Do Particles in Wastewater Protect Pathogens from Disinfection

by

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Declaration

I certify that this work does not contain any material which has been accepted for the award of any degree or diploma, in any University or tertiary institution; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

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Charndeep Singh Chahal

Abstract

Ultraviolet (UV) radiation and chlorination are widely used in drinking water and recycled water disinfection. Both organic and inorganic particles can limit disinfection efficiency by protecting pathogens in various ways, such as physical shielding and increasing disinfectant demand. The negative impact of particles is well documented, and the particle size required to offer protection differs for and is dependent on each pathogen type. This increases the disinfectant demand and can require additional treatment, such as filtration, thereby increasing the treatment costs. The extent of association of pathogens (bacteria, viruses or protozoans) with the particles vary widely and is dependent on factors such as the nature (organic or inorganic) and size of particles, wastewater environment and treatment type. A better understanding of the various pathogen associations and their impact on disinfection kinetics is required to devise alternate cost-effective treatment options. This thesis investigates the association behaviour of indicator organisms and the effect of particles on UV and chlorine disinfection in treated wastewater effluent from South Australian and Victorian wastewater treatment plants.

Scanning electron microscopy coupled with elemental analyser was used to analyse the shape, structure and composition of various particles. Additional parameters, such as nutrients, solids, and particle surface charge, were monitored to characterise the water quality and particulate environment. The surrogate organisms used in this study were *E. coli*, FRNA bacteriophage (or MS-2 phage) and anaerobic bacterial spores, and the fraction of organisms associated with particles was determined using centrifugation, serial filtration and homogenisation (for spores and *E. coli* only). The association behaviour of *C. parvum* oocysts was also studied using the same techniques and compared with anaerobic spores. Disinfection experiments using UV irradiation and chlorination were conducted to determine if there were any differences in the inactivation of particle-associated and free organisms, or if particles interfered in disinfection.

Unique particulate environments displaying specific elemental, size and charge characteristics for particles in the different effluents were identified. A new SEM protocol was developed to semi-quantitative the contribution of biological (organic) and inorganic particles in samples. Adelaide lagoon effluent had the highest monthly average number of particles in all the size classes, which were mostly inorganic in nature (70 %) compared to the Adelaide secondary effluent and Melbourne lagoon effluent. Adelaide lagoon effluent had higher numbers of larger particles than the other two samples. *E. coli* and anaerobic spores were associated with 1.2 and 10 µm particles respectively. FRNA bacteriophage did not show any association with particles. Anaerobic spores were not ideal surrogates for *C. parvum* oocysts; the oocysts showed some evidence of particle association, but the extend of removal due to potential particle association was much lower than that observed for spores. The presence of particles negatively impacted disinfection efficiency.

I conclude that particle association is greatly dependent on the characteristics of the pathogen / surrogate, particle size, and potentially other factors that impact particle charge and surface interactions. Homogenisation and filtration can be used as effective methods for particle removal or particle / pathogen disaggregation. For the doses tested, particles negatively impacted the efficiency of UV disinfection, and filtration greatly increased the efficiency disinfection by removing particles. Our findings will help selective designing of effective filtration methods for pathogen removal.

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Abbreviations

ANOVA	Analysis of variance
ARB	Antibiotic resistant bacteria
ASP	Activated sludge process
ATCC	American type cell culture
AWQC	Australian Water Quality Centre
BWWTP	Bolivar wastewater treatment plant
BOD	Biological oxygen demand
CBF	Chemical-biological flocculation
CDEO	Conductive-diamond electrochemical oxidation
CEPT	Chemically enhanced primary treatment process
CFU	Colony forming units
COD	Chemical oxygen demand
CT	Chlorine Contact time
DAFF	Dissolved air floatation and filtration
DALY	Disability adjusted life year
DLVO	Derjaguin, Landau, Verwey and Overbeck
DOC	Dissolved organic carbon
DOM	Dissolved organic matter

EDX	Energy dispersive X-ray
ELS	Electrophoretic light scattering
EPS	Extracellular polymeric substances
FISH	Fluorescent in situ hybridisation
F	Filtrate
HCl	Hydrochloric acid
HRAP	High rate algal pond
HSWWTP	High salinity wastewater treatment plant
LISST	Laser in situ scattering and transmissometry
MI	MuGal and IBDG
NGS	Next generation sequencing
NH_3	Ammonia
NO_2^-	Nitrite
NO_3^-	Nitrate
NTU	Nephelometric turbidity units
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PSD	Particle size distribution

RAS	Return activated sludge
SA	South Australia
SEM	Scanning electron microscope
SS	Suspended solids
TCCA	Trichloroisocyanuric acid
TOC	Total organic carbon
Total P	Total phosphate
TSA	Tryptic soy agar
TSC	Tryptose sulphite cycloserine
TSS	Total suspended solids
UV	Ultraviolet
VS	Volatile solids
WSP	Waste stabilisation pond
WWWTP	Western wastewater treatment plant
WWTP	Wastewater treatment plant
25W	25 West
55E	55 East
115E	115 East

Chapter 1: Introduction:

Two disinfection processes commonly used to treat wastewater are ultraviolet irradiation and chlorination. Disinfection guidelines exist for potable water which recommend low turbidity and therefore low concentrations of particles. As drinking water with turbidity ≥ 5 is not fit for purpose, but recycled water can contain higher concentrations of particles which can limit disinfection efficiency (Emerick et al., 2000, Templeton et al., 2008). During chlorination or UV disinfection of drinking water or potable water, a linear relationship between dose and rate of inactivation of pathogen exists, however, in the case of recycled water the relationship may be non-linear. This deviation from first order kinetics is known as tailing and is thought to be caused by protection afforded to the pathogens by particles in the water (Amoah et al., 2005, Torres-Palma et al., 2017). Suspended particles can offer various modes of protection, such as scattering or attenuation of ultraviolet (UV) light, shielding particle-embedded pathogens from light or chemical disinfectant or by absorption of the disinfectant. Processes such as filtration can achieve acceptable levels of disinfection, but at an increased cost. Many microorganisms such as coliform bacteria, *Cryptosporidium* spp. and viruses can be associated with particles and this association may allow them to survive disinfection processes and cause a health hazard. The extent of association of pathogens with particles varies widely and is dependent on factors such as the nature and size of particles, the wastewater chemistry (parameters such as pH, nutrients, solids composition etc) and treatment process (Amoah et al., 2005, Madge and Jensen, 2006a, Templeton et al., 2005). An Improved understanding of the mechanisms which promote attach of microorganism to particles will enable us to develop cost-effective treatment methods, thereby producing safe recycled water with minimal by-products. The major aim of this study was to understand and identify the association behaviour of selected pathogens and surrogates and analyse their effect on the disinfection processes. The specific objects of this project were:

- To understand and review the current state of knowledge regarding wastewater treatment, particle changes during various treatment processes and the mechanisms of particle-pathogen interactions (Chapter 1).
- To characterize and quantify (various properties including physiochemical properties, size, shape, structure and charge) the various particles in wastewater effluents from two geographically separated treatment plants utilising similar wastewater treatment processes (Chapter 3).
- To investigate the particle association behaviour of pathogen surrogate organisms (*E. coli*, FRNA bacteriophage) for pathogens (Chapter 4)
- To examine the particle association of oocysts of the protozoan pathogen *Cryptosporidium parvum* and spores of *Clostridium* spp, as a potential surrogate for the oocysts (Chapter 5).
- To determine the impact of particles and particle association on the chlorine or UV disinfection kinetics for pathogen surrogates and *C. parvum* oocysts (Chapter 6).

1.1 Synopsis

This research is focused on identifying and validating the impacts of particle-pathogen associations on the disinfection of various microorganisms in the treated wastewater effluents. The research is conducted in three main sections: 1) characterisation of the nature of particles in wastewater effluents, 2) investigation of the particle association behaviour of selected surrogate microorganisms and 3) measurement of the impact of particles on disinfection processes. The outcomes of the studies are described in the following six (6) chapters.

Chapter 1 provides a review of the prevalence of pathogens and pathogen surrogates in the wastewater; wastewater treatment processes commonly used in Australia; the nature and types of particles found in wastewater and the changes in the type (size and nature) of particles following different stages of treatment; the particle association behaviour of various pathogens and the impact of association on disinfection. The review - examines the major knowledge gaps regarding the nature of particles in the wastewater and the association behaviour of pathogens with these particles.

Chapter 3 addresses the first objective of this study, to enumerate and characterise the various particles in different types of wastewater effluent. Particles were analysed in samples collected from the Bolivar wastewater treatment plant in Adelaide, South Australia and the Western treatment plant in Melbourne, Victoria. The samples were collected from points upstream and downstream of stabilisation lagoons from Bolivar and downstream of the 55E lagoon system at the Western treatment plant. The study characterised the major particle types, particle size, composition and charge, and identified differences related to location and stages of treatment processes.

Chapter 4 will addressed the second major objective of this study; to determine the partitioning behaviour of indicator microorganisms for bacteria and viruses (*E. coli* and FRNA bacteriophage) in different types of wastewater. The study employed two important fractionation techniques; centrifugation and serial filtration to determine which indicator microorganisms were associated with which particle size.

Chapter 5 continue exploring the second objective by analysing the association behaviour of *C. parvum* by using anaerobic bacterial spores as a potential surrogate for *C. parvum* oocysts. The association behaviour of *C. parvum* in wastewater was investigated by using three different partitioning techniques; serial filtration, centrifugation and homogenisation. The association

behaviour of anaerobic bacterial spores was compared to *C. parvum* oocysts and the study was therefore able to provide important information on the behaviour of oocysts in highly turbid wastewater.

Chapter 6 addressed the major objective of this study; to determine the impact of particle-pathogen associations on the disinfection processes. The study compared the effect of particles on the inactivation kinetics of microbes exposed to UV irradiation and chlorination in the absence or presence of particles. Particle-associated microbes were separated by filtration and by homogenisation.

The tested wastewater treatment plants utilise similar yet distinct treatment processes, which may cause differences between the two wastewaters. This research programme will lead to an improved understanding of particle- pathogen behaviour with the potential to be broadly applied by utilities that use lagoons in their treatment trains particularly when disinfecting high turbidity wastewater or devising alternative cost-effective treatment options. My results will inform the development of improved disinfection processes which will maximise pathogen inactivation by minimising disinfection by-product formation and energy usage.

Publications associated with this Thesis

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Chapter 1.2: Pathogen and Particle Associations in Wastewater: Significance and Implications for Treatment and Disinfection Processes

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1.2.1 Abstract

Disinfection guidelines exist for inactivation of pathogens in potable water and recycled water, but wastewater with high numbers of particles can be more difficult to disinfect, making compliance with the guidelines problematic. Disinfection guidelines specify that drinking water with turbidity ≥ 1 Nephelometric Turbidity Units (NTU) is not suitable for disinfection and therefore not fit for purpose. Treated wastewater typically has higher turbidity(1 – 10 NTU for secondary treated effluent). Two processes widely used for disinfecting wastewater are chlorination and ultraviolet radiation (UV). In both cases, particles in wastewater can interfere with disinfection and can significantly increase treatment costs by increasing operational expenditure (chemical demand, power consumption) or infrastructure costs by requiring additional treatment processes to achieve the required levels of pathogen inactivation. Many microorganisms (viruses, bacteria, protozoans) associate with particles, which can allow them to survive disinfection processes and cause a health hazard. Improved understanding of this association may enable development of cost-effective treatment, which will become increasingly important as indirect and direct potable reuse of wastewater water becomes more widespread in both developed and developing countries. This review provides an overview of wastewater and associated treatment processes, the pathogens in wastewater, the nature of particles in wastewater and how they interact with pathogens, and how particles can impact disinfection processes.

1.2.2 Introduction

Although water is abundant and covers 75% of the Earth's surface, most of the freshwater "is available at the wrong place, at the wrong time or with the wrong quality" (Falkenmark and Lindh, 1974). The availability of freshwater is greatly impacted by global climatic changes and increases in human population, urbanization and pollution (Vörösmarty et al., 2010), to the extent that the United Nations predicts that by 2050 more than half of the world's population will be living in water deficient countries (Pigram, 2007).

Australia is considered to be the driest continent after Antarctica, with less than 1% of the world's available fresh water (Pigram, 2007). Increasing population growth and demand, combined with reductions in available freshwater due to climate change and drought (specifically the Millennium drought from 1995 – 2009), have driven developments in wastewater recycling and water management within Australia (Lazarova et al., 2001, Greenway, 2005, Moe and Rheingans, 2006, Pigram, 2007, Chiew et al., 2011). Similar challenges have affected other countries, including the U.S.A, Western Europe and Israel, leading to an increased focus on wastewater reuse internationally (Wade Miller, 2006). The State of California has been impacted particularly by water shortages, and an extreme 5 year drought prompted local water resource authorities to make regulatory provisions for direct and indirect potable reuse of wastewater (ORDER-WQ-2016-0068-DDW, 2016). In Australia, the percentage of wastewater reuse varies in different states, but overall has increased from 2001-2015 (Table 1.1). The non-potable reuse of treated wastewater includes irrigation of crops and parklands, dual reticulation within domestic and commercial buildings (Moe and Rheingans, 2006), recreation and mining (Dillon, 2000). Wastewater can also be treated to high standards for direct or indirect potable reuse applications, such as the supplementation of surface or ground waters for drinking (Moe and Rheingans, 2006).

Table 1.1 Wastewater recycling rates

Table 1.1 Wastewater recycling rates. Comparison of the rates of wastewater recycling (expressed as a percentage of total wastewater produced) in major Australian cities during 2001-15

Capital City	Recycling %					
	2001-2	2005-6	2007-8	2009-10	2012-13	2014-15
Adelaide	11.1	18.1	30.6	28.7	31.3	33.7
Melbourne	2.0	14.3	23.2	22.8	16.1	15.8
Perth	3.3	5.3	6.4	6.1	8	NA
Sydney	2.3	3.5	4.4	7.3	8	NA[a]
Total	3.3	8.4	11.3	16.8	17	17

a - Information is not available or could not be calculated from available data

Irrespective of the intended use, wastewater must be treated sufficiently so that it is fit for purpose and will not adversely affect human health or the environment. The Australian Guidelines for Wastewater Recycling (AGWR) use a risk management framework incorporating hazard analysis and critical control point principles to identify and manage human or environmental health hazards in wastewater (AGWR, 2006). Hazards can be managed by limiting entry into wastewater destined for reuse (e.g. selective harvesting of wastewater sources to avoid high risk contaminants), by the use of treatment processes to remove or reduce microbial or chemical hazards, and by preventative measures at the point of use to limit exposure to any microbial or chemical hazards.

The AGWR place particular emphasis on the control of microbial contaminants to protect human health and uses the measure of disability adjusted life years (DALYs), in combination with end use and exposure scenarios, as the basis for setting health-based treatment targets for wastewater (AGWR, 2006). In Australia, regulatory authorities have deemed that the tolerable risk from any given pathogen in reuse water is no more than 1 microDALY per person per year. The removal or inactivation of pathogens by various treatment and disinfection processes can be impaired by particles in the wastewater, leading to non-compliance with health standards

and increased risk to end-users. It is therefore critical to understand the interactions between particles, pathogens and wastewater treatment processes to enable optimal removal of pathogens and the production of safe reuse water. This paper provides an overview of wastewater, the pathogens and indicator organisms of interest in wastewater, the treatment processes commonly used for the production of reuse water, the interactions between wastewater particles and pathogens and how these can affect treatment processes and impact upon wastewater reuse.

1.2.3 Wastewater

Today's rapidly growing societies generate wastes that enter water bodies (Parr et al., 2002). Different types of wastewater (Figure 1.1) include those derived from domestic, commercial, industrial and agricultural sectors, as well as surface run-off (storm water) from urban areas (Abdel-Raouf et al., 2012, Metcalf and Eddy, 2003). Domestic wastes are derived from human communities and contain human wastes (faeces and urine) as well as water from laundry, kitchen, bathing and other household chores (Mara, 2004).

Water usage adds many natural organic, inorganic and man-made compounds to the wastewater, such as grit, dirt, oil, nutrients, chemicals, metals, plant and animal wastes (Abdel-Raouf et al., 2012). Inorganic solids present in wastewater include salts, metals and surface sediments (Templeton and Butler, 2011b). Organic compounds are generally biodegradable and comprise body and food wastes that can be metabolized by microorganisms in a process which reduces the oxygen available for other life forms (Templeton and Butler, 2011b). Hence organics in wastewater can be quantified by measuring biological oxygen demand (BOD) and chemical oxygen demand (COD) (Henze et al., 2008).

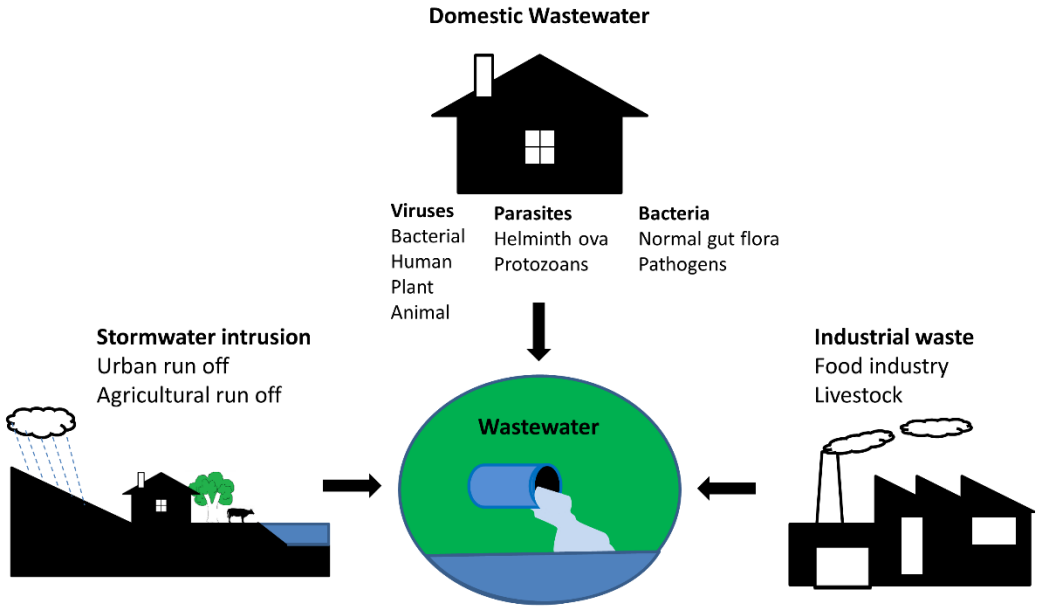

Figure 1.1 Wastewater contamination

Figure 1.1 Wastewater contamination: Major sources of wastewater contamination

The wastewater environment is an ideal medium for both pathogenic and non-pathogenic microorganisms (Abdel-Raouf et al., 2012). Dangerous pathogens include enteric bacteria, viruses, protozoa, parasitic worms and their eggs (Abdel-Raouf et al., 2012). Faecal matter is a major component of domestic sewage and the source of the majority of human pathogens in wastewater (Symonds and Breitbart, 2014). Industrial waste from food production, particularly from animal processing, can also be a source of pathogenic microorganisms.

