosporidium spp.* and *Cryptosporidium parvum* was custom synthesised by IDT custom gene synthesis (Coralville, IA, USA). Purified recombinant plasmids containing target amplicon was produced by Integrated DNA Technologies (pIDTSmart with ampicillin; IDT), and cloned into a vector followed by plasmid extraction (Coralville, IA, USA). A 10-fold dilution ranging from 1×10^6 to 1×10^0 copies per µL of DNA extract was prepared from the synthesized plasmid DNA. A 3-uL template from each dilution was used to prepare a standard curve. For each standard, the genomic copies were plotted against the cycle number at which the fluorescence signal increased above the quantification cycle value (C_q value). The amplification efficiency (E) was determined by analysis of the standards and was estimated from the slope of the standard curve as $E = 10^{-1/slope}$.

PCR reproducibility and limit of detection qPCR performance characteristics

qPCR standard was analysed in order to determine the amplification efficiencies (E) and the correlation coefficient (r^2). The repeatability (intra-assay agreement) and reproducibility (inter-assay agreement) of the each qPCR assay were assessed by determining the precent coefficient of variation (CV) (Bustin *et al.,* 2009). The CV values were calculated from the Cq values of each standard ranging from 3×10^6- 3×10^0 gene copies. The intra-assay repeatability was calculated based on the Cq values by testing each dilution 10 times in the same qPCR run. The inter-assay reproducibility was calculated based on the Cq values by testing each standard on five different days. The qPCR lower limit of quantification (LLOQ) was also determined from the Cq values obtained for each standard. The lowest amount of diluted standards detected in 100% triplicate assays was considered qPCR LLOQ. All qPCR reactions were performed in triplicate. For each qPCR assay, a negative (sterile water) control was included. To minimize qPCR contamination, DNA extraction and qPCR setup were performed in separate laboratories.

Appendix D. PCA and PLS-DA analysis of data from WWTPs

Beenyup WWTP

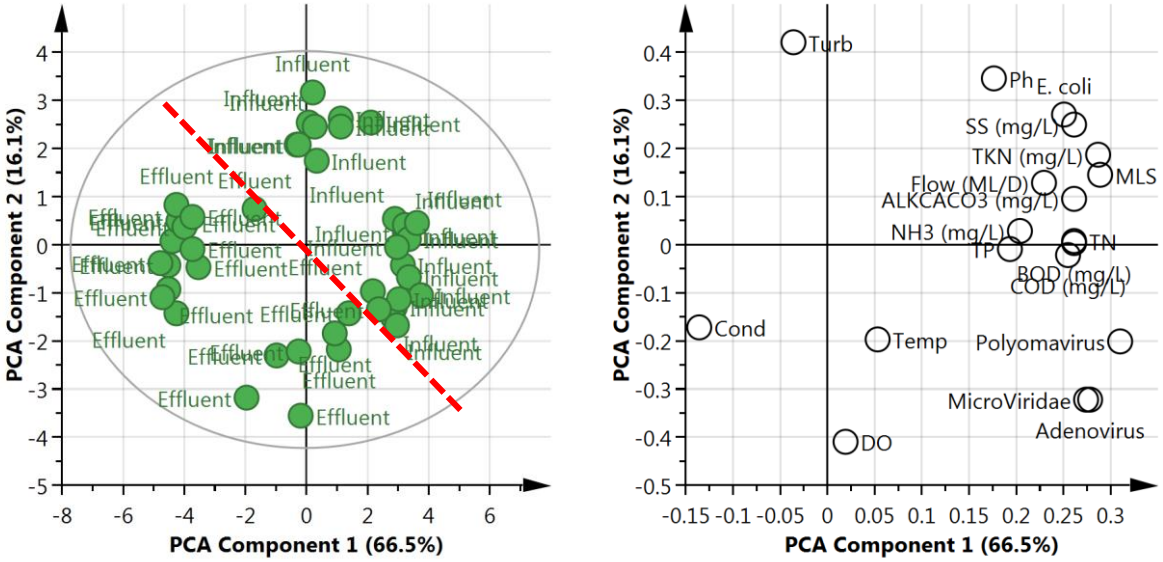

Figure D.1. PCA Score Scatter Plot and Loading Scatter Plot comparing Beenyup influent and effluent samples.

The red dotted line highlights the separation within the data. Note: the PCA eclipse (solid line) represents the 95% confidence interval, with R^2X and Q^2(cum) values of 0.825 and 0.650, respectively.
Within group analysis, PLS-DA applied with variables with a 50% cut off limit excluded.