Although solid materials constitute only 0.1% of the total volume of wastewater (Middleton, 1977), suspended solids can alter the light penetrance and temperature of water bodies, impact benthic plants and clog waterways (Bilotta and Brazier, 2008, Templeton and Butler, 2011b). Excesses of some nutrients, such as nitrogen and phosphorous, can be toxic for fish and other animals including humans, and also cause eutrophication of receiving waters, thus contributing to the formation of algal blooms that can present further human or environmental health hazards (Templeton and Butler, 2011b). If untreated, wastewater will go septic and the decomposition of matter will create unhygienic and hazardous conditions. On the other hand,

municipal wastewaters are also a rich source of nutrients that can be directly recovered or provide additional benefits if present in reuse water for agriculture, horticulture, forestry and domestic gardening applications (Greenway, 2005).

Hence there are compelling reasons to treat wastewater in order to reduce the risk of transmitted diseases and environmental pollution (Mara, 2004), and to retrieve valuable nutrients and fresh water that would otherwise be lost in the waste-stream.

1.2.3.1 Pathogens in wastewater

Wastewater streams contain many different types of pathogens that present a major health risk (Table 1.2). Human pathogens include bacteria, viruses, parasitic protozoans and helminths (Cai and Zhang, 2013). Pathogens can enter wastewaters from many sources. Enteric pathogens enter wastewater from human and animal fecal wastes or from fecally contaminated water from other household uses such as bathing or laundry (Gerardi and Zimmerman, 2004). Livestock and poultry can be infected with zoonotic enteric pathogens and so wastewater from food processing also represents a human health risk (Hill, 2003, Gerardi and Zimmerman, 2004). The major pathogens and diseases or illnesses they cause are shown in Table 2 (Ashbolt, 2004, Gerba and Smith, 2005). For a summary of pathogens and representative indicator organisms in wastewater, including their geographical distribution, numbers in primary and secondary treated wastewater and summary information of detection methods, readers are directed to the report by Keegan et al. (2010).

1.2.3.2 Bacteria

Bacteria constitute the most diverse group of human pathogens in wastewater. Many types of bacteria colonize the human intestine and are shed in feces. While many of these bacteria are commensal and beneficial to their hosts, some are pathogenic and these enteric bacterial

Table 1.2 Pathogens in wastewater

Table 1.2 Pathogens in wastewater: The major pathogens of concern in municipal wastewater and diseases or illness associated with them

	Name of Pathogen	Major disease or symptoms
Bacteria	*Salmonella* spp.	Salmonellosis, typhoid, paratyphoid
	Shigella spp.	Bacillary dysentery
	Yersinia spp.	Gastroenteritis
	Vibrio cholera	Cholera
	Campylobacter jejuni	Gastroenteritis
	E.coli	Gastroenteritis
Viruses	Polio virus	Poliomyelities
	Coxsackie virus	Meningitis, hepatitis, pneumonia, fever
	Echovirus	Meningitis, paralysis, encephalitis, fever,
	Hepatitis A virus	Infectious hepatitis
	Rotavirus	Acute gastroenteritis with severe diarrhoea
	Human calicivirus	Epidemic gastroenteritis with severe diarrhoea
	Reovirus	Respiratory infections, gastroenteritis
	Hepatitis E virus	Infectious hepatitis, miscarriage and death
	TT hepatitis	Hepatitis
	Astrovirus	Gastroenteritis
	Adenovirus	Upper respiratory infection and gastroenteritis
Protozoa	*Cryptosporidium* spp.	Cryptosporidiosis
	Entamoeba histolytica	Acute dysentery
	Giardia duodenalis	Giardiasis
	Balantidium coli	Balantidosis
	Toxoplasma gondii	Toxoplasmosis
Helminth Worms	*Ascaris lumbricoides*	Ascariosis
	Ascaris summ	Coughing and chest pain
	Trichuris trichiura	Diarrhoea, anaemia, weight loss
	Toxocara canis	Fever, abdominal pain, muscle ache
	Taenia sasginata	Insomnia, anorexia
	Taenia solium	Insomnia, anorexia
	Necator americanus	Hookworm disease
	Hymenolepis nana	Teaniasis

pathogens constitute the majority of bacterial pathogens in wastewater (Varela and Manaia, 2013). Major human bacterial pathogens in wastewater include *Salmonella* spp., *Escherichia* spp., *Shigella* spp., *Yersinia* spp., *Klebsiella* spp., *Leptospira* spp., *Vibrio cholerae, Aeromonas hydrophila*, *Legionella pneumophila*, *Mycobacterium* spp. and *Pseudomonas* (Stevik et al., 2004, Maynard et al., 2005, Cai and Zhang, 2013). Enteric bacterial pathogens such as *Salmonella* spp., *Escherichia* spp., *Shigella* spp., *Yersinia* spp. and *V. cholerae* typically cause gastrointestinal infections such as diarrhea, dysentery and gastroenteritis (Okoh et al., 2007, Anastasi et al., 2010, Varela and Manaia, 2013). *Helicobacter pylori*, which causes gastric ulcers and is linked to some cancers, might also be waterborne but transmission pathways for this pathogen have not been conclusively demonstrated (Anastasi et al., 2010). Other diseases caused by bacteria in wastewater include wound infections (*Pseudomonas aeruginosa*), respiratory infections (*Legionella pneumophila Mycobacterium avium*) and leptospirosis (*Leptospira*) (Gerardi and Zimmerman, 2004, Levy et al., 2010). Some bacteria, such as *Legionella pneumophila*, *Mycobacterium avium*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila*, are environmental and are opportunistic rather than frank pathogens, since they cause disease in a host with a predisposing factor, such as reduced immunity or with reduced physical barriers to infection due to burns or wounds (Gerardi and Zimmerman, 2004).

Escherichia coli are genetically diverse and predominantly harmless bacteria that are part of the normal gut flora of warm blooded animals, including humans. This species has been widely used as an indicator of fecal contamination and is found in densities of 10^5 to 10^{10} colony forming units (CFU) per liter of raw sewage (Matthews et al., 2010). Some strains of *E. coli* possess additional genes, encoding virulence determinants such as adhesion factors and toxins, which allow them to be pathogenic and cause intestinal or extra-intestinal diseases. These pathogenic strains can cause gastroenteritis, diarrhea, urinary tract infections hemolytic uremic syndrome and meningitis (Anastasi et al., 2010).

The increased production or use of antibiotics in countries such as the United States, India, China and Greece has led to an increased awareness of the presence of antibiotics and antibiotic resistant bacteria (ARB) in wastewater (Bitton, 2005, Bouki et al., 2013). Many reports have described the persistence of ARB through the wastewater treatment train and there is currently much interest in the environmental fate of both ARB and the genetic elements encoding antibiotic resistance, particularly if these genes are passed to and persist in environmental bacteria (Bouki et al., 2013).

1.2.3.3 Viruses

Viruses are another diverse group of waterborne human pathogens. Untreated wastewater can have as many as 10^3 to 10^7 virus particles per liter of wastewater (Okoh et al., 2007, Keegan et al., 2010). However, the density of viruses in treated wastewater depends on various factors: type of treatment process, season and geographical area (Kitajima et al., 2014). Many of the viruses are poorly removed by the secondary treatment processes used to remove bacterial pathogens (Ottoson et al., 2006). The major viral pathogens in wastewater are enteric viruses such as hepatitis A, norovirus, rotavirus, adenoviruses, astroviruses and the various enteroviruses (Cai and Zhang, 2013, Ashbolt, 2004). The site of virus multiplication in the host is generally related to the type of disease caused, with most enteric viruses multiplying in the host's intestine, although in some cases other tissues can be infected (Wyn-Jones et al., 2011). Consequently, enteric viruses can cause a variety of diseases in humans, such as gastroenteritis, meningitis, hepatitis and myocarditis (Ashbolt, 2004). These enteric viruses are shed in high quantities, 10^5-10^{11} virus particles/gram of feces, by infected individuals (Okoh et al., 2010, Fong and Lipp, 2005). Several emerging viruses, such as severe acute respiratory syndrome coronavirus and human parechovirus, can also be excreted in feces and found in wastewater (Wyn-Jones et al., 2011). Industrial waste from slaughterhouses can add zoonotic viruses to wastewater, such as animal adenoviruses, sapoviruses and hepatitis E (Wyn-Jones et al., 2011).

A variety of pathogenic plant viruses, such as pepper mild mottle virus and tobacco mosaic virus, have been identified in human feces and wastewater (Symonds and Breitbart, 2014).

1.2.3.4 Protozoa

Protozoans are another important group of microorganisms in wastewater and can be up to ten times larger than bacteria (Boztoprak and Özbay, 2013). Protozoan parasites, such as *Cryptosporidium parvum* (10^2-10^4/L), *Cryptosporidium hominis* and *Giardia duodenalis* (10^4-10^5/L), are commonly detected in sewage (Li et al., 2009), although their abundance may be seasonal, depending on country and climate. *Cryptosporidium* and *Giardia* are intestinal parasites that infect humans and animals. In the case of *Cryptosporidium*, there is no effective drug treatment that directly kills this parasite in the host and so infection can be fatal in patients lacking a functional immune system (Abrahamsen et al., 2004). The transmissive stage of *Cryptosporidium* is a non-reproductive and dormant oocyst (Searcy et al., 2005), which is shed in feces and is highly infective. *Cryptosporidium* infects host enterocytes, causing diarrhea, nausea and abdominal pain, a condition commonly referred to as cryptosporidiosis (Fletcher et al., 2012). *G. duodenalis* is a unicellular flagellate eukaryote that is another major waterborne pathogen causing intestinal infection (Adam, 2001). The transmissive stage of *G. duodenalis* is a dormant cyst (Adam, 2001) and infection, known as giardiasis, can cause acute diarrhea, abdominal pain, nausea, malabsorption, and weight loss (Fletcher et al., 2012). Other pathogenic protozoa, including *Cyclospora*, *Entamoeba histolytica* (which causes amoebic dysentery), *Isospora belli* and *Enterocytozoon bieneusi*, have also been reported in outbreaks of waterborne illness (Khanum et al., 2013).

1.2.4 Pathogen surrogates for measuring treatment process performance

Wastewater is a reservoir for pathogens and poses a major health risk, particularly when discharges enter recreational waters on in the case of reuse. Culture-based methods are

traditionally used for detecting microorganisms, and because many are not easy to culture, cannot be cultured, or are otherwise expensive to isolate and enumerate (Gilbride et al., 2006, Keegan et al., 2010). Since it is not practical (or in some cases possible) to detect and monitor all known pathogens, indicator organisms are employed as surrogates for the presence of fecal contamination and hence possible presence of pathogens (Harwood et al., 2005). A good indicator should be present in the pathogen source and absent from unpolluted areas. It should be present in abundance, non-pathogenic, easy to culture and show similar behavior as the pathogen (Bosch, 2010).

The most widely used indicator organisms are enteric bacteria, primarily due to the ease and low cost of the relevant culture detection methods. *E. coli*, in particular, is considered a mandatory fecal indicator by the United States Environmental Protection Agency (USEPA) and European Union (EU) for risk characterization of drinking water sources, compliance monitoring of drinking water and monitoring of recycled water and wastewater discharges (Stevens et al., 2003). Other bacteria, such as enterococci, have also been used as fecal indicators (Stevens et al., 2003). However, given differences in size and structure, it is unlikely that all groups of pathogens (bacteria, viruses, protozoa and helminths) will behave in the same way, therefore it is not ideal to have a single, universal microbial indicator (Ashbolt et al., 2001).

Bacterial fecal indicators have been shown to be poor surrogates for viruses and protozoans, highlighting the need for different indicators for different groups of pathogens (Duran et al., 2003). Bacteriophage, such as somatic coliphage, F-specific RNA coliphage and *Bacteroides fragilis* bacteriophage have been suggested as potential viral fecal indicators, as well as indicators for monitoring the effectiveness of treatment processes (Monis et al., 2015, Duran et al., 2003). Fecal indicator organisms are only linked to the presence or absence of fecal

contamination (and hence risk of pathogen presence) but they do not necessarily give any information about the movement, removal or inactivation of pathogens. Therefore, there is a need for process indicators or model organisms, which are defined as groups of organisms that are indicative of pathogen behavior in similar environments (Ashbolt et al., 2001). Process indicator organisms include *E. coli* and FRNA bacteriophage (Monis et al., 2015). Alternative targets, such as particles or measuring particle size distributions, are also potential process indicators. Analysis using particle size distribution (PSD) was found to be a useful surrogate method for helminth ova detected in wastewater influents and effluents (Chavez et al., 2004) and was also predictive of pathogen removal in a pilot-scale activated sludge reactor (Keegan et al., 2010).

1.2.4.1 Bacteria

Shigella, Campylobacter and *Salmonella* are the most common bacterial causes of gastroenteritis in Australia and industrialized countries but these organisms are present in low numbers in wastewater (Matthews et al., 2010). Therefore, indicator organisms such as *E. coli* have been used as an indicator for other enteric bacteria. The majority of enteric pathogenic bacteria respond to water treatment in a similar fashion to *E. coli* (Keegan et al., 2010) and hence it was used as a model organism for assessing UV inactivation (McElmurry et al., 2011). *E. coli* is a gram negative rod shaped bacterium approximately 1.8µm long and 0.8µm in diameter, which lives in the gut of warm blooded animals (Berg, 2004, McElmurry et al., 2011). It is safe and easy to culture, inexpensive to analyze and does not generally regrow in wastewater, making it an ideal process indicator for bacterial pathogens when monitoring the efficacy of wastewater treatment processes.

1.2.4.2 Viruses

Adenoviruses and noroviruses have been suggested as representative viruses due to their abundance in wastewaters (Keegan et al., 2010, Hewitt et al., 2011). However, these viruses are pathogenic and norovirus is not readily cultured, requiring highly specialized three-dimensional cell culture methods to produce the fully differentiated enterocytes needed to support infection and virus propagation. It has been suggested that fecal bacteriophage (viruses infecting enteric bacteria), such as somatic coliphage and F-specific coliphage, can be indicators for pathogenic viruses (Skraber et al., 2004, Monis et al., 2015). However, there is not a universally accepted enteric virus indicator, in part because there is such diversity in virus size, shape and genome type. Bacteriophage have been used as model organisms because they show similar resistance to disinfection processes as most of the enteric viruses and they also have similar or higher abundance compared to enteric viruses in natural water and wastewater (Ashbolt et al., 2001, Duran et al., 2003, Grabow, 2004). Phage have been used as models to examine raw and treated drinking water supplies (Grabow, 2004). FRNA bacteriophage, which are viruses that infect coliform bacteria that possess an F plasmid and are actively expressing conjugative F pili, are the most attractive surrogates because their structure, morphology and composition resembles that of human enteric viruses (Grabow, 2004). In addition, they are unable to multiply in natural water environments in the absence of actively growing host cells and have similar responses towards disinfectants as human enteric viruses (Grabow, 2004). MS-2 phage is a FRNA coliphage that has been used as a model organism for norovirus (Dawson et al., 2005). *E. coli* is the host for MS-2 phage, which bypasses the need for complex mammalian cell culture for enumeration of MS-2 phage (Dawson et al., 2005). In addition, MS-2 phage is relatively easy to propagate using *E. coli* cultures and so can be used for challenge testing if higher numbers of virus are required to validate the performance of treatment processes.

1.2.4.3 Protozoa

Cryptosporidium and *Giardia* are important pathogens that are problematic due to their resistance to chlorine (*Cryptosporidium* in particular), environmental persistence, low infectious dose and relatively high cost for detection and measurement of infectivity. The bacterium *Clostridium perfringens*, a spore-forming obligate anaerobe, has been considered as an indicator for pathogenic protozoa (Ashbolt et al., 2001). *Clostridium* is found in abundance in sewage as it is associated with the feces of warm blooded animals (Ashbolt et al., 2001). The small (1 μm) spores, which are 4 – 10 times smaller than protozoan oocysts or cysts, do not interact with soil grains and in some ways behave like colloids, making them highly resistant to degradation and inactivation (Schijven et al., 2003). These spores have been associated with the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in wastewater (Cheng et al., 2012) and have similar partitioning behavior to *Cryptosporidium* and *Giardia* in storm water (Cizek et al., 2008a). It has been suggested that they are conservative indicators for the removal of *Cryptosporidium* and *Giardia* by wastewater treatment processes (Keegan et al., 2010).

1.2.5 Pathogen detection methods

Increases in population, habitat encroachment, international travel and the globalization of world trade have all contributed to the emergence of new pathogens or re-emergence of known pathogens of human health significance (Gilbride et al., 2006). There are many techniques for isolating and detecting pathogenic microorganisms in wastewater, ranging from simple culture-based techniques to next generation sequencing (NGS). Some of these are standard methods and their use may be mandated in different countries for regulatory compliance. It is beyond the scope of this review to discuss these techniques in any detail, but there are many useful review papers describing or evaluating molecular techniques (Monis et al., 2005, Gilbride et al., 2006, Ramirez-Castillo et al., 2015, Yergeau et al., 2016) and research reports are also a good source of information for both conventional and molecular detection protocols for

pathogens or surrogates in wastewater (Keegan et al., 2010, Monis et al., 2015, Francy et al., 2011).

All techniques have advantages and limitations and a list of some traditional and modern techniques is shown in Table 3. Emerging techniques include NGS, which has been used to detect pathogenic bacteria in wastewater (Cai and Zhang, 2013, Ye and Zhang, 2011, Yergeau et al., 2016). Next generation 454 pyrosequencing has also been used successfully to characterize microbial communities from different wastewater samples (Ye and Zhang, 2013). Caution needs to be used when interpreting NGS data, particularly when identification is based on the sequencing of relatively small amplicons, which makes misidentification possible, particularly for closely related species. The technique is also very sensitive and a thorough understanding of the level of background contamination from the laboratory environment or between samples is required to determine if a result is the detection of a rare taxon or an artifact.

Table 1.3 Pathogen detection

Table 1.3 Pathogen detection. List of detection methods used to study different pathogens found in wastewater

Technique	Benefits	Limitations
Culture based methods	Easy to perform Low cost	Majority of bacterial population cannot be artificially cultured. Not a direct measurement if using indicator organisms.
Microscopy	Fast Direct Observation	Limited options for species identification Requires expertise
Fluorescent in-situ hybridization (FISH)	Quantitative Direct visual resolution of cells including non culturable bacteria	Labor intensive Limited ability to identify multiple target species
FISH and Confocal scanning laser microscope (CLSM)	Direct visual resolution of cells including slow growing and non culturable bacteria	Expensive
Polymerase Chain Reaction (PCR)	Culture independent Rapid Highly sensitive Accurate	False positive results Inhibition by contamination Requires knowledge of target organisms sequences for assay detection
Multiplex PCR	Rapid and simultaneous detection of target microorganisms	Primer dimers may function as a single reaction
Amplified Ribosomal DNA restriction Analysis (ARDRA)	Culture independent	DNA extraction and PCR biases Not quantitative
Terminal-restriction fragment length polymorphism (t-RFLP)	Suitable for wide range of microorganisms	DNA extraction and PCR biases
Denaturing gradient gel electrophoresis (DGGE)	Fast and semi-quantitative	Specificity can be an issue due to short target sequences
Ribosomal RNA intergenic spacer analysis (RISA)	Use of r-RNA gene sequence heterogeneity Heterogeneity in length and sequence among bacteria	
Nucleic Acid Microarray	High throughput design Various applications	Low sensitivity for environmental samples Sample processing complexity
On Chip Technology	PCR and hybridization on a single chip Less interference between parallel reactions	Integration and packaging
Next Generation sequencing	Culture independent Rapid community analysis Versatile (community function or composition)	DNA extraction and PCR biases Not quantitative Expertise for bioinformatics analysis Expensive equipment

1.2.6 Wastewater treatment

It is important to treat wastewater cost effectively while ensuring the quality is sufficient to enable safe disposal or reuse. The majority of countries utilize conventional wastewater treatment processes in which physical, chemical and biological reactions remove suspended solids, biodegradable organics and pathogenic microorganisms (Metcalf and Eddy, 2003, Middleton, 1977). These processes are grouped into preliminary, primary, secondary and tertiary stages and form a treatment train (Figure 2.2).