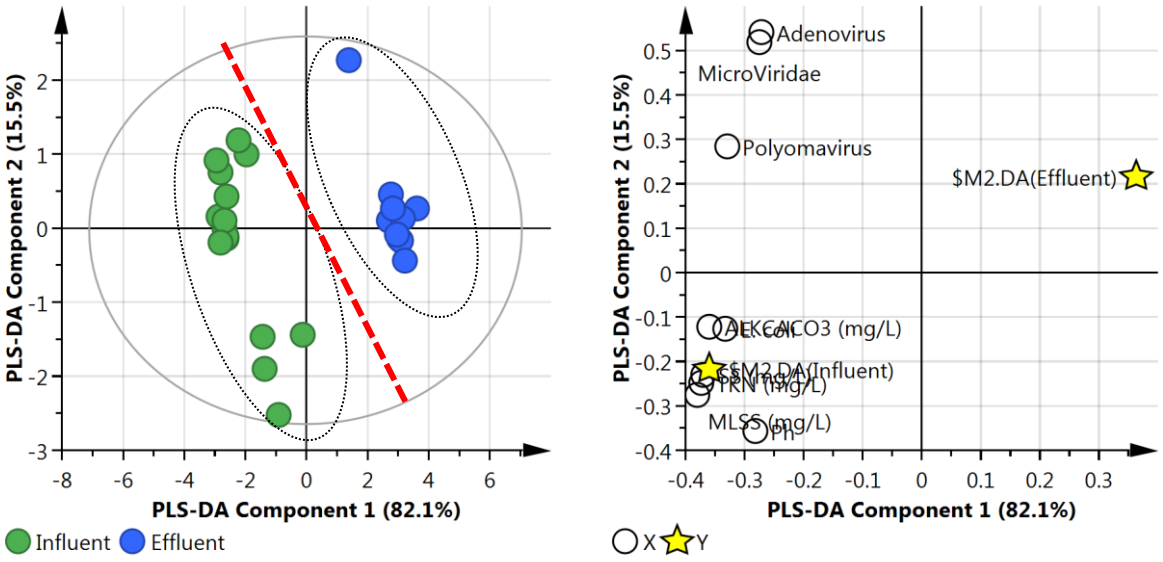

Figure D.2. PLS-DA Score Scatter plot and PLS-DA Loading Scatter plot of the Beenyup influent and effluent data.

Note: the PLS-DA eclipse (grey solid line) represents the 95% confidence interval. The red dotted line highlights the separation within the data. The black dotted highlights the influent and effluent groupings. PLS-DA R^2X, R^2Y and Q^2 values are 0.961, 0.991 and 0.980 respectively.

Boneo WWTP

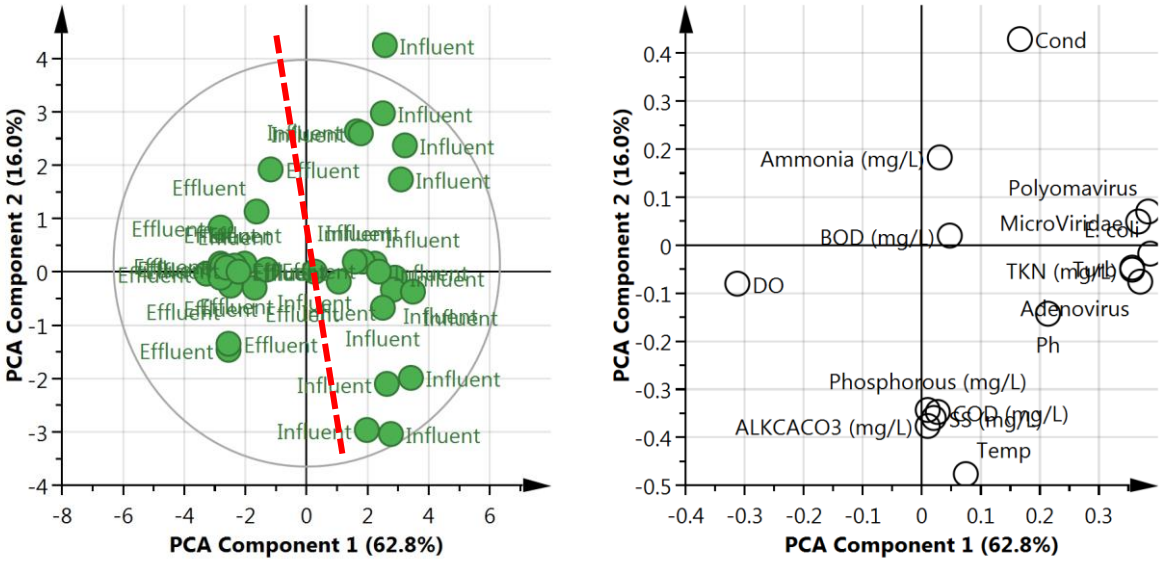

Figure D.3. PCA Score Scatter Plot and Loading Scatter Plot comparing Boneo influent and effluent samples.