1.2.6.1 Preliminary treatment

The first wastewater treatment stage is designed to remove large objects such as bottles, cans and plastics which can clog and block downstream processes (Okoh et al., 2007). Preliminary treatment typically consists of screening and grit removal and can use bar, drum, cutting or band screens that are inclined towards the inflowing water and trap objects as the sewage water flows thorough them (Templeton and Butler, 2011b). The captured debris can be manually or mechanically removed and fibrous materials can be further dewatered. Grit removal involves removing abrasive inorganic materials such as sand, gravels and other heavy particulate matter and is necessary to avoid clogging and abrasive damage to the equipment and sewage pipes downstream (Templeton and Butler, 2011b). There are different types of grit channels; velocity channels or aerated channels, which reduce the velocity of influent and allow the heavy abrasives to settle to the bottom before removal.

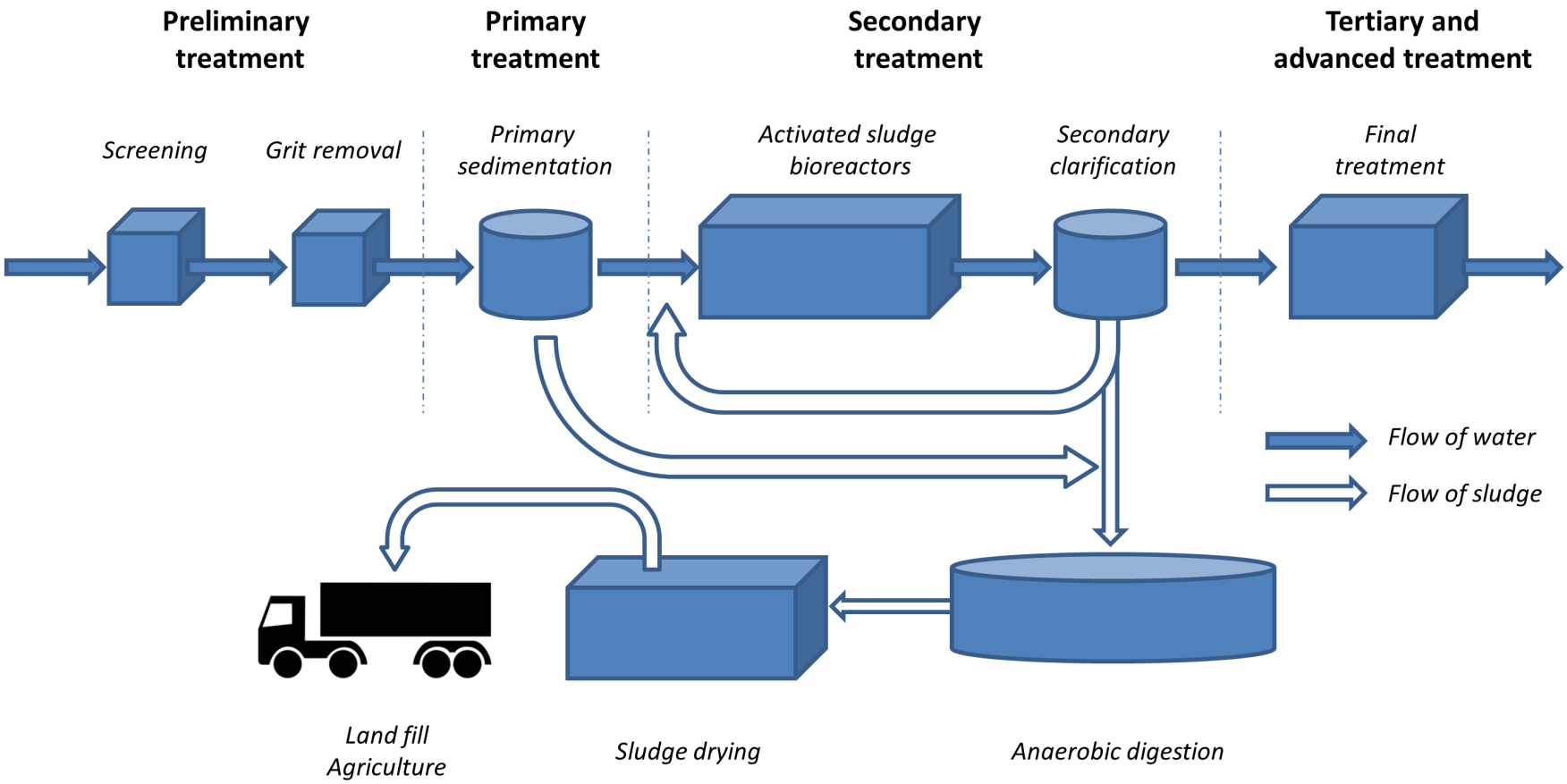

Figure 1.2 Wastewater Treatment

Figure 1.2 Wastewater Treatment: Schematic of a typical wastewater treatment

1.2.6.2 Primary treatment

Primary treatment processes are designed to remove suspended solid wastes and reduce particulate forms of biological oxygen demand (BOD). It is generally described as the first level of treatment and removes approximately 50-70% of total suspended solids, 65% of oil and grease and 25-50% of BOD (Sonune and Ghate, 2004). Major physical modes for separating solids from wastewater are flocculation and sedimentation, which involves settling solids under the influence of gravity (Templeton and Butler, 2011b). The most common sedimentation tanks (also known as clarifiers) are rectangular or circular (Figure 2.3) in shape. In rectangular tanks, water enters from one end and leaves from the other end (Fig 2.3a) whereas in circular tanks water enters from the center and moves outwards radially (Fig 2.3b). An important feature of these tanks is a weir. In sedimentation, the speed of water affects settling of solids. Therefore, weirs are carefully designed physical barriers which determine the flow rate (Templeton and Butler, 2011b). Dissolved and colloidal substances are not removed at this stage (Sonune and Ghate, 2004).

As well as basic primary treatment methods, advanced methods separate dissolved organic matter by the addition of coagulants or flocculants (Odegaard, 2000). The flocculent is a metal salt which aggregates the suspended colloids and facilitates separation by settling or filtration (Odegaard, 2000). The outflow water is known as primary effluent and it contains mainly dissolved organic and inorganic solids. Once clarified, the primary effluent enters secondary treatment.

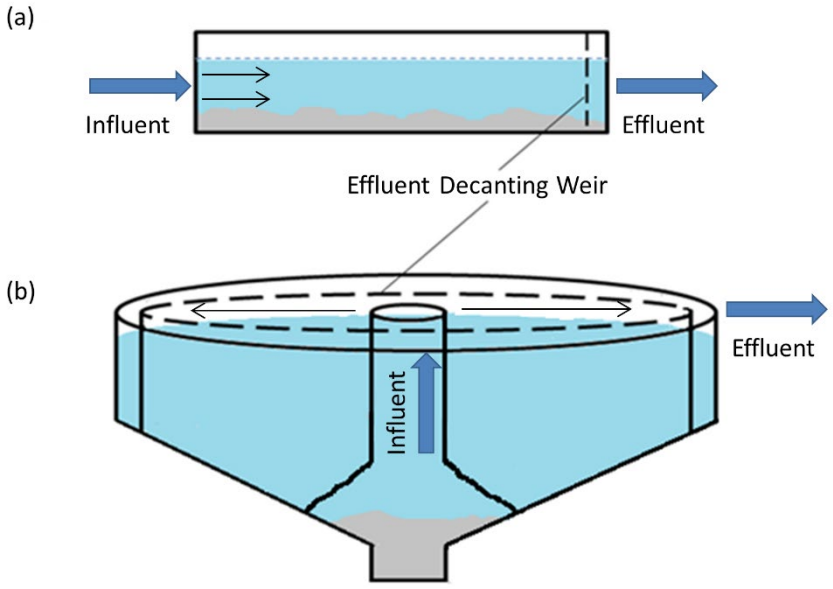

Figure 1.3 Wastewater sedimentation tanks

Figure 1.3 Wastewater sedimentation tanks: Illustration showing the most common designs of wastewater sedimentation tanks (clarifiers): (a) rectangular or horizontal flow clarifier and (b) circular or radial flow clarifier

1.2.6.3 Secondary treatment

Secondary treatment processes remove nutrients and dissolved organic and inorganic solids from the primary effluent by the application of various biological treatment processes (Sonune and Ghate, 2004, Spellman, 2013). The different functional operations that occur during secondary treatment are carbon oxidation and nutrient removal. Carbon oxidation is mediated by microorganisms and involves the oxidation or metabolism of organic matter into carbon dioxide, water and cellular biomass (Grady et al., 2011). The energy produced is utilized by microorganisms for growth and reproduction (Davies, 2005).

The two key nutrients that must be removed prior to discharge of wastewater to the environment are nitrogen and phosphorous. The biological processes of nitrification and denitrification remove inorganic nitrogen (Gerardi, 2010). Nitrification converts ammonia (NH_3) to nitrate (NO_3^-), while denitrification converts the NO_3^- to nitrogen gas. Incomplete nitrification/denitrification can lead to the production of nitrous oxide, which is a potent

greenhouse gas. A combination of biological and physiochemical processes can be used to remove phosphorus. Biological processes include enhanced biological phosphorus removal plants, which are designed to selectively support the growth of phosphate accumulating organisms (PAO) that are capable of storing orthophosphate (Gerardi, 2010). There are a number of different types of secondary treatments that can be used to mediate biological nutrient removal (BNR) processes (Table 1.4).

1.2.6.3.1 Activated sludge process

The activated sludge process (ASP) is commonly used for biological removal of nutrients from wastewater. An ASP involves two major stages. The first stage is the decomposition of pollutants by an heterogeneous and highly diverse culture of microorganisms, which metabolizes organic matter and inorganic nutrients to more simplified and environmentally benign end products such as carbon dioxide and nitrogen gas (Tong et al., 1980). The heterogeneous microbial culture is termed 'activated sludge' (Okoh et al., 2007) and the biomass is normally arranged in microbial aggregates called flocs, which are kept in suspension by aeration and mechanical mixing (Sustarsic, 2009, Seviour and Nielsen, 2010). The most basic ASP set-up for this first stage comprises an aeration tank and an aeration source. However, there are many modifications to this basic design (Figure 1.4) to include anoxic and/or anaerobic zones to improve total nitrogen removal by nitrification/denitrification and phosphorous uptake (Sustarsic, 2009, Seviour et al., 2003, Fux and Siegrist, 2004, Vaiopoulou et al., 2007, Okoh et al., 2007).

Table 1.4 Secondary wastewater treatment

Table 1.4 Secondary wastewater treatment options and their key features (Parr et al., 2002, ESCWA, 2003, Liu et al., 2003)

Treatment Process	Description	Key features
Activated sludge Process (ASP)	Aerobic digestion of organic matter by bacteria, can also include anaerobic and anoxic zones for N and P removal	• Multi chamber reactor unit • Different configurations can be employed for specific treatment • Efficient treatment method
Aerated lagoons	Mechanically aerated 1-4 meters deep ponds	• Performance impacted by temperature • More complicated than waste stabilisation lagoons • High operational costs
Land treatment	Sewage is supplied in controlled conditions to soil	• Pollutants such as phosphorous are not easily removed • Three main types-Slow rate, rapid infiltration and overland flow
Oxidation pond	Modified ASP with long retention times	• Easy to maintain and control • Low initial costs • Less sludge production • Unable to treat toxic wastes
Constructed wetlands	Sewage flows through artificial vegetative pond systems	• Treatment by combined action of soil matrix and soil root interface • No oxygenation required • Requires large land areas
Rotating biological contactor	Attached growth biological process with vertical rotating discs partially submerged in wastewater	• Rotating plates forms slime microbial layer on surface • High degree of organic removal • Simple and effective • External aeration needed
Trickling filters	Aerobic attached growth-Sewage flows through a fixed bed of filter media covered with biomass	• Aerobic digestion • Pre-treatment essential • No external aeration needed
Up-flow anaerobic sludge blanket (UASB)	Anaerobic process uses a blanket of bacteria to absorb sewage load	• Suitable for hot temperatures • Produces less sludge • No aeration needed • Rich microbial diversity • Long start up times
Waste stabilisation ponds	Large surface area earthen basins use mixed biological processes	• Different types on basis of biological activity • Removal of soluble organic matter • Effective in removing pathogens • Low costs

The second stage of the ASP is separation of the biomass from the treated water in a secondary clarifier, which uses gravity sedimentation (Sustarsic, 2009, Seviour and Nielsen, 2010). The clarified supernatant is sent for tertiary treatment, while a large portion of the settled biomass (termed return activated sludge or RAS) is recycled back to the head of the ASP. While the main purpose of an ASP is biological removal and stabilization of nutrients, it is also recognized to be an effective treatment barrier against pathogens via predation (by higher organisms) and by attachment, adsorption or entrapment to or within the biological floc (Bitton, 2005, Okoh et al., 2007, Keegan et al., 2010). Reports describing the efficacy ASP for pathogen removal vary and this may be related to operational differences between wastewater treatment plants (WWTPs) and also the effect of season on pathogen density and treatment performance. Removal of *E. coli* has been reported to be between 1.5 – 2.5 $\log_{10}$, while *Cryptosporidium* removal was reported to be between 1 – 3 $\log_{10}$ (Keegan et al., 2010, King et al., 2015a). In contrast, removal of viruses varied between different WWTPs and also appeared to be different for some viral species (Keegan et al., 2010). For example, removal of rotavirus was 7-8 $\log_{10}$, whereas norovirus removal ranged from 1 – 6 $\log_{10}$ (Keegan et al., 2010).

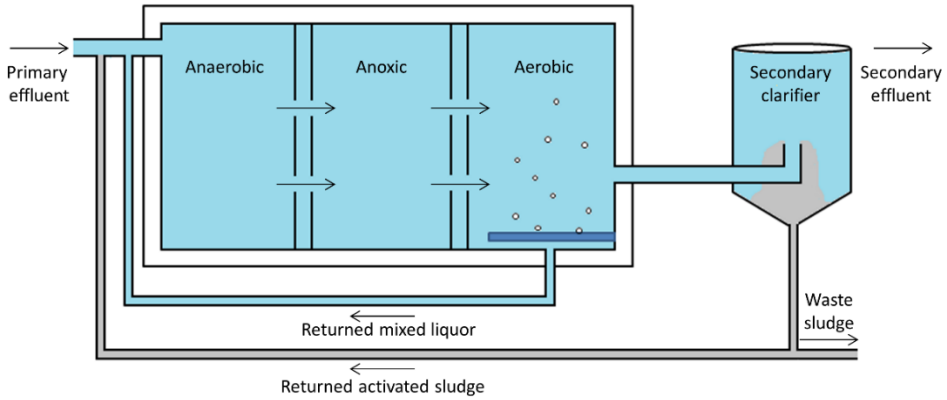

Figure 1.4 Modified Activated Sludge

Figure 1.4 Modified Activated Sludge: Schematic of a modified activated sludge process that promotes biological removal of nitrogen and phosphorous

1.2.6.3.2 Waste Stabilization ponds

Waste stabilization ponds (WSP's) are large shallow basins in which wastewater is stored for extended periods of time to enable biological treatment by communities of bacteria and algae species (Alexiou and Mara, 2003). WSP's provide a green treatment technology with the advantages of low energy demand and low operational costs combined with highly efficient removal of organic matter and pathogens (Faleschini and Esteves, 2011). Waste stabilization ponds are often used in small rural communities as the sole treatment option for sewage, or as a polishing step after ASP or other secondary treatments prior to discharge or reuse.

Three major mechanisms contribute to the elimination of pathogens from WSPs; a) adverse conditions in the ponds (e.g. temperature, sunlight and predation), b) long residence times for microorganisms in ponds leading to natural death and c) adsorption to particles and sedimentation (Campos et al., 2002, Greenway, 2005, Karim et al., 2004). The removal of pathogens and the final effluent density of pathogens is also related to pond depth, detention time, number of ponds and pond geometry (Von Sperling, 2005). Waste stabilization ponds remove fecal coliforms, *E. coli* and other pathogenic microorganisms through photo-oxidative DNA damage arising from sunlight, as well as through other physicochemical factors such as temperature and pH (Davis-Colley et al., 2000). Sunlight and temperature have also been found to inactivate *Cryptosporidium* suspended in a WSP (King et al., 2015a). Predation by other microorganisms or zooplankton can also contribute to removal of pathogens, especially bacteria and protozoan parasites (Stott et al., 2001, King et al., 2015a). Waste stabilization ponds have shown removals of 2–4 $\log_{10}$ for viruses, 3–6 $\log_{10}$ for bacteria, 1–2 $\log_{10}$ for protozoan cysts (Templeton et al., 2005) and up to a 3 $\log_{10}$ for helminth eggs (Jiménez et al., 2010). Protozoan removal in WSPs can be highly seasonal, with higher removal in the summer/autumn months (2.5 – 3 $\log_{10}$) and lower removal in the winter/spring months (0.5 – 1.2 $\log_{10}$) reported for an Australian pond system (King et al., 2015a).

There are many types of pond designs (Symonds et al., 2014), with the most common configuration being a sequence of facultative and maturation ponds (Shilton, 2005). In a relatively simple configuration (Figure 1.5a) there is no pre-treatment and only one primary facultative pond is connected to the maturation ponds. However, more advanced facilities include an anaerobic pretreatment step before the facultative pond (Figure 1.5b). The different types of pond systems are described in the following sections.

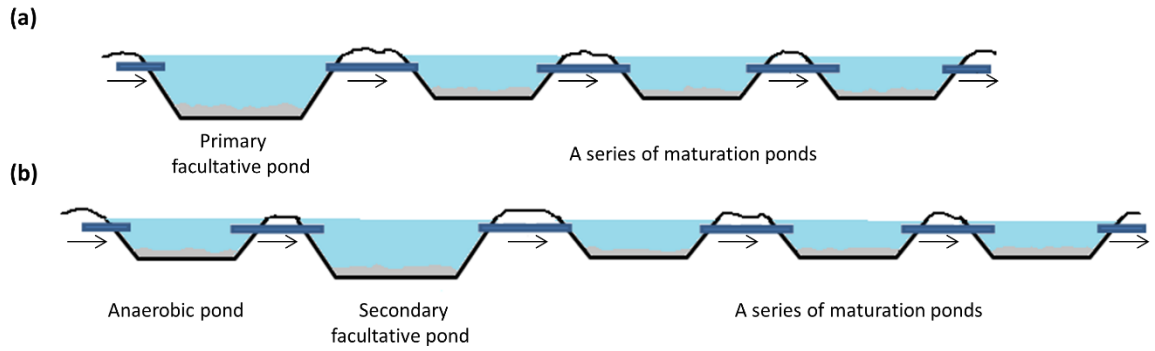

Figure 1.5 Waste stabilization ponds

Figure 1.5 Waste stabilization ponds: Schematics of two common variations of standard pond systems. (a) one primary facultative pond with no pretreatment and (b) pretreatment using an additional anaerobic pond

1.2.6.3.2.1 Anaerobic ponds

Anaerobic ponds operate without oxygen and function to remove organic bulk. They have short retention times and can remove 40-70% of the organic load in wastewater (Shilton, 2005). Sedimentation is a major mechanism of pathogen elimination in these ponds. In general, the density and hence settling velocity of microorganisms is low (e.g. 30 mm/day for *Cryptosporidium* oocysts (Medema et al., 1998a)), so attachment of pathogens to denser particles is required for sedimentation to occur. Helminth eggs, which are large and relatively dense, readily settle under gravity and are removed in these ponds (Campos et al., 2002).

1.2.6.3.2.2 Facultative ponds

Facultative ponds operate under both aerobic and anaerobic conditions. Pathogen removal in these ponds is a complex process involving factors such as sedimentation, sunlight-mediated inactivation, high pH, low carbon dioxide and high oxygen concentrations (Campos et al., 2002). These ponds consist of different functional layers or zones, namely anaerobic, heterotrophic and photic zones (Bitton, 2010). The lowest zone is anaerobic and removes sedimented organic matter in the absence of oxygen while producing gases such as methane and carbon dioxide (Faleschini and Esteves, 2011). In the heterotrophic zone, carbon dioxide stimulates algal growth, which provides oxygen for heterotrophic aerobes to decompose organic matter (Bitton, 2010). The top or surface zone, also known as the photic zone, is characterized by high rates of algal photosynthesis causing the water to become highly oxygenated. This oxygen is utilized in the aerobic decomposition of organic matter by heterotrophic bacteria (Von Sperling, 2007). Facultative ponds are generally shallow and range from 1-2.5m in depth, with detention times that range from 5-30 days (Shilton, 2005, Bitton, 2010)

1.2.6.3.2.3 Maturation ponds

Maturation ponds are 1-2m deep with a detention time of approximately 20 days (Bitton, 2010). Their major function is pathogen removal but they also serve to remove nutrients (Shilton, 2005, Von Sperling, 2007). A series of small maturation ponds is usually used instead of a single maturation pond (Shilton, 2005) because it easier to prevent short circuiting. Maturation ponds tend to be shallower than other ponds since this allows the efficient removal of pathogens by solar radiation (UV penetration), high pH, high dissolved oxygen and low nutrient content (Von Sperling, 2007, Symonds et al., 2014). Maturation ponds can achieve 100% removal of protozoans and helminth eggs (Amahmid et al., 2002) and 99% removal of coliforms (Von Sperling, 2007).

Waste stabilization ponds are commonly used in developing countries such as India and Bolivia, as well as in developed nations such as Australia (Phuntsho et al., 2016), but mechanisms for the removal of enteric viruses are not well understood and require further study (Symonds et al., 2014).

1.2.6.3.2.4 High Rate Algal Ponds

A less commonly used pond format for treating primary effluent is the high rate algal pond (HRAP). These are generally shallow ponds that are well mixed to promote the growth of green microalgae (Craggs et al., 2014), which provides reductions in the organic load and pathogen numbers (Araki et al., 2001). An added benefit of HRAP is it can also cause pathogen inactivation, with one study measuring a 97% reduction in *Cryptosporidium* infectivity (Araki et al., 2001). In addition to secondary treatment, HRAP provides some tertiary treatment, with the algae removing contaminants such as heavy metals (Ramanan et al., 2016). While HRAP has a higher energy demand compared with other pond systems, it is relatively low energy and cost-effective compared with other secondary treatment options, especially if energy-efficient paddle mixers are used. HRAP can be used either directly with primary effluent or with wastewater that has been pretreated by anaerobic ponds or clarifiers to remove solids (Craggs et al., 2014). If carbon is limiting in the wastewater then the performance of HRAP can be enhanced by aeration with CO_2 (Craggs et al., 2014). There has been increased interest in HRAP as an option for culturing algae for biofuel production, as well as a treatment option for limiting blooms of cyanobacteria since HRAP allows better control of the bacterial/microalgal community compared to WSPs (Ramanan et al., 2016). The smaller footprint of HRAP systems (compared with WSPs) makes them an attractive option for urban or semi-urban regions that are rapidly expanding and require a decentralized sewage treatment option that is low cost with minimal land use.

1.2.6.4 Tertiary treatment and disinfection

Tertiary treatment is the final polishing step required to achieve the desired quality of reclaimed water (Guardabassi et al., 2002) and is mediated by a variety of chemical, biological and physical processes. The selection of treatment processes is dependent upon the desired end-use. In the case of applications such as woodlot or sub-surface irrigation, where human contact with the reuse water is unlikely, secondary-treated effluent might be suitable without the need for further treatment. However, the tertiary treatment requirements for reuse water increase as the likelihood of human exposure to the reuse water increases. In general, the effluent needs to be treated and/or disinfected sufficiently to reduce pathogen numbers to levels that meet public health safety requirements. These target numbers are determined by risk assessments that consider exposure routes, exposure amounts, infectious doses and disease outcomes (AGWR, 2006). Nutrients such as phosphorous can be precipitated out by the addition of lime or alum (Templeton and Butler, 2011b) and, less commonly, microalgae (e.g. using HRAP) have also been reported to effectively remove nitrogen and phosphorous (Aslan and Kapdan, 2006). Pathogens can be physically removed by filtration methods, such as dissolved air flotation filtration or microfiltration for bacteria and protozoans and ultrafiltration for virus removal. Membrane filtration methods are highly effective for the removal of pathogens, especially larger organisms like protozoa and bacteria (Ottoson et al., 2006). Filtration has the added benefit of removing particulates to improve downstream disinfection processes that are required to inactivate remaining pathogens.

The final and possibly most important step in tertiary treatment (in terms of microbial safety at least) is the disinfection of the wastewater prior to reuse. Ultraviolet (UV) radiation and chlorination are widely used and well characterized disinfection processes. Chlorine is added to treated wastewater for predetermined periods of time designed to optimize microorganism exposure and inactivation (described in more detail in 7.1 Chlorination), following which any

residual chlorine is neutralized prior to discharge to the environment or aquifer storage (Templeton and Butler, 2011b). By convention, chlorine disinfection targets are set by contact time, or CT, which is measured as the product of the chlorine dose (in mg/L) and time (in minutes). It is therefore possible to achieve the same CT using a high dose/short time or low dose/long time. The CT is affected by the level of free available chlorine, which is determined by temperature and pH. This is an important consideration since the required CTs for pathogen inactivation are much higher in cold water (e.g. a CT of 8 mg.min/L for viruses 5□C, (EPA, 2003)) than in warmer water (e.g. a CT of 3 mg.min/L for viruses 20□C, (EPA, 2003)). The CTs for chlorine disinfection of drinking water or wastewater have been determined for the major enteric pathogens and these are defined in many guidelines (AGWR, 2006, WHO, 2006, EPA, 2003). Achieving the desired CT in reuse water can be more difficult compared with drinking water on account of higher chlorine demand and also due to the formation of chloramine in cases when NH_3 is present, both of which make the CT calculation more complex (Keegan et al., 2012c). Chloramine is a far less potent oxidant compared to chlorine and requires orders of magnitude higher CTs to achieve the same level of disinfection as chlorine (Keegan et al., 2012c). Common enteric bacterial pathogens, such as *Salmonella*, *Campylobacter* and *E. coli*, have relatively low chlorine CTs, of 1 mg.min/L or less (WHO, 2006). Viruses are also effectively inactivated by chlorine, although they are slightly more resistant compared with enteric bacteria. Chorine is ineffective against some protozoan parasites, particularly *Cryptosporidium* (CT 15,300 mg.min/L for 3 $\log_{10}$ inactivation, (WHO, 2006)) and *Toxoplasma* (CT > 144,000 mg.min/L, (Wainwright et al., 2007)).

UV radiation is often preferred to chlorination because it requires fewer steps, is safer (compared with handling chlorine gas or other methods of generating chlorine) and avoids the production of disinfection by-products (Templeton and Butler, 2011b). However, UV disinfection also tends to be more expensive than chlorination, especially for building the

required infrastructure. UV treatment involves exposure of wastewater to a UV-C light source (described in more detail in 7.2 Ultraviolet Radiation), usually a UV lamp enclosed in a quartz sleeve within a stainless steel pipe or suspended in a concrete channel (Gadgil et al., 2002). UV is particularly effective against bacteria and enteric protozoans (*Giardia* and *Cryptosporidium*) but some viruses, in particular adenovirus, have high UV resistance (Hijnen et al., 2006). A combination of UV and chlorination can be particularly effective, using suitable doses of chlorine for virus inactivation and UV for *Cryptosporidium* inactivation, allowing the most cost-effective use of each treatment technology. Other disinfection methods have also been developed and tested. Ozone has been proven to be effective against viruses, protozoan cysts and helminth eggs (Paraskeva and Graham, 2002) and peracetic acid has also been considered a strong disinfectant because of its effective bactericidal, fungicidal, sporicidal and virucidal properties (Kitis, 2004). Conductive-diamond electrochemical oxidation (CDEO) has proven effective in disinfecting wastewater and a combination of CDEO with ultrasound technology increased the disinfection efficacy by reducing the agglomeration of *E. coli* cells (Llanos et al., 2015). However, this technology does not appear to have been adopted for large-scale commercial use.

1.2.7 Turbidity and particles in wastewater

Turbidity has been considered an important indicator of water quality for many years (Mccoy and Olson, 1986) and is related to other water quality parameters such as total suspended solids (TSS) and microbial load, although the relationships are variable (Joannis et al., 2008, Hannouche et al., 2011). It is always essential to monitor turbidity when treating drinking water or wastewater because it interferes with disinfection processes, such as chlorination and UV radiation (Rowe and Abdel-Magid, 1995, Berman et al., 1988, Dietrich et al., 2003, Madge and Jensen, 2006a). Turbidity can be defined as the optical property of water that causes light shone through the water sample to be scattered rather than absorbed or transmitted (Hannouche et al., 2011). Turbidity also defines the clarity or cloudiness of water (Madhavi and Rajkumar, 2013). In wastewater, the suspended matter (particles) contributing to turbidity includes clay, grit, organic or inorganic matter and algae or other microorganisms (Madhavi and Rajkumar, 2013). In the effluent of a wastewater treatment plant after different treatment steps, turbidity provides a measure of the remaining suspended solids or particulate matter in the treated effluent and can provide a measure of treatment performance. High turbidity is associated with the availability of a support medium for the transport of attached pathogenic microorganisms and with increased disinfection demand (Mccoy and Olson, 1986). Turbidity is an indicator of particulate pollution and can be used to measure and control effluent quality, which is particularly important for effluent discharge since suspended solids can have adverse environmental impacts (Hannouche et al., 2011). Increases in wastewater turbidity have been associated with increased densities of bacteria, *Cryptosporidium* oocysts and *Giardia* cysts (Crittenden et al., 2012).

1.2.7.1 Turbidity measurement

Turbidity is measured using a nephelometer and expressed in Nephelometric Turbidity Units (NTU) (Rowe and Abdel-Magid, 1995, Davies-Colley and Smith, 2001). The basic principle

involves passing a light beam through a sample and measuring any scattered light at a 90 degree angle from the incident light path (Figure 1.6). The amount of light measured reflects the number of particulates scattering the light beam (Joannis et al., 2008).

1.2.7.2 Particles in wastewater

Wastewater is made up of wastes from municipal, industrial and, in some cases, agricultural sources. All of these sources can contribute particulate matter that is either inert (inorganic) or of biological origin (organic) and can be different sizes, shapes and densities (Madge and Jensen, 2006a). Particles can be categorized based on their size (Figure 1.7) and are either dissolved (<0.001 µm), colloidal (0.001–1 µm), supracolloidal (1–100 µm) or settleable (>100 µm) (Azema et al., 2002, Pallarès et al., 2011). Particles in water can be of various shapes such as spherical, semi-spherical, ellipsoid, rod-shaped, strings or random coils (Crittenden et al., 2012).

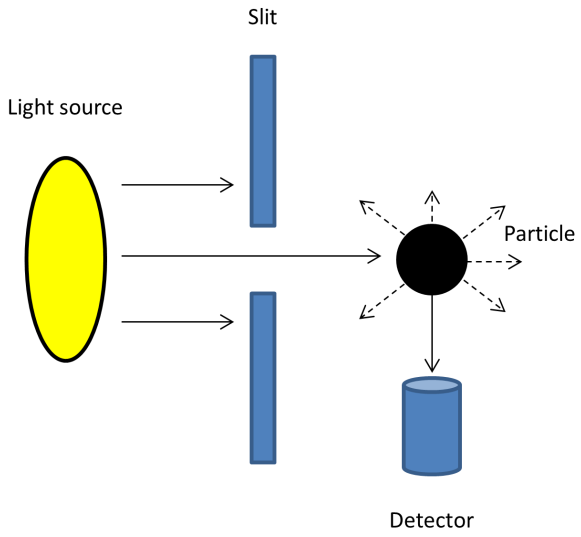

Figure 1.6 Nephelometer

Figure 1.6 Nephelometer: Simplified representation of the operating principles of a nephelometer. Light is directed from a light source to the sample through a narrow slit and the reflected light is collected by a detector and analyzed.

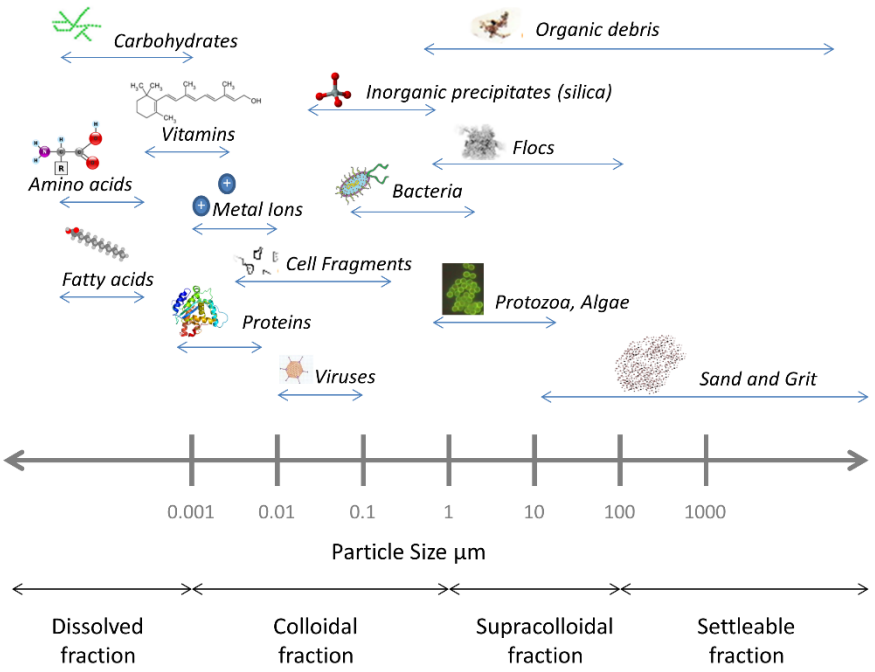

Figure 1.7 Particles in wastewater

Figure 1.7 Particles in wastewater: Comparison of the size distribution of different types of particles in wastewater.

The organic matter in wastewater is of high molecular weight, up to 10^3 Da, and can be classified into different fractions, such as carbohydrates, fatty acids and proteins (Sophonsiri and Morgenroth, 2004). Depending on their composition, organic particles can be readily biodegradable, slowly biodegradable, soluble non-biodegradable, or particulate non-biodegradable (Sophonsiri and Morgenroth, 2004). Municipal wastes are the source of many of these organic fractions and contain small colloids to large particles of up to 63 µm (Sophonsiri and Morgenroth, 2004). Industrial and agricultural wastes mainly comprise soluble organic matter and large particles of > 1.2 µm and > 10 µm respectively (Sophonsiri and Morgenroth, 2004). Wastewater contains many inorganic constituents such as metals, salts and surface sediments. Toxic heavy metals such as Copper (Cu), Zinc (Zn), Cadmium (Cd), Nickle (Ni) and Chromium (Cr) mostly enter wastewater via industrial wastes (Barakat, 2011).

Domestic wastewater also includes contaminants such as soaps, detergents and other household chemicals.

1.2.8 Effect of wastewater treatment on particles

Particles in wastewater are affected by physical, chemical and biological processes during treatment (Garcia-Mesa et al., 2012). The particle size distribution (PSD) is different for primary, secondary and tertiary treated water (Neis and Tiehm, 1997b). Dense, readily settleable inorganic particles, such as sand and grit larger than 0.01 mm, are removed in preliminary treatment stages and less dense organic and inorganic particles in the size range of 0.1mm to 35 µm are removed in primary sedimentation tanks (Shon et al., 2007b). Measurement of the PSD for 3 different municipal primary effluents using a combination of differently sized membrane filters or sieves showed that primary effluent is dominated by small particles <8 µm, which comprise 70 – 88% of the particles by mass (Neis and Tiehm, 1997b). Parallel analysis of primary effluent PSDs using laser scanning was shown to compare favorably with physical sizing by straining (Neis and Tiehm, 1997b).

Traditional primary treatment applies sedimentation under gravity and can remove particles of <50 µm, whereas smaller particles are more efficiently removed by chemically enhanced primary treatment processes (CEPT) and chemical-biological flocculation (CBF) (Zhang et al., 2007). CEPT has been shown to be effective in removing particles in the size range of 20-80 µm; however, it was not effective in removing particles <10 µm, whereas CBF was highly effective at removing particles >5 µm (Zhang et al., 2007). The majority of organic particles in wastewater are colloidal and supra colloidal; however, after biological treatment (CBF), the remaining organic matter is in the soluble fraction (García-Mesa et al., 2010). Particles can transform during biological treatment, with most of the settleable and suspended organic matter metabolized and incorporated into sludge mass or active organisms suspended in the bulk water. This biotransformation of particles also occurs in subsequent secondary or tertiary

treatment steps. Most of the settleable and suspended inorganic particles are entangled in the sludge mass, while non-settleable, non-biodegradable or dissolved organic and inorganic particles pass out in the primary effluent (Henze et al., 2008).

During secondary treatment, (e.g. in activated sludge plants), fine particulates, colloidal particles and large molecules become entangled to form flocs (Davies, 2005). Flocs are made up of a diverse community of microorganisms and non-living organic matter, such as extracellular polymeric substances (EPS), which are secreted by microorganisms and play an important role in floc formation (Figure 1.8). Bacteria, fungi and protozoans attach to the internal and external surfaces of the flocs, which are typically in the size range of 10 μm to 1000 μm (Davies, 2005). Secondary clarifiers remove most of the flocs and the clarified water can be disinfected and discharged to the environment or subjected to tertiary treatment. Particle size analysis of effluent from a conventional secondary clarifier identified with a size range of 1-10 μm (Wu et al., 2009). Particle size and load within clarified effluent is influenced by the settling performance of the activated sludge biomass, as poor settling biomass (termed sludge bulking) results in the carryover of high concentrations of suspended solids and attached pathogens, which can also impact negatively on downstream tertiary treatment processes such as filtration and disinfection. Tertiary treatment, depending on the process, further reduces the loads of particulates and organic contaminants, and disinfection is used as the final step to reduce pathogen numbers to below guideline levels for reuse (Shon et al., 2007b).