The red dotted line highlights the separation within the data. Note: the PCA eclipse (solid line) represents the 95% confidence interval, with R2X and Q2(cum) values of 0.788 and 0.549, respectively.

Within group analysis, PLS-DA applied with variables with a 50% cut off limit excluded.

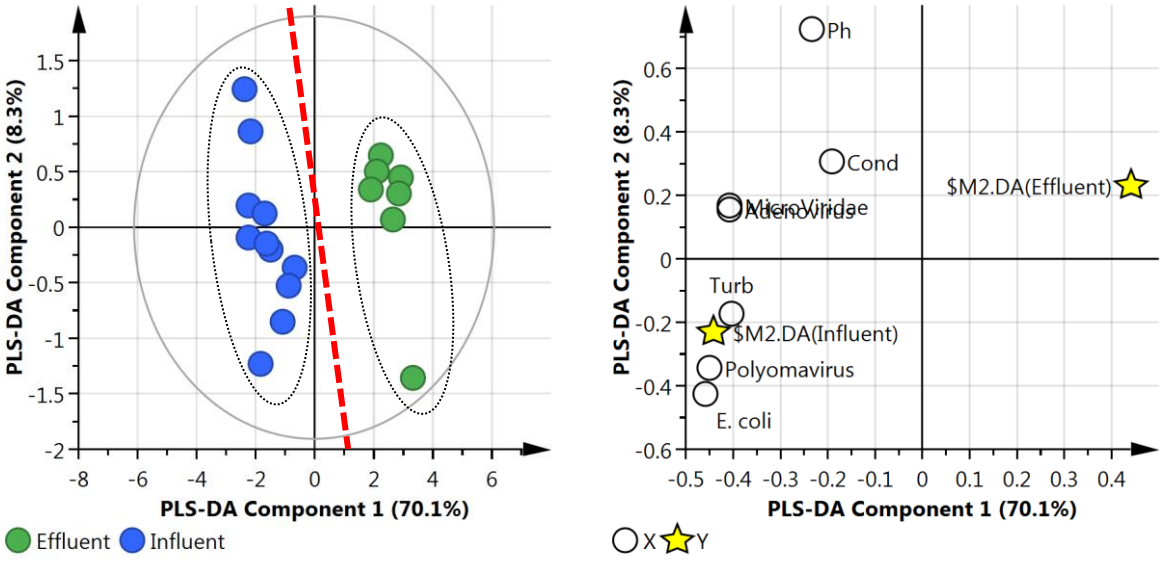

Figure D.4. PLS-DA Score Scatter plot and PLS-DA Loading Scatter plot of Boneo influent and effluent data.

Note: the PLS-DA eclipse (grey solid line) represents the 95% confidence interval. The red dotted line highlights the separation within the data. The black dotted highlights the influent and effluent groupings. PLS-DA R2X, R2Y and Q2 values are 0.784, 0.965 and 0.934 respectively.