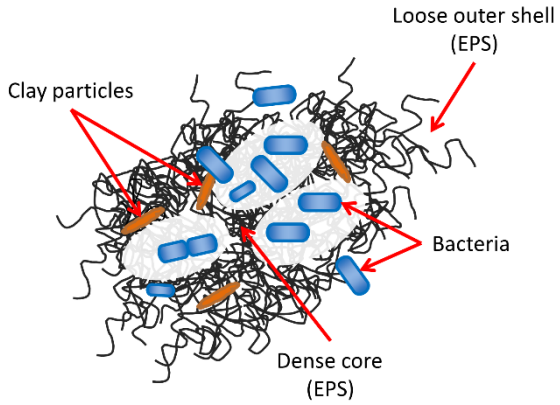

Figure 1.8 Bacterial Floc

Figure 1.8 Bacterial Floc: A typical structure of a bacterial floc held together by extrapolymeric substances (EPS) associated with inorganic clay particles. (Reproduced (Farnood, 2014)).

1.2.9 Particle characterization techniques

Organic matter in wastewater is characterized by its biochemical oxygen demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC) and volatile suspended solids (VSS) (Sophonsiri and Morgenroth, 2004). The major issue with studying the size distribution of particles in wastewater is that they are diverse, ranging from a thousand Daltons in the case of organic molecules to hundreds of microns in the case of biological flocs. Therefore, multiple methods are required in combination, such as filtration and chromatography (Sophonsiri and Morgenroth, 2004). Different techniques for measuring the size of particles include microscopy, electrical sensing, light scattering, light obstruction and membrane filtration (Aguilar et al., 2003). Analysis by microscopy allows direct visualization of the particles, with measurements typically made using computer-aided image analysis, which can automate both particle sizing and counting from the captured images (Emerick et al., 1999). The only issue with microscopy is that the sample needs to be dispersed in such a way that large and small particles are uniformly distributed on the slide (Aguilar et al., 2003). The electrical sensing technique applies voltage across a small orifice through which the particles are passed. The changes in electric potential are directly related to the volume of particles passing (Aguilar et

al., 2003). Coulter counters and multi-sizers are such instruments, which have been used previously for measuring PSD in raw untreated wastewater samples (Chavez et al., 2004).

Particle interactions with light can also be used to estimate particle size. In the case of light obstruction, the amount of light blocked by a particle is measured as it passes through a sensing zone. A particle analyzer uses this principle and measures the size and quantity of particles that pass through the diode (Jolis et al., 2001). The light scattering method uses a similar principle to flow cytometry, with the magnitude of light deflection around the particle (equivalent to forward scatter) used to estimate particle size (Aguilar et al., 2003). Instruments such as the Coulter laser granulometer and Sequoia LISST (laser *in situ* scattering and transmissometry) use this method and have been used to measure PSD in wastewater (Azema et al., 2002, Keegan et al., 2010). A limitation of the light-based methods is that the optical properties of the particles can affect sizing. Apart from size, the refractive index of a particle will also influence how the light scatters, and some diffraction-based particle analyzers use specific calibration factors for different compounds to improve the accuracy of particle sizing. As a consequence, when measuring the PSD of a water or wastewater sample using light diffraction, it is important to understand the nature of the particles being studied (e.g. are the organic or inorganic, if inorganic what type of material?) to enable the selection of appropriate calibration factors.

Particles can be analyzed using methods that separate them on the basis of size or density, such as membrane filtration, sieving or centrifugation (Dietrich et al., 2003, Characklis et al., 2005a). These methods are cheaper and simpler than using PSD analyzers and have the added advantage of collecting the particles for further characterization. This approach is useful for studying the partitioning behavior of chemical or microbial contaminants, which can be measured in the different fractions. For example, particles in a water sample can be stained with a colored dye and filtered using a series of membranes with different pore sizes. Particles captured on the filters can then observed under a microscope and analyzed by an image

analyzer (Dietrich et al., 2007). When using flat-bed membranes for size exclusion, caution must be employed to avoid overloading the filter and blocking the membrane pores because this results in the capture of particles smaller than the nominal pore size of the membrane, which would lead to erroneous results.

Particle structure is another important parameter to study. Scanning electron microscopy has been used to study the structure of mixed liquor particles (Figure 1.9). There are various compartments and complexities within these particles (Figure 1.9) and characterizing such structural aspects can help to elucidate the nature of particle-pathogen associations.

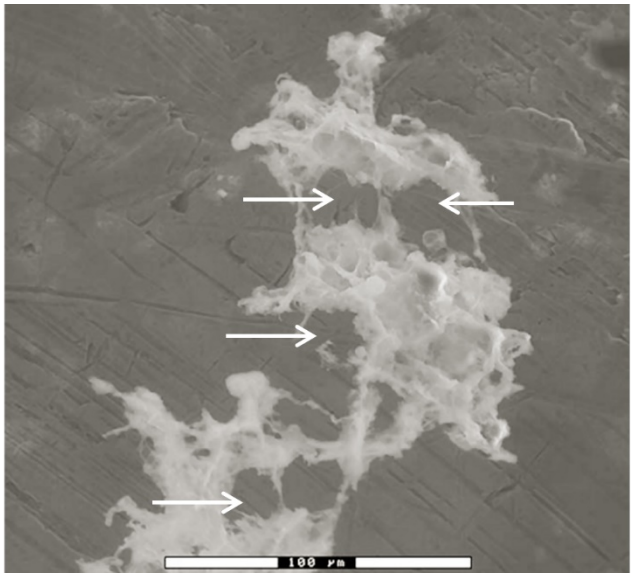

Figure 1.9 Mixed Liquor particle

Figure 1.9 Mixed Liquor particle: an environmental scanning electron microscope image of a mixed liquor particle in the size range of 90-106 um describing its structure. Arrows show different compartments outlined by fibrils. Reproduced from (Gibson et al., 2009).

1.2.10 Pathogen-Particle Associations

Bacteria, viruses and protozoans, from a diverse range of water types (wastewater, freshwater, marine, estuarine), can be free in suspension or associated with particles (Characklis et al., 2005a, Dietrich et al., 2007, Malham et al., 2014). There are two different types of particle-microorganism interactions; particles physically associated with microorganisms as clumps, and particles not physically associated with microorganisms but providing protection by

shielding them from UV light or by contributing to disinfectant demand (Sophonsiri and Morgenroth, 2004). It has been found that the shielding effect of particles increases with increasing particle size (Madge and Jensen, 2006a). The binding of microorganisms to particles can be through electrostatic attractions, hydrophobic interactions or physical entrapment (Templeton et al., 2005). The association between microorganisms and particles can change with time, as the formation and disaggregation of biological flocs is a continual process in environmental waters (Malham et al., 2014). Microorganisms associated with denser particles settle quickly, whilst microorganisms associated with lighter particles tend to stay suspended in water and survive for longer (Characklis et al., 2005a). Figure 1.10 shows bacteria attached to different types of wastewater particles (Ben van den Akker, unpublished data).

The binding of pathogens to particles has been studied using either direct visualization or physical separation techniques. High resolution visualization using confocal microscopy or fluorescent microscopy has been used to image the attachment of protozoan oocysts to inorganic particles and river sediments (Searcy et al., 2005, Li et al., 2009). The same techniques used to characterize particles (described above) can be used to study pathogen-particle binding. Both size exclusion and centrifugation have been used to determine the partitioning behavior of protozoans (Cizek et al., 2008a). Centrifugation has the advantage over filtration because it separates microorganisms and particles using both size

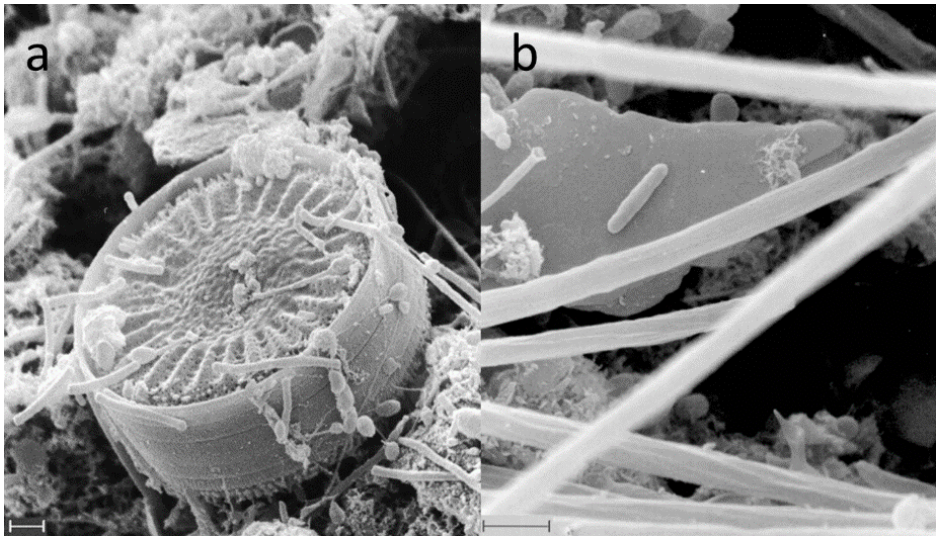

Figure 1.10 *E. coli* Association

Figure 1.10 *E. coli* Association: Attachment of *Escherichia coli* to organic and inorganic particles: Scanning electron microscopic image of (a) growth of *E. coli* attached to a diatom in a biofilm (b) *E. coli* attached to a clay particle. Scale bars indicate 1 µm.

and density (Cizek et al., 2008a). Centrifugation has been used to analyze the partitioning behavior of *E. coli*, enterococci, *C. perfringens*, *Cryptosporidium*, *Giardia* and coliphage in storm water (Characklis et al., 2005a, Krometis et al., 2007a, Cizek et al., 2008a). There are few reports characterizing the nature of the wastewater particles with attached pathogens and this is a knowledge gap that requires further investigation.

1.2.10.1 Bacterial associations with particles

There can be different types of associations between bacteria and particles. Nutrients released from the surface of different types of particles may attract bacteria, which can migrate to the particles, attach and colonize the particle surface (Winkelmann and Harder, 2009). Bacteria have an affinity for inorganic particles and can be adsorbed onto the surface of these particles (Stevik et al., 2004). Various factors influence bacterial association with particles (summarized in Table 4), for example, particle size, particle composition and the age or growth status of the bacteria (Stevik et al., 2004, Madge and Jensen, 2006a). The adsorption of bacteria to the surface of a particle can be explained using the Derjaguin, Landau, Verwey and Overbeck

(DLVO) double layer theory (Stevik et al., 2004, Hipsey et al., 2006). According to the DLVO theory, bacterial attraction occurs at two zones around a particle, the first ("primary energy") is within 1nm of the particle surface and the second ("secondary energy") is within 5-10 nm of the particle surface (Stevik et al., 2004, Hipsey et al., 2006). There are consequently two steps involved in the adsorption of bacteria to a particle surface. The first step occurs within the secondary energy zone and is weak and can be reversed (Stevik et al., 2004, Hipsey et al., 2006). In this step, the bacterial cell overcomes any repulsive electrostatic forces and adsorbs to the particle's surface. Weak Van der Waal and electrostatic forces contribute to this adsorption and can be easily overcome by other physical forces such as a change in the ionic composition of the medium or hydraulic shear forces (Stevik et al., 2004, Hipsey et al., 2006). The second adsorption step, also known as adhesion, occurs within the primary energy zone and is stronger and irreversible. It occurs when the bacterial cell forms a permanent bond with the surface and involves a large amount of energy (Stevik et al., 2004). The adhesion can be mediated by extracellular polymers such as EPS, via the formation of by dipole-dipole interactions or hydrogen bonding (Stevik et al., 2004). Apart from direct adsorption to a particle surface, bacteria can associate with particles by either harboring in the cracks of particles or by adhering to biofilms (LeChevallier et al., 1984, Winkelmann and Harder, 2009).

The formation of biofilm requires actively growing bacteria. Considering that pathogenic bacteria are unlikely to replicate under the nutrient and temperature conditions typical of wastewater treatment systems (Keegan et al., 2010), it is more likely that heterotrophic bacteria will mediate the formation of biofilm or production of EPS and bacterial aggregates, which form a substrate for the binding of pathogenic bacteria, viruses or protozoans.

In unchlorinated drinking water, culture independent methods (cell counts by flow cytometry and estimates of cell numbers using ATP measurement) were used to determine that there were 25-50 bacterial cells associated with each particle (Liu et al., 2013b). However, this study did

not determine the particle size, capturing particle-associated bacteria using 1.2 µm membranes. Analysis of rainfall runoff flowing into a drinking water reservoir identified a relationship between turbidity and coliform / *E. coli* counts, with the strongest correlation (R^2=0.8) being with particles in the size range of 3.2-17 µm (Hipsey et al., 2006). While the authors speculated that this strong correlation was due to association between the particles and bacteria, analyses (such as size fractionation or microscopy) were not conducted to verify the association. Analysis of bacteria in estuary water demonstrated that the numbers of bacteria associated with particles (measured using 3 µm filters) increased with increased turbidity (Bidle and Fletcher, 1995). Few equivalent studies have been conducted for wastewater particles.

Coliform bacteria, which fall in the size range of 1-10 µm, have been shown to be associated with particles greater than 10 µm in diameter (Templeton et al., 2005). Fluorescent *in situ* hybridization (FISH) has been used to detect particle-associated microorganisms in wastewater and allows the examination of organisms while they are in contact with the particles, providing a better understanding of their associations. Particle-associated coliform bacteria in wastewater have been enumerated using 1.2 µm membrane filtration and *in situ* hybridization (Loge et al., 2002). The association of coliforms with particles in activated sludge appeared to decrease with increased mean cell residence time through the ASP (Loge et al., 2002), although it was not clear if the residence time affected coliform-particle binding, or if the decrease in coliforms reflected natural die-off in the ASP as a function of time.

1.2.10.2 Viral associations with particles

Enteric viruses (15 – 80 nm) are much smaller than bacteria (1 – 10 µm) and are generally associated with much smaller particles, less than 10 µm in size (Madge and Jensen, 2006a). The association of viruses with particles (Figure 1.11) depends upon the surface charge of the particle and virus charge, morphology and size (Madge and Jensen, 2006a). Different virus species have different proteins that protect their genome (called a capsid or virus coat), these

differences are likely responsible for differences in charge and removal through treatment processes and mediate adsorption to particles. Viruses, depending on species, can be readily adsorbed onto sand particles, clay particles, suspended colloids, transparent extracellular polymer and fecal matter via electrostatic and hydrophobic interactions (Templeton et al., 2007, Mari et al., 2007). These associations increase their survival rates and render them more resistant to disinfection processes (Templeton et al., 2007).

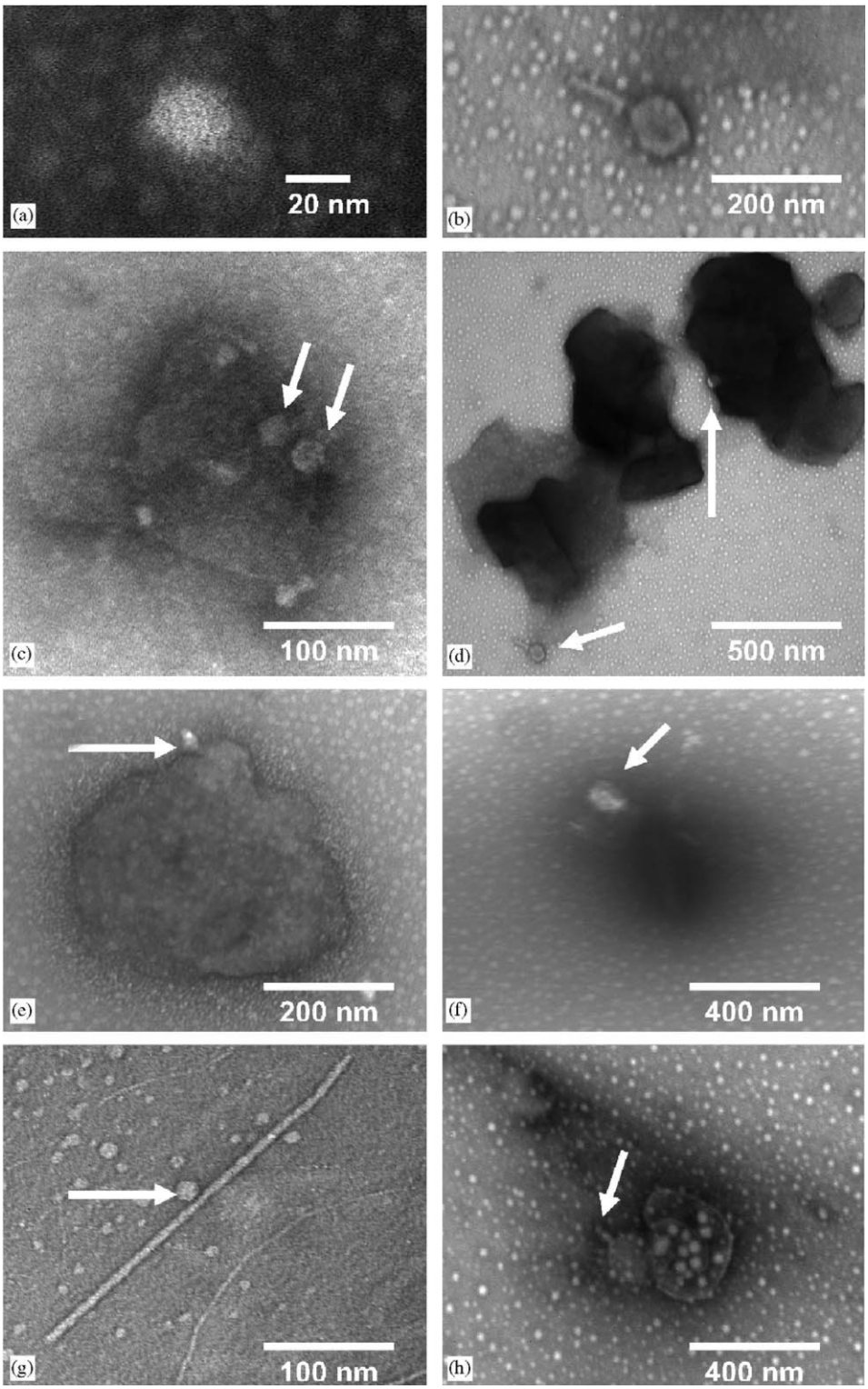

Figure 1.11 Particle associated Viruses

Figure 1.11 Particle associated Viruses Particle associated viruses: Transmission electron microscopy images of MS-2 phage (left panels) and T4 bacteriophage (right panels). (a and b) Phage free in suspension; (c and d) phage associated with kaolin clay particles; (e and f) phage associated with humic acid flocs; (g) MS-2 phage associated with a bacterium flagellum; (h) T4 associated with a sludge particle. Arrows indicate the bacteriophage. Reproduced from (Templeton et al., 2005).

Although there have been many studies of the occurrence of viruses in wastewater (reviewed by Keegan et. al. (2010)), there have been few studies of the association of viruses with particles in wastewater. The association of norovirus with wastewater particles has been successfully demonstrated using cascade filtration of water from a waste stabilization pond (Silva et al., 2008). In this report, sets of different sized filters were used to capture particle-associated norovirus. The filters were then analyzed for the presence of virus to determine the size of particles to which the virus was attached (Silva et al., 2008). Norovirus, detected using reverse-transcription PCR, were found free in solution and on a wide range of particles sizes, including large settleable particles >180 µm, smaller particles captures on a 0.45 µm filter and colloidal particles. This finding contrasts with a study of virus-particle associations in wastewater from an ASP, which used continuous flow centrifugation of filtration to size separate particles and detected enteroviruses using cell culture of Buffalo green monkey kidney cells (Hejkal et al., 1981b). This study reported minimal association between viruses and large particles, with 72% of virus particles associated with particles <0.3 µm in primary effluent and 96.6% of virus particles associated with particles <0.3 µm in clarified secondary effluent. As discussed above, a possible reason for the differences between the two studies could be the physical differences between the different virus species (norovirus versus enterovirus), although it is also possible that the differences could be due to differences in the particles in activated sludge and pond systems.

1.2.10.3 Protozoan associations with particles

Parasites such as *Cryptosporidium* and *Giardia* exist as oocysts and cysts in wastewater streams. There are few studies about the association of either oocysts or cysts with suspended wastewater particles. With the exception of bacterial specific characteristics such as the production of EPS and the presence of pili/fimbriae, many of the factors that influence attachment of bacteria to surfaces (Table 5) might also play a role in (oo)cyst attachment to

Table 1.5 Factors affecting bacterial association

Table 1.5 Factors affecting bacterial association: Summary of factors affecting the association of bacteria with particles

Parameters	Physical
DLVO	Derjaguin, Landau, Verwey and Overbeck double layer theory There are attractive and repulsive electrostatic forces between bacteria and particle surfaces that are stratified into three zones; a near zone ("primary minimum") within 1 nm of the particles surface, in which bacteria are attracted to the particle, then an electrostatic repulsion zone, then a "secondary minimum" attractive zone 5e10 nm from the particles surface. The strength of attraction and repulsion between the bacterium and particle is affected by the ionic strength of the matrix such that increased ionic strength increases the repulsion.
Temperature	Decreased temperature decreases the energy available for adsorption and increases the viscosity of the bacterial cell wall or capsular polymers which also decreases adhesion.
Water flow	Higher flow/velocity reduces the contact time between bacteria and particles and also increases hydraulic shear which can disrupt the first stage of binding under DLVO.
DOM	Dissolved organic matter (DOM) Organic matter attached to particle surfaces may increase bacterial adsorption if positively charged, but organic material in the water may compete with bacteria for adsorption sites. The type and concentration of DOM might also influence bacterial chemotaxis/biofilm formation.
Comparison and size of particle (s)	The size, surface area, volume, and surface roughness can all influence the number of adsorption sites and rate of disassociation.
Particle Type	**Chemical**
pH	The effect of pH on bacterial adsorption is related to the characteristics of the adsorbing surfaces (e.g., carboxyl and amino groups on bacterial surfaces) and the ionic strength of the matrix. Particle surfaces commonly have a negative electrostatic charge, as do bacteria at neutral pH. Bacterial adsorption to inorganic particles increases as their zeta potential decreases.
Hydrophobicity	Bacterial hydrophobicity and charge increase during exponential growth, and this promotes adhesion to particles.
Ions	The ionic strength of the matrix affects electrostatic interactions between pathogens and particles. Divalent cations (e.g., $Ca^{2+,}$ Mg^{2+}, Cu^{2+}, Zn^{2+}) promote adsorption by acting as a bridge between negatively charged particles/bacteria, more so than monovalent cations (e.g., Na^{+}). Anions do not affect adsorption.
Gouy-Chapman	The charge on the surfaces of particles or bacteria is neutralized by oppositely charged ions in the water. This

	causes formation of a Gouye Chapman diffuse electric double layer. Bacterial-particle adsorption is affected by the thickness of this layer, which is a function of ionic strength.

Particle Type	**Microbiological**
Cell surface	Flagella, fimbriae, and pili have the effect of increasing diameter and promote the breach of electrostatic barriers. Motility increases the likelihood of bacterial-particle contact, and can overcome electrostatic repulsion.
Bacterial size and shape	Smaller bacteria more likely to be lodged in crevices in particles.
Extracellular polymeric substances (EPS) and Biofilm	Extracellular polymeric secretions are often polysaccharides with the potential for hydrogen bonding and dipole-dipole-type interactions, and these characteristics promote irreversible adhesion even in the absence of favorable DVLO association conditions. Rarely applicable to pathogenic bacteria in the environment which do not actively grow and produce EPS. The presence of EPS may affect the adsorption of pathogenic bacteria to particles.
Chemotaxis	Bacteria are attracted to many chemicals, and this may play a role in particle adsorption.
Bacterial concentration	Particle adsorption may be proportional to cell concentration. The numbers of pathogenic bacteria are in turn related to factors that affect survival, such as pH, temperature, nutrient availability, and predators.

particles. The attachment of oocysts to inorganic particles in soil is variable and greatly influenced by any organic molecules present, with oocysts showing significant attachment to clay loam in the presence of manure (Kuczynska et al., 2005). Oocysts have a negative surface charge and both steric and electrostatic forces can contribute to association with particles (Searcy et al., 2005). In contrast *Giardia* cysts are hydrophobic and may consequently interact with particles in different ways compared with *C. parvum* oocysts (Dai et al., 2004). Oocysts or cysts can interact with surface attached microbial communities (biofilms) and such attachment can influence the transport of (oo)cysts during water or wastewater treatment, contributing to increased sedimentation and removal (Searcy et al., 2006a, Helmi et al., 2008b). Considering this and other studies of the factors influencing oocyst and cyst attachment to

suspended particles in surface waters (Medema et al., 1998a, Searcy et al., 2005), it is likely that there is some level of association between (oo)cysts and wastewater particles.

In one study, the association of oocysts with particles in surface water was thought to aid in the recovery efficiency of oocysts from the water sample, but this was dependent on particle size and concentration method (Feng et al., 2003). Oocysts and cysts are not thought to attach to inorganic particles in the water column (Dai and Boll, 2003b); however, considering the effect of organics on oocyst binding in soil it is possible that the organics in wastewater could similarly facilitate binding of oocysts to inorganic particles. The surface charge characteristics of particles and microorganisms can alter during the wastewater treatment processes (Medema et al., 1998a) and certain surface macromolecules can hinder the attachment of oocysts to surfaces (Kuznar and Elimelech, 2006). It is therefore possible that the nature of the interactions can change depending on the stage of treatment. Oocyst age or integrity might also play a role in particle associations. Characterization of oocysts in raw sewage and clarified secondary effluent suggested that damaged, non-infective oocysts were preferentially removed during ASP treatment (King et al., 2015a). In this study, the total number of oocysts decreased following ASP treatment, but the proportion of infectious oocysts in the clarified effluent (31%) increased compared with the proportion of infectious oocysts in the raw sewage (10%), suggesting selective removal of non-infectious oocysts (King et al., 2015a). However, this study did assess if particle binding was responsible for the oocyst removal. While it is possible for oocyst-particle association, a PCR-based detection study suggested that oocysts in secondary effluent were not particle-associated (Tsuchihashi et al., 2003). The association of protozoan parasites with wastewater particles still requires further investigation to determine if this occurs at different treatment stages and how this might impact oocyst removal and inactivation.

1.2.11 Impact of pathogen-particle associations on disinfection processes

1.2.11.1 Chlorination

Chlorination has been used for many decades and is the leading technology for disinfection of recycled water. Chlorine has high oxidizing capacity and is mostly used in high concentrations to kill pathogens, although high dosages can cause the formation of harmful by-products (Virto et al., 2005). Excessive use of chemicals beyond that required to achieve target levels of disinfection is also not cost-effective and increases the cost of producing reuse water. Chlorine reacts with the cell membrane and alters or damages vital cell functions (Venkobachar et al., 1977). Exposure to chlorine causes stress to microorganisms via irreversible cell injuries and in some cases it causes bacteria (e.g. *S. typhimurium*) to enter a viable non-culturable state if the dose is not high enough to cause outright cell death (Oliver et al., 2005). A major disadvantage of chlorination is that the majority of protozoans with cyst forms (*Toxoplasma*, *C. parvum and G. duodenalis*), helminths, and certain strains of bacteria are highly or moderately resistant to chlorine (Liberti et al., 2003). After disinfection, dechlorination is generally carried out to remove residual chlorine, which increases the overall cost of the process (Lazarova et al., 1999). This step is critical to protect the environment that receives any wastewater discharges because chlorine and derivatives (e.g. chloramines) are toxic to many aquatic organisms.

Microorganisms (bacteria and viruses) associated with particles are more resistant to chlorine compared with microorganisms free in suspension (Winward et al., 2008). The protective effect is related to the nature of the particle, with organic particles providing more protection compared with inorganic particles (Berman et al., 1988). Chlorine is able to penetrate particles by radial diffusion (Dietrich et al., 2003, Winward et al., 2008). in a two-step process in which it passes through different boundary layers of the wastewater particle (Figure 1.12). Chlorine

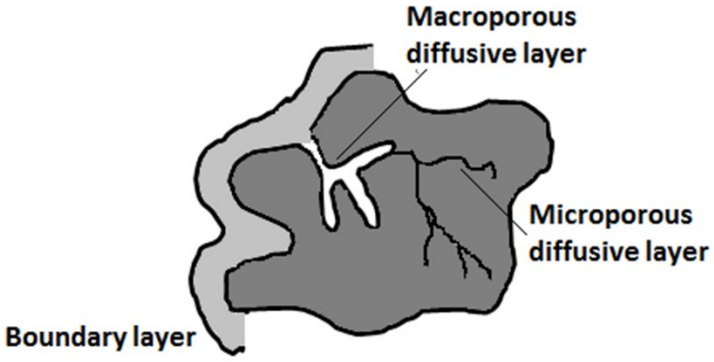

Figure 1.12 Wastewater particles structural pathways

Figure 1.12 Wastewater particles structural pathways: various interstitial diffusive layers of a wastewater particle. Modified from (Dietrich et al., 2003).

penetration of particles is therefore controlled by the initial chlorine concentration, which influences the diffusion rate (Winward et al., 2008). The presence of organic matter increases the chlorine demand of wastewater; the residual free chlorine reduces with an increase in the amount of organic matter and reduces the availability of free chlorine for disinfection (Winward et al., 2008). The presence of organic matter can also stabilize the cell membrane and reduce the sensitivity of bacteria to chlorine by reducing the access of chlorine to the cell membrane (Virto et al., 2005).

Pathogens embedded in a particle are further protected from chlorine due to the presence of the extracellular materials that surround it (Templeton et al., 2005). The protective effects of particles in terms of chlorine (or other oxidants) therefore can be linked to chlorine demand, membrane stabilization or the incomplete penetration of the chemical into the particle in the case of embedded pathogens (Dietrich et al., 2007).

The presence of particle-associated bacteria has been linked to a phenomenon known as tailing (Figure 1.13), which is the deviation of any disinfection process from first order kinetics at

relatively high doses of disinfectant (Loge et al., 2002, Dietrich et al., 2003, Winward et al., 2008, Liang et al., 2010). Tailing is characterized by no further increase in the inactivation of microorganisms even though increased amounts of disinfectant are applied (Liang et al., 2010). This phenomenon is problematic for the production of reuse water, since the survival of any pathogens or key process indicators will mean that the water is not fit for use and will require additional treatment to make it safe. Therefore, it is important to tailor the treatment processes so that disinfection processes can work with maximum efficiency, minimizing the chlorine dose required and any the residual chlorine, as well as minimizing the number of surviving cells.

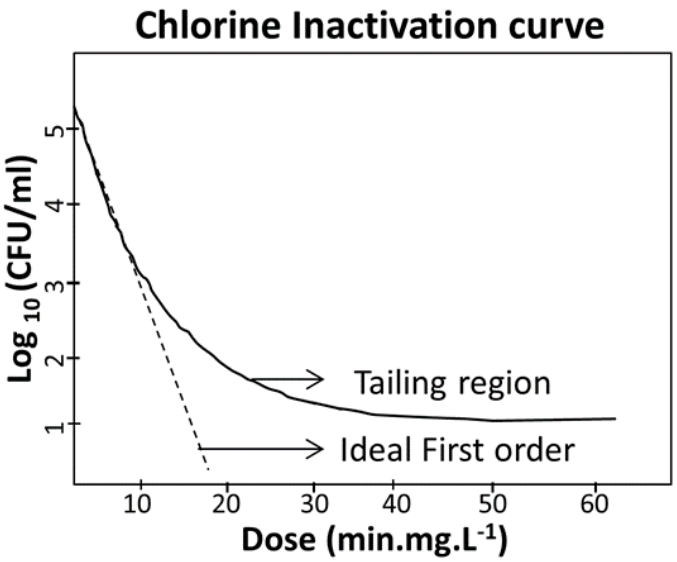

Figure 1.13 Typical Chlorine inactivation Graph

Figure 1.13 Typical Chlorine inactivation Graph: Graph of chlorine inactivation of microorganisms illustrating a first order disinfection curve (dashed line) and disinfection with tailing (solid line).

1.2.11.2 Ultraviolet Radiation

An effective alternative to chemical disinfection is UV radiation, which is a physical process that involves exposing water to a UV light source. UV radiation is preferred to chlorine because it does not cause the formation of toxic by-products (Lazarova et al., 1999, Hassen et al., 2000). The UV light spectrum can be split into UV-C (200 – 280 nm), UV-B (280 – 320 nm) and UV-

A (320 – 400 nm), with only UV-C used for disinfection (King et al., 2008). The other components of UV (UV-B and UV-A) occur in natural sunlight. Key biological components required by microorganisms are DNA and RNA, these molecules absorb light at 260 – 280 nm and so can be damaged by UV. The germicidal wavelength of UV light is 254 nm, which is the wavelength that causes maximum DNA damage by inducing DNA adducts called thymine dimers, which hinder normal transcriptional and DNA replication processes and prevent cell division (Gehr et al., 2003). Other wavelengths of UV across the spectrum cause cell death by damaging critical proteins that are required for cell function (King et al., 2008). There are two types of UV lamps used for disinfection, low-pressure UV lamps, which produce UV light around 254 nm, and medium-pressure lamps, which produce UV light from 200 – 300 nm (Chen et al., 2009, Craik et al., 2001).

UV radiation provides effective inactivation of bacteria, protozoa and some viruses (Sangsanont et al., 2012, Chen et al., 2009, Craik et al., 2001). There are two main formats for UV reactors, open channels, where UV lamps encased in quartz sleeves are suspended in the channel as water flows through it, and closed pipe systems, which are normally constructed of stainless steel with the lamps enclosed in a quartz sleeve and sited in the middle of the pipe (Lazarova et al., 1999, Hassen et al., 2000, Templeton and Butler, 2011b). UV disinfection has been shown to be very effective for the inactivation of pathogenic protozoans such as *C. parvum and G. duodenalis*, with UV doses of 25mJ/cm^2 resulting in 3 $\log_{10}$ reduction of *Cryptosporidium* oocysts and doses of 40 mJ/cm^2 have shown 4 $\log_{10}$ reduction of *G. duodenalis* (Craik et al., 2001, Linden et al., 2002). Many studies have shown that low pressure UV doses of 30 to 40 mJ/cm^2 can cause 4 $\log_{10}$ inactivation of pathogenic viruses; however, a high dose of 200 mJ/cm^2 is required to inactivate (4 $\log_{10}$) adenoviruses (Eischeid et al., 2009). In the case of adenovirus medium pressure UV is more effective, with a lower wavelength around 220 nm associated with the inactivation (Chen et al., 2009). UV has also been shown

to cause 0-1.5 $\log_{10}$ reduction of *Ascaris lumbricoides* eggs, which are one of the most resistant pathogens to other disinfection processes (Brownell and Nelson, 2006).

There are some limitations with UV disinfection. The first is that it does not provide any residual disinfection, which means that any surviving microorganisms can regrow post disinfection and also that if there is any subsequent contamination of the water (e.g. due to a pipe break) then there is no disinfectant to inactivate any introduced contaminants. Another is that many microorganisms have systems for the repair of UV-induced DNA damage, which means that they can regain the capacity to grow or cause infection if the level of UV damage is not enough to overwhelm the capacity of these repair systems (Hassen et al., 2000).

Factors that affect the efficiency of UV include turbidity, suspended solids, dissolved organic carbon, lamp sleeve fouling and lamp aging (Hassen et al., 2000). The presence of organics causes attenuation of the light, which can be overcome by the use of sufficient lamp power. However, as with chlorine, particles (or cell aggregation) can also cause tailing. An example of a UV dose response curve is shown in Figure 1. 14, with the initial steep slope indicating the inactivation of free in suspension microorganisms, followed by a plateau in inactivation representing tailing that is caused by particles (Gehr et al., 2003, Farnood, 2014).

Particles can shield microorganisms in different ways, by providing shading or partial absorption of the UV energy to reduce the effective dose, or by scattering the light (Figure 1.15). Large particles have been shown to affect the disinfection process more than smaller particles (Jolis et al., 2001) and particles of 50 μm or greater can completely shade pathogens from UV (Blume et al., 2002). Particles around 10 μm affect disinfection because they are capable of shielding embedded bacteria from UV radiation. In addition, some smaller particles (such as inorganic silica) can scatter the UV light and limit light penetration through the reactor, protecting microorganisms. Larger particles can be easily removed by filtration prior to

disinfection indrinking water treatment facilities (Templeton et al., 2005). Therefore, if filtration can be applied upstream at wastewater treatment process, then large particles can be easily removed and the effectiveness of disinfection can be improved for both chlorination and UV.

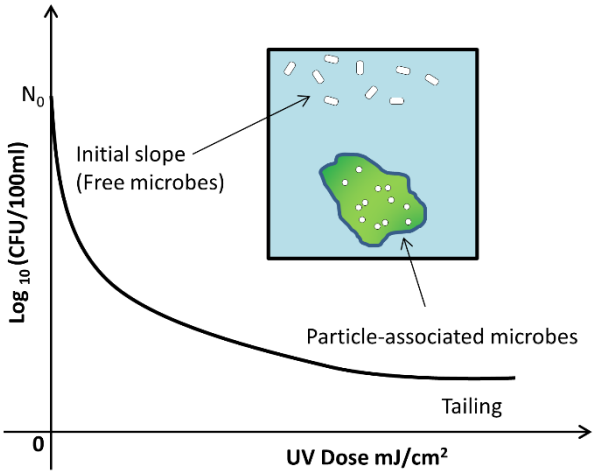

Figure 1.14 Typical UV inactivation curve

Figure 1.14 Typical UV inactivation curve for microorganisms comparing log inactivation versus UV dose, highlighting the initial shoulder at lower UV doses, the steep inactivation slope representing inactivation of free microorganisms and a shallow slope representing tailing. Reproduced from (Farnood, 2014).

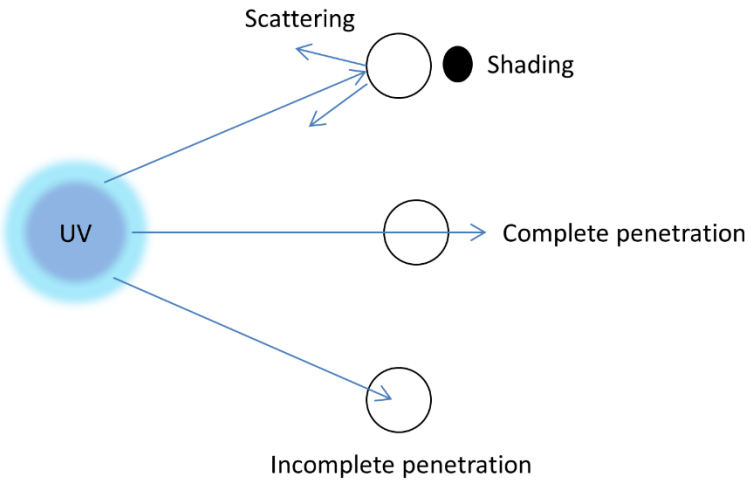

Figure 1.15 Limitations of UV radiation

Figure 1.15 Limitations of UV radiation: different protective effects on inactivation of pathogens by UV radiation.