Rosny WWTP

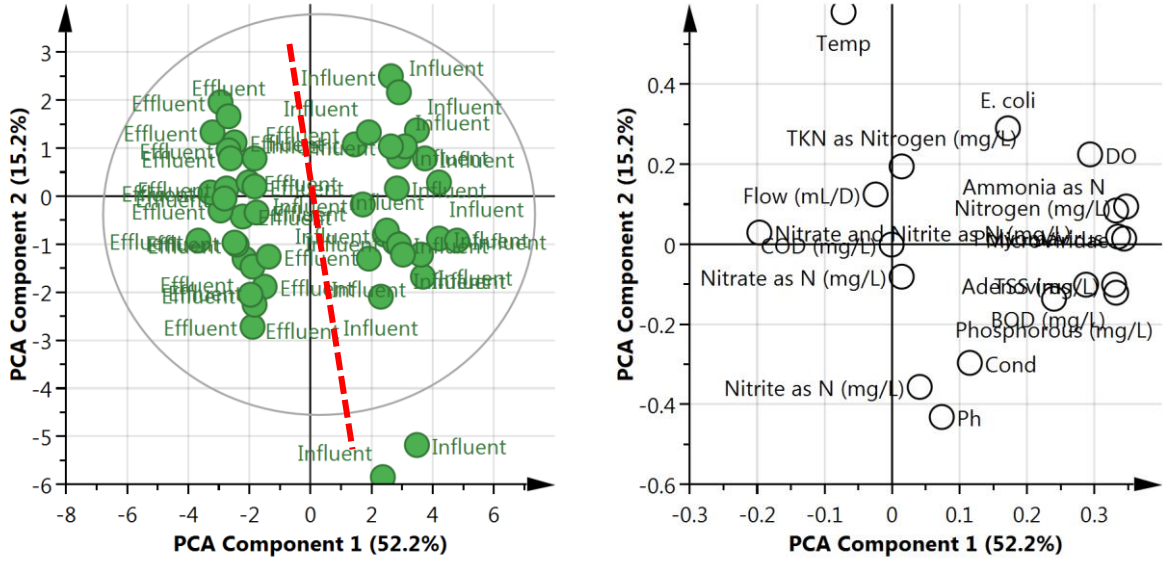

Figure D.5. PCA Score Scatter Plot and Loading Scatter Plot comparing Rosny influent and effluent samples.

The red dotted line highlights the separation within the data. Note: the PCA eclipse (solid line) represents the 95% confidence interval, with R2X and Q2(cum) values of 0.674 and 0.420, respectively.

Within group analysis, PLS-DA applied with variables with a 50% cut off limit excluded.

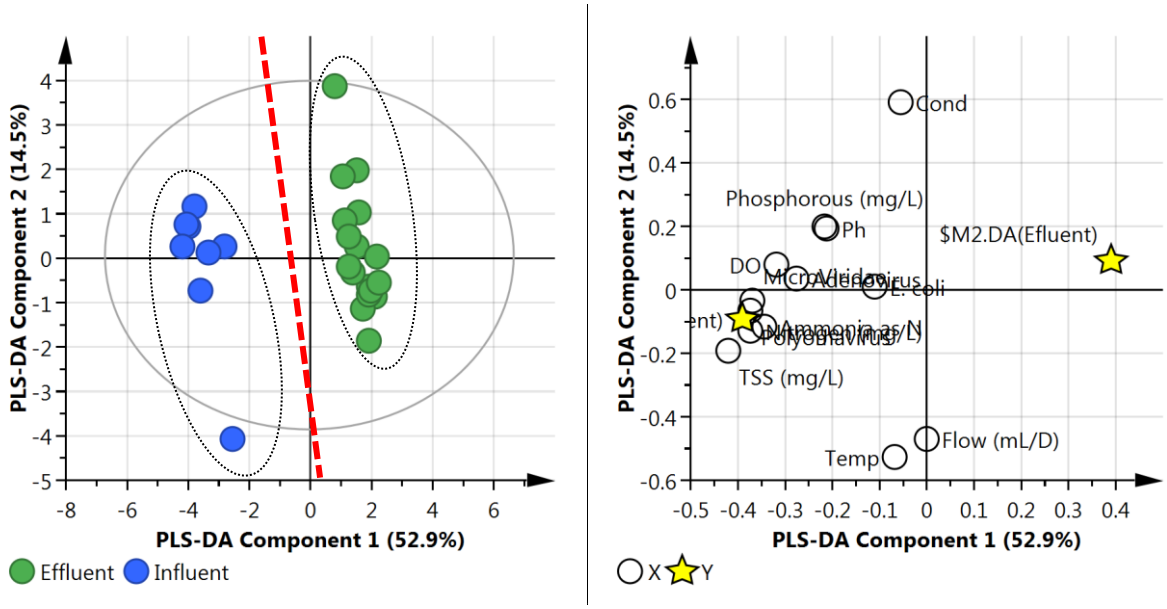

Figure D.6. PLS-DA Score Scatter plot and PLS-DA Loading Scatter plot of Rosny influent and effluent data.

Note: the PLS-DA eclipse (grey solid line) represents the 95% confidence interval. The red dotted line highlights the separation within the data. The black dotted highlights the influent and effluent groupings. PLS-DA R2X, R2Y and Q2 values are 0.674, 0.983 and 0.958 respectively.

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CONTACT US

t 1300 363 400
+61 3 9545 2176
e enquiries@csiro.au
w www.csiro.au

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Land and Water Flagship
Jatinder Sidhu
t +61 7 3833 5576
e Jatinder.Sidhu@csiro.au

w www.csiro.au/LWF