Smaller particles can easily pass through filters and viruses are associated with these smaller particles (Hejkal et al., 1981b). However, these smaller particles tend to provide less shielding, although this is dependent on whether the particle is organic or inorganic (Jolis et al., 2001, Templeton et al., 2005). Bacteria can contribute to the formation of particles by forming aggregates, a natural phenomenon known as bioflocculation. This is often mediated by EPS, which not only holds the bacterial floc together but also provides protection to the enmeshed bacteria by absorbing UV radiation. Consequently, bacterial flocs are also a cause of tailing (Farnood, 2014). The formation of flocs can be related to the density of microorganisms, and similar aggregation can also occur in the absence of EPS when there are high densities of virus particles or protozoan (oo)cysts. This aggregation of microorganisms presents a challenge for measuring UV dose responses – if the number of organisms used in experimental systems is too high then tailing caused by aggregation occurs and the dose response will not be correctly determined. In addition, the aggregation of microorganisms confounds culture-based enumeration and affects the accuracy of direct counting methods such as microscopy of flow cytometry.

1.2.12 Concluding remarks

Wastewater is becoming increasingly important to society, not only because it can be used to augment dwindling fresh water supplies, but also because it can be used for energy production and the recovery of nutrients and other resources. In order to realize these benefits, wastewater needs to be treated sufficiently to ensure that it is affordable while still protecting public health. This is an important consideration because without public confidence in recycled water it will not be accepted, but at the same time if it is too expensive then consumers will use the cheapest water available, which is often surface water or ground water.

One of the major costs associated with the production of reuse water is treatment for pathogen removal or inactivation. While chemical contaminants are also important, health regulators tend to focus on contaminants that cause acute disease, especially in the context of non-potable reuse of wastewater when chronic human exposure is unlikely. With this in mind, an understanding of the fate of pathogens through wastewater treatment and disinfection processes, as well as knowledge of the factors that influence these processes, is required to ensure optimal treatment for managing the risk from pathogens in wastewater. One of the major influencing factors on the fate of pathogens in water is association with particles. It is therefore important to understand the nature of pathogen-particle associations, the factors influencing formation and stability of the association and how the association affects treatment disinfection processes.

Much of the knowledge regarding pathogen-particle interactions has been gathered from studies of fresh water or storm water systems and we know that various particle characteristics such as shape, size, composition and structure all play important roles in the association process. However, there have been few studies characterizing particles in wastewater and the nature of the associations between pathogens and particles in wastewater remains a knowledge gap. Similarly, there have been studies on the impact of particles on the disinfection of some pathogens or pathogen indicators in wastewater, but these have not examined the nature of the pathogen-particle interactions and how these affect disinfection. Future studies need to characterize particles and pathogen-particle interactions along different stages of the wastewater treatment train, to provide a better understanding of how the different treatment steps influence the nature of the particles present and their interaction with pathogens. A better understanding of this behavior may identify ways to modify processes to alter pathogen partitioning or identify other treatment strategies for dealing with particle-associated pathogens. Controlling pathogen-particle associations provides an opportunity to enhance

wastewater treatment and reduce treatment costs; increased levels of association can enhance removal by sedimentation processes, reduced levels of association can enhance disinfection.

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Chapter 2

General Methods:

2.1 Bolivar Wastewater treatment plant (BWWTP)

The Bolivar Wastewater treatment plant was commissioned between 1964 and 1969 (Sweeney et al., 2005b).There are three different treatment plants located at the Bolivar site; the Bolivar WWTP (BWWTP), the Bolivar high salinity WWTP (HSWWTP) and the Bolivar dissolved air floatation and filtration plant (DAFF) (Holmes et al., 2010). The BWWTP is South Australia's largest WWTP (Wen et al., 2009) and it serves a population of 750,000 Equivalent persons every day (Liu et al., 2012). It is located 18 Km north of Adelaide, South Australia at 34°45'23"S and 138°34'15"E (Sweeney et al., 2003, Sweeney et al., 2005a). The plant processes almost 60% of Adelaide's domestic and industrial wastewater with a running capacity of 130 ML of wastewater per day (Smernik et al., 2004) with a mix of approximately 75% domestic and 25% industrial (Liu et al., 2012). The climatic conditions vary from hot and dry Mediterranean summers (16°C/29°C) to mild and moderate winters (7°C/16°C). The direction of the wind varies and the average wind speed is 4.3 m/s (Sweeney et al., 2003).

The BWWTP was originally established as a three-stage treatment process. The primary treatment incorporates screening, grit removal, aeration and sedimentation, and is followed by secondary treatment using trickling filters and clarification. The effluent is tertiary treated through a series of six waste stabilisation ponds (WSPs) (Cromar et al., 2005). To enable the production of Class A recycled water, a DAFF plant was built and commissioned in the year 1999. Due to issues with odour and changes to the Environmental Protection Act limited nutrient discharge to St Vincent Gulf, the trickling filters were replaced with an activated sludge plant (ASP) in the year 2001 (Sweeney et al., 2005b). The current operating plant (Fig 2.1 (reproduced from (Liu et al., 2012)) has four primary sedimentation tanks, four aeration tanks, eight secondary circular settlers/clarifiers, six stabilisation lagoons (generally only three are operating in summer) and four rectangular flocculation tanks (Wen et al., 2009, Lee et al., 2010).

Wastewater influent enters pre-treatment, where it flows through screens and grit removal aeration tanks. Each of the circular clarifiers has a volume of 5278 m^3 and is 40m in diameter, 3m high at the perimeter and 6.6m high at the centre. Each of the rectangular flocculation tanks has a volume of 6693 m^3 and is 68.4m long, 23.3m wide and 4.2m high (Lee et al., 2010). The aeration tanks have total retention time of 18 hours, which includes 12 hours of aeration and 6 hours of no aeration (Wen et al., 2009). Six stabilisation lagoons are arranged in two parallel rows of three ponds each. The total surface area of the lagoons is 346 ha with an average depth of 1.4 m and a residence time of around 27 days (Cromar et al., 2005, Sweeney et al., 2005b, Holmes et al., 2010). Lagoon 1 is the largest of all the six ponds with a depth of 1.4 m and a residence time of 12 days (Sweeney et al., 2003, Sweeney et al., 2005a). The secondary effluent from clarifiers typically enters stabilisation ponds with a retention time of 27 days (Liu et al., 2012). The tertiary effluent is either released via a marine outfall or polished by the dissolved air floatation/filtration (DAFF) plant, which can process 120 ML of lagoon effluent per day. This DAFF plant uses alum coagulation, flocculation, dissolved air floatation, dual media (sand and coal) filtration and chlorination. This treated water meets class A standards and is used as recycled water for agriculture and domestic use.

There are two stages of sludge treatment; anaerobic digestion and stabilisation of in the sludge lagoons. Mesophilic anaerobic digesters operate at 34°C with a retention time of 7 days and the Biogas produced during this process is harvested and used for energy generation. Sludge flows from the digesters to the sludge stabilisation lagoons for settling, evaporation drying and finally stock piling (Smernik et al., 2004, Liu et al., 2012). Following appropriate holding times (stockpiling), the Biosolids are beneficially reused as a soil conditioner in farms.

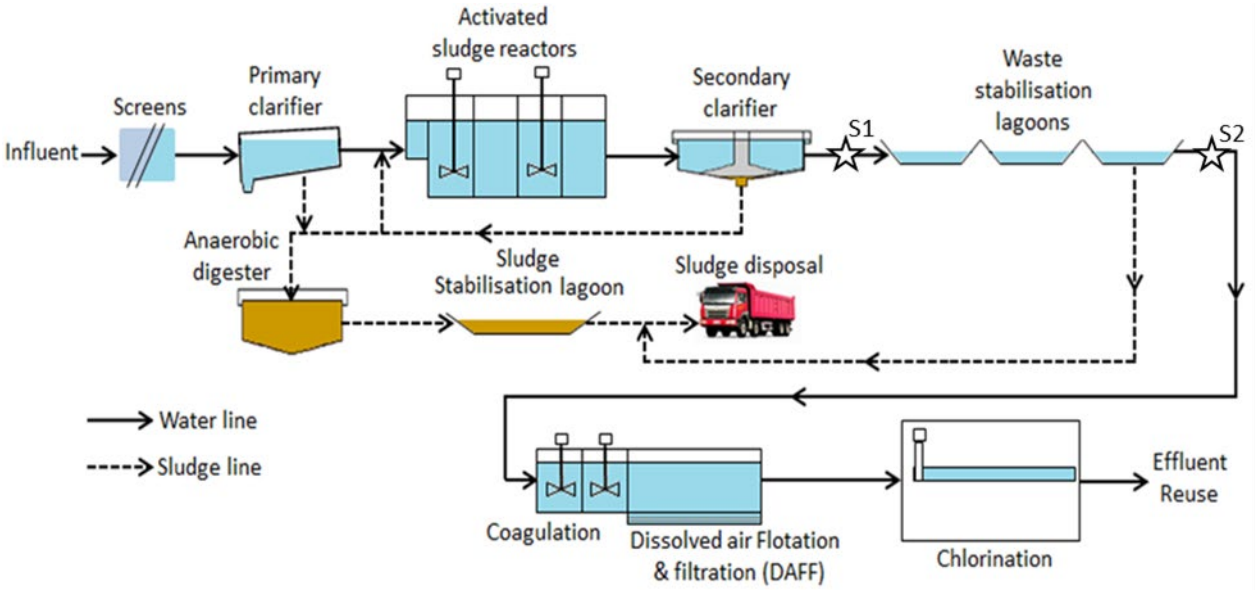

Figure2.1 Schematics of Bolivar wastewater Treatment Plant

Figure 2.1 Schematics of Bolivar wastewater Treatment Plant located in Adelaide, SA. Various components and process flow diagram of BWWTP (reproduced from (Liu et al., 2012)). S1- Adelaide secondary effluent collection point, S2- Adelaide lagoon effluent collection point.

2.2 Western wastewater treatment plant (WWWTP)

The Western wastewater treatment plant is one of the biggest in the world and covers an area of 10800 ha near Werribee in the Port Philip region, 32 km southwest of Melbourne, Victoria (Loyn et al., 2014). Western WWTP treats 485 ML/day for 2 million people, more than half of Melbourne's population (Goodman et al., 2013). Western WWTP lies in a temperate climatic zone at 37°51'0.00 S and 144º 33' 59.99 E with an annual rainfall of 515 mm (Paul, 1996). Western WWTP, formerly known as the Werribee farm or Werribee treatment complex, first became operational in the year 1897 (Barker et al., 2011) and combines ponds, land filtration and grass filtration for treatment (DeGarie et al., 2000). Ponds operate the entire year, while land treatment operates in the summer and grass filtration operates in the winter (DeGarie et al., 2000). Land filtration was the first treatment method used, starting in 1897. Large open paddocks are filled with 10 cm of sewage and the soil acts as a filter (Barker et al., 2011). Grass filtration started in the 1930's and comprised two-step filtration (Barker et al., 2011). Sewage

is first allowed to settle in large sedimentation tanks. Large heavy rubbish settles to the bottom and lighter rubbish floats on the top layer. The middle layer of primary treated effluent flows on slanting bays of grass lands. Sewage filters through the grass and the clear effluent flows out through earthen drains (Barker et al., 2011). The first treatment lagoon came into existence in 1936 and the first modern lagoon was started in 1986 (Barker et al., 2011).

Almost 70 % of sewage is treated in waste stabilisation ponds and the rest by land and grass filtration. The plant consists of a series of multi-pond systems covering an area of 1160 ha (McLean et al., 2000); 25 West (25W), 55 East (55E), 115 East (115E) and Old Lagoons (De Maria et al., 2006). The new generation lagoons 115E, 55E and 25W consist of 10 ponds in series and occupy areas of 200, 280 and 271 ha respectively (Paul, 1996). The first pond comprises two sections; anaerobic and aerobic (De Maria et al., 2006). The rest of the ponds are facultative and maturation ponds (Fig 1.7 reproduced from (Miettinen et al., 2004)). The lagoon systems have been upgraded from time to time. Before upgrading, 25 W comprised 10 ponds in series. A recent upgrade involved conversion of a part of pond 1 of 25W to include an ASP to increase nitrogen removal (Mitchell Laginestra and van-Oorschot, 2009, De Maria et al., 2006). The ASP is configured in a Modified LudzackEttinger format comprising an anoxic zone and an aeration basin with 5 separate aeration zones. The sewage from the ASP flows into 5 clarifiers (45 m wide) and effluent from the clarifiers is returned back to Pond 2 and then moves to the maturation ponds (Mitchell Laginestra and van-Oorschot, 2009, De Maria et al., 2006). The maturation ponds then provide 25 days hydraulic retention time for inactivation (De Maria et al., 2006).

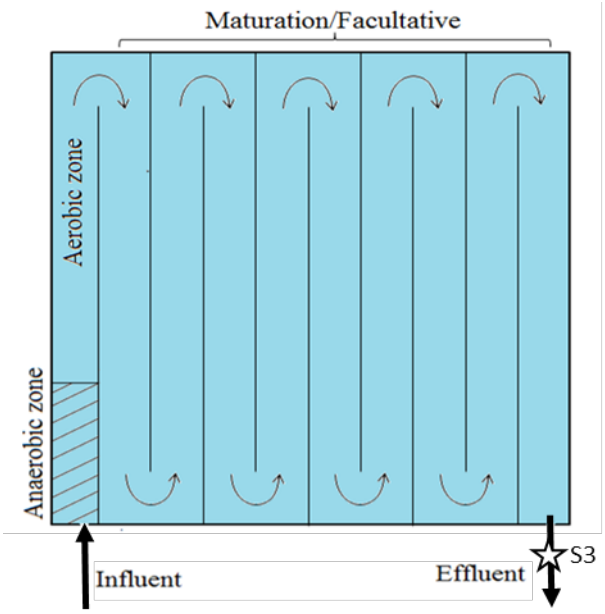

Figure 2.2 Multi pond systems at Western treatment plant

Figure 2.2 Multi pond systems at Western treatment plant. A series of 10 anaerobic, aerobic and maturation ponds describing a typical pond system at western treatment plant Melbourne (reproduced from (Miettinen et al., 2004)). S3- Melbourne lagoon effluent collection point.

2.3 Overview of methods

Three research themes were examined by applying a suite of complimentary methods (Fig 2.3).

This framework is used to describe the methods in detail below.

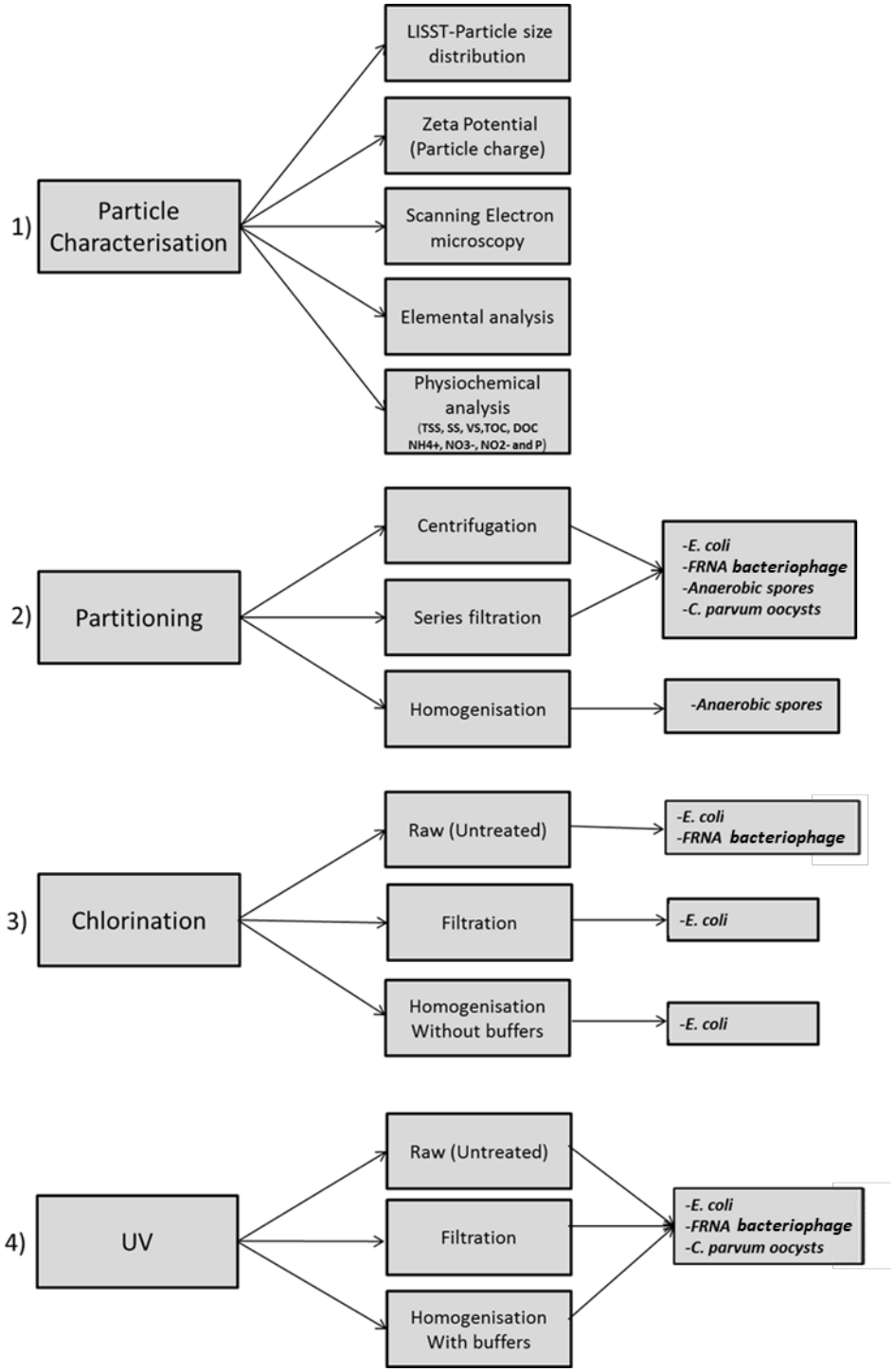

Figure 2.3 Methods overview

Figure 2.3 Methods overview: Schematics of the various methods used in the study.

2.3.1 Sample collection

Treated effluents collected from BWWTP were collected from points both upstream (Adelaide secondary effluent) and downstream (Adelaide lagoon effluent) of wastewater lagoons, whereas only lagoon effluent from a point downstream of 55E lagoon was collected from the WWWTP (Melbourne lagoon effluent). Grab samples (10L) were collected once a month in thiosulphate (20%) dosed jerry cans, transported on ice and stored at 4°C before analysis. All the analyses were performed in duplicate or triplicate within 24 hours of sampling.

2.3.2 Wastewater characteristics

Three sub-samples were taken from each 10 L parent sample and characterised by examining suspended solids (SS), total suspended solids (TSS) and volatile solids (VS) according to the standard methods for the examination of wastewater (A.P.H.A., 1998). Concentration of NH_3, NO_2^-, NO_3^- and total P within the samples was measured using the respective HACH calorimetric test kits 10031, 10019, 10020 and 8048. Samples were sent to the Australian Water Quality Centre (AWQC) for total organic carbon (TOC) and dissolved organic carbon (DOC) analysis. Scanning electron microscopy coupled with elemental analysis were used to identify various particles and their composition in the samples (details in section 3.4). Surface charge of the particles was determined by measuring zeta potential of the samples in triplicate (Section 3.5).

2.3.3 Particle size distribution

Triplicate 100 mL subsamples of each sample were analysed for particle size distribution using laser in situ scattering transmissometry (LISST-XR, Sequoia Instruments). The LISST measures the volume of each particle size class as a function of the sample volume (µL of particles / L of sample). This was converted to a count by assuming particles were spherical and using the volume of a sphere to estimate the number of particles in each size class. The particle counts for each size class were then grouped into size ranges; <1.2 µm, 1.2-10 µm, 10-100 µm and >100 µm. The particle count of each group was $\log_{10}$ transformed for analysis.

The means were then compared with Tukey's multiple comparison test using Graphpad Prism (version 7.03) and two-way analysis of variance was performed by setting the interaction between sample and month for each size group.

2.3.4 Partitioning Techniques

2.3.4.1 Partitioning by centrifugation:

Samples were removed from refrigeration and inverted gently to resuspend the settled particles. Slow-speed centrifugation was used to separate pathogens attached to denser particles from lower density free floating microbes and from microbes attached to lighter particles. It involved centrifuging 2 X 900 mL aliquots of each sample at 931 X g for 10 minutes at 4° C with a brake speed of 4 using a Beckman Coulter Allegra X-12 R centrifuge. The conditions were optimised by testing different speeds from 200-1200 X g and analysing particle profiles to observe separation. Centrifugation at 931 X g removed 81% of particles and increasing the speed had no further effect on particle removal. After centrifugation, 600 mL of supernatant was removed for analysis and the pellet was resuspended in the remaining 300 mL of supernatant. The organism loads in the supernatant were estimated by multiplying the average numbers recorded on the plates by the total supernatant volume (900 mL). The organism load in the pellet (only for validation experiments) was calculated by multiplying the average numbers recorded on the plates by the total pellet volume (300 mL) and for the following set of experiments, the number of organisms in the pellet was estimated by subtracting the number of organisms in 300 mL supernatant from the number of organisms in the resuspended pellet (which = pellet + 300 mL supernatant).

2.3.4.2 Partitioning by serial filtration:

It was performed using 10 µm (Millipore K99CP04700), 1.2 µm (Millipore RTTP04700), 0.4 µm (Millipore HTTPP04700) and 0.2 µm (Millipore GTTPP04700) polycarbonate filter

membranes to determine particle-microbe association at various size ranges. Caking of filter membranes was minimised by selecting filtration volumes based on their turbidity; 200 mL, 120 mL and 160 mL for Adelaide secondary effluent, Adelaide lagoon effluent and Melbourne lagoon effluent respectively. Monthly samples were filtered in series and the filtrate was collected after each filtration step for further analyses. The filter retentate was recovered by placing filter membranes into tubes with 40 mL phosphate buffered saline (PBS), followed by shaking using a Vortex Genie 2 vortex mixer. The tubes were shaken for 5-10 minutes with a break of 30 seconds after every 2 minutes to dislodge and analyse the retained particles and microbes. The organism loads were calculated by multiplying the average numbers of organisms recorded on the plates with the volume used for the experiment.

2.3.4.3 Partitioning by homogenisation:

Two dispersion protocols, using a WiseTis Digital homogenisor (HG15D), were followed to disaggregate larger particles and release particleassociated or aggregated spores into the samples. Technique 1 involved homogenising 100 mL wastewater samples (90 mL sample + 10 mL of 10X dispersion buffer) for 1.5 minutes at 13,500 rpm in the presence of a dispersion buffer (Buffer A), which consisted of 1 µM Zwittergent 3-12, 1 mM EGTA, 0.1 M Tris buffer (final concentration) (Li et al., 2009). Technique 2 involved homogenising 100 mL wastewater samples (90 mL sample + 10 mL of 10X dispersion buffer) for 4 min at 8000 rpm in the presence of a dispersion buffer (Buffer B), which consisted of 100 mg/L M Zwittergent 3-12 only (final concentration) (Caron et al., 2007b). Triplicate samples were processed using a WiseTis Digital homogeniser (HG-15D).

2.3.5 Microbial analysis:

E. coli and FRNA bacteriophage were selected because they are accepted surrogates for pathogenic bacteria and enteric viruses, respectively, in wastewater systems (van den Akker et

al., 2014, Young et al., 2016). *E. coli* and FRNA bacteriophage were measured in raw (untreated) and treated samples, supernatants and pellets after centrifugation, and filtrate and filter membrane retentate after filtration. Standard EPA method 1604 was used for detection of *E. coli*. This involved filtering 100 mL of a sample or prepared dilution of each sample (in triplicate) through a 47 mm diameter, 0.45 µm filter membrane and incubating the membrane on MI (MuGal and IBDG) agar (Becton Dickinson 214882) plates overnight at 35 °C (Oshiro, 2002). The total numbers of *E. coli* were determined by counting visible blue colonies that also fluoresced under UV light, recorded as colony forming units (CFU) per 100 mL. FRNA bacteriophage were quantified on Tryptic Soy Agar (TSA) medium (Oxoid CM0131) using a double-layer agar plaque assay adopted from(Noble et al., 2004). Triplicate 1 mL sub-samples were added to a concentrated molten TSA agar overlay, poured onto the base agar plate, allowed to set and then incubated inverted overnight at 37 °C using *E. coli* (American Type Cell Culture (ATCC) 700891) as a host. Plaques (visible zones of clearance) were then enumerated across the whole plate and represented as plaque forming units (PFU) per mL. Anaerobic spores were enumerated using the standard method AS/NZ 4276.17:2000 (detailed in section 5.2.4). *C. parvum* oocysts were enumerated using fluorescent microscopy (detailed in section 5.2.6).

2.3.6 Disinfection

Chlorine and UV radiation were used to disinfect the samples and analyse the disinfection kinetics of *E. coli*, MS-2 phage and *C. parvum* oocysts (Detailed in section 6.2.6 and 6.2.7).

Chapter 3: Particle characterisation of wastewater effluents from two Australian treatment plants

3.1 Introduction:

Wastewater is a reservoir for human pathogens that present a major health risk (Cai and Zhang, 2013, Ajonina et al., 2015, Zhou et al., 2015). The quality and quantity of wastewaters vary but they are generally comprised of microorganisms, organics, nutrients, metals and inorganics (Henze and Yves, 2008, Abdel-Raouf et al., 2012). Particulate matter in wastewater can be either organic or inorganic and of various shapes and sizes (Crittenden et al., 2012). The nature and types of particles in wastewater are affected by various physicochemical and biological processes in a particular treatment process (Shon et al., 2007a). Treatment systems differ by the types of particles that they remove or produce; for example, waste stabilisation ponds are usually dominated by phytoplankton and the density and type varies with the type of pond design, nature of waste and season (Mara, 2013, Buisine and Oemcke, 2003). On the other hand, the activated sludge process, which is a suspended growth biological system that is widely used to remove nutrients, is dominated by microorganisms that exist in a microbial floc, which include bacteria, viruses, fungi, protozoa, metazoa and algae (Schmit et al., 2009, D'Antoni et al., 2017).

Pathogens such as bacteria, viruses and protozoa can either be dispersed (free floating) or associated with particles in wastewater (Emerick et al., 2000, B. Ormeci, 2003, Templeton et al., 2008). Pathogens in water can range from viruses (20 - 80 nm diameter), bacteria (0.5 - 3 µm), protozoans (4 - 18 µm) and larger organisms (Ferguson et al., 2003). The size and surface characteristics of particles affect the particle-pathogen associations (Madge and Jensen, 2006a, Templeton et al., 2008) and particles (both inert and biological) can affect pathogen removal by causing tailing and reducing the efficacy of disinfection. This can occur through different mechanisms, such as shading or shielding (Madge and Jensen, 2006a). Particle shielding can affect disinfection by oxidants such as chlorine or by ultraviolet (UV) irradiation. Dietrich et al. (2003) reported that higher chlorine penetration within a wastewater particle can be

achieved by increasing the initial chlorine concentration. However, this might also increase the resulting residual chlorine concentration and disinfection by-product formation. Similarly, increasing the UV dose may improve the inactivation of particle-associated pathogens but increase capital requirements and operational costs. Ideally, a targeted treatment system will balance removal of particles with optimal disinfection. However, depending on the treatment system, particle profiles can be dynamic and change during the course of a day or as a function of season. Different types of particles may appear at different times of year due to various seasonal changes, which would again create different possibilities for interactions. Given that each treatment plant might have a unique profile of particulate material, it is important to characterise the particles from different stages of treatment and elucidate the ways microbes associate with particles. Such data will inform the design of cost-effective treatment options with the potential to produce recycled water at a lower cost.

There are a variety of ways to characterise particles, including visualisation by microscopy, particle size distribution analysis, charge analysis and physicochemical measurements. Scanning electron microscopy coupled with elemental analysis has been used previously as a visualisation tool to observe the shape of particles and also to analyse chemical composition (Adin, 1999). The presence of certain elements can help identify the type of effluent (Adin, 1999). Particle size distribution is an important tool in wastewater characterisation and has been used previously for a number of different applications, including characterising contaminants and evaluating treatment process performance (Levine et al., 1991, Dogruel et al., 2009, García-Mesa et al., 2010). Electrostatic charges have been found to influence the interaction of bacteria and viruses with particles (Daniels, 1980, Rijnaarts et al., 1999, Stevik et al., 2004, Templeton et al., 2008, Wong et al., 2012). Zeta potential provides an indication of the surface charge and has also been used previously to characterise wastewater particles and evaluate the influence of particle charge on treatment processes (Adin, 1999, Yap et al.,

2012). Other gross parameters important in the characterisation of wastewater are chemical oxygen demand, biological oxygen demand, organic carbon, turbidity and solids (Bisschops and Spanjers, 2003).

This study was designed to characterise particles within treated wastewater samples from two geographically separated treatment plants with different treatment processes, catchments and environmental factors. This quantitative analysis of wastewater particles was conducted in the context of a broader aim to examine the interactions between particles and pathogens in order to optimise pathogen removal and overall treatment efficiency.

3.2 Methods:

3.2.1 Sample collection:

Grab samples (10L) were collected once each month throughout the year (May 16 –Apr 17) in thiosulphate (20%) dosed jerry cans, transported on ice and stored at 4°C before analysis. All the analyses were performed in duplicate or triplicate within 24 hours of sampling.

3.2.2 Physicochemical parameters: Concentrations of TSS, VS, SS,

NH_3, NO_3^-, nitrite (NO_2^-) and total P were measured in duplicate as described in section 2.3.2 (Chapter 2). Other parameters such as Turbidity and the pH were also recorded in duplicate as described in section 2.3.2 (Chapter 2).

3.2.3 Particle size distribution (PSD) was determined using Laser *in situ* Scattering Transmissometery (LISST) on a portable XR (Sequoia Instruments, Washington, USA) as described in section 2.3.3 (Chapter 2).

3.2.4 Scanning Electron Microscopy (SEM) and elemental analysis. Three samples were collected from each site in each of three months; May (autumn), June and July (winter). These months were selected to avoid overestimation of the type of particles caused by algal blooms

in the warmer months. The samples were brought out of refrigeration and left at room temperature for an hour prior to analysis. Each sample (n=9) was filtered in triplicate through 13 mm diameter (area 132.67 mm^2) and 0.8 μm pore size polycarbonate filter membranes (n=27 filters, Rowe Scientific 025351). Filters were loaded with 3-5 mL of sample to maximise the coating of the membrane surface and to avoid background noise from the filter during elemental analysis. Filters were dried overnight on the slides in a desiccator. No dehydration, fixing or freezing was performed on samples and dried filters were directly mounted on aluminium stubs using double sided carbon tape. Samples were splutter coated with a 5 nm layer of platinum and viewed with an Inspect FEI F50 scanning electron microscope under an operating voltage of 10-20 kV. Digital images were obtained using SEM combined with the software TEAM™.

The elemental composition of the particles was investigated with an EDAX energy dispersive X-ray (EDX) micro-analyser coupled with SEM. Counting time of the X-ray spectra was 30 live seconds. The average area scanned on each of the 27 filter membranes was $1mm^2$, in approximately six sub-areas. Large areas of each 132.67 mm^2 filter did not contain particles, and the SEM was used to find particles for elemental analysis. The six areas analysed on each filter ranged from approximately 10 to 450 μm^2 to encompass different sized particles. The particles selected for elemental analysis and imaging were representative of all the particles on that filter and there were no analyses included of singleton or rarely found particles. The replication (9 filters from each site and each month) and numbers of areas scanned (n=6 per filter) therefore constituted a semi-quantitative analysis. Blank filter membrane was used as a control to identify background elements from the filter. The Y-axis shows proportion of each element in the area of the image that was analysed and includes the background matrix.

The elemental analysis supported visual and morphological identification of particles as being 'biological' or 'inorganic'. Digital images obtained from different filters were magnified a further 300x and overlain with a grid approximately 5x5 µm. The percentage of area in each square in the grid was assessed as being occupied by material that was 'biological', 'inorganic' or empty (Fig 5). When the quality of the image was too poor to be able to assess individual particles, that square was not included, hence the numbers of squares assessed in each image varied from 55 to 85. Four images from each lagoon were examined, and a total of 40540 μm^2 (Adelaide) and 51492 μm^2 (Melbourne) was scored for particle type. The mean±stdev percent values of four images (filters) were calculated for each parameter: 'biological', 'inorganic' and 'empty'. The number of large particles with dimensions in the 20 - 100µm range, and the area of the image they appeared in, was also recorded.

3.2.5 Surface charge: The zeta potential of the particles in samples was measured by electrophoretic light scattering (ELS) using a Malvern Zetasizer WT. Triplicate samples (1 mL) were injected into cuvettes and the surface charge was measured by a laser which scans the particles as they move towards the oppositely charged electrode in the presence of an electric field (Jiang et al., 2009). The zeta potential measured by the analyser was related to surface charge of particles in water. To analyse charge distribution over different size ranges of particles, samples were serially filtered through 0.2, 0.4, 1.2 and 10 µm polycarbonate filter membranes. The zeta potential of all the filtrates was measured in the same way.

3.2.6 Statistical analysis:

The average particle size distribution profile obtained from three subsamples was used to group particles into different size classes, and the mean±stdev number of particles x 10^{12} per L (n=12) of different size classes shown. All the physiochemical parameters were plotted as mean±stdev.

For each location, the area occupied by 'inorganic' or 'biological' particles in SEM images was presented as mean±stdev particle areas in four images from different filters.

For zeta potential analysis, the interactions were set between each filtrate and month for each sample, and sample and month for each filtrate. Two-way analysis of variance was used to compare the differences between samples and months by performing Tukey's multiple comparison test using Graphpad prism 7.03 with significance assigned at $p<0.05$. Pearson's correlation test was performed to analyse any relationships between particle size and turbidity for each of the sample.

3.3 Results:

For all effluent types, the smallest particles were present in the highest numbers, with the number of particles decreasing as the particle sizes increased (Fig. 3.1). The average numbers of particles smaller than most enteric bacteria (0.37-1.01 µm) ranged from 7.2 x 10^9 particles / L for Adelaide secondary effluent to 6.3 x 10^{10} particles / L in Adelaide lagoon effluents (Fig 1). Particles in the size range of bacteria (1.19 – 3.7 µm) ranged from 2.9 x 10^8 particles / L to 2.5 x 10^9 particles / L (Fig. 3.1). Enteric protozoa such as *Giardia* (~12 – 15µm long and 6-8 um wide (Adam, 1991), and *Cryptosporidium* (~4-6µm) (Xiao et al., 2004), are in the 3.7 - 14µm size range; the average number of particles in the size range was between from 2.8 x 10^7 particles / L to 3.9 x 10^8 particles / L (Fig. 3.1). Particles in the largest size class (14 – 104 µm), which includes larger organisms such as protozoans, diatoms, colonial algae and zooplankton, ranged from 3.6 x 10^6 particles / L to 1.8 x 10^7 particles / L (Fig. 3.1). Particles in Melbourne lagoon effluent ranged from 6.9 x 10^9 particles/L (0.37 to 1.01 µm), 1.9 x 10^8 (1.19-3.7 µm), 1.6 x 10^8 (3.7 -14 µm) and 1.8 x 10^7 (14-104 µm). Overall, Adelaide lagoon effluent had the

highest monthly average number of particles in all size classes, followed by Melbourne lagoon and Adelaide secondary effluent (Fig 3.1).

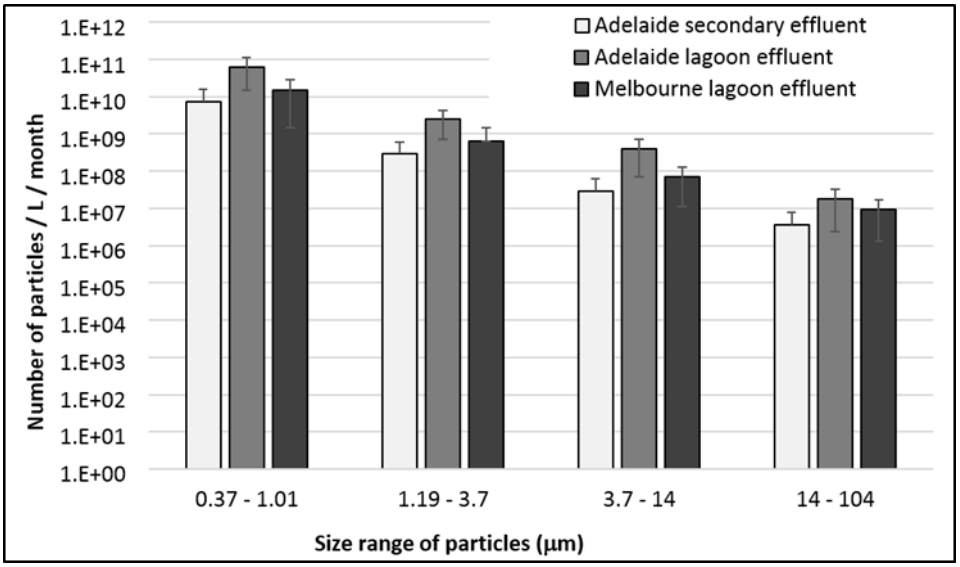

Figure 3.1 Number of particles in wastewater

Figure 3.1 Number of particles in wastewater. Each month for a year, three 100 mL subsamples of Adelaide secondary effluent, Adelaide lagoon effluent and Melbourne lagoon effluent were collected and analysed by Laser In Situ Scattering (LISST). The average particle size distribution profile obtained from three subsamples was used to group particles into different size classes (x-axis), and the mean±stdev number of particles x 1012 per L (n=12) shown.

Contour plots were used to compare the particle composition of effluent samples collected on different months, for both particle counts (Fig 3.2) and particle volumes (Fig 3.3). Due to the large difference in counts / volume for the different size classes, the data were log transformed to allow them all to be visualised in the same graph. While small particles (<1 µm) accounted for >90% of the particle count (Fig 3.2), it was particles in the 10 – 100 µm range that accounted for most of the particle volume (Fig 3.3). The particle profiles were similar between Adelaide and Melbourne lagoon samples for winter (June – August, Fig 3.2 and Fig 3.3). In the case of the Adelaide lagoon samples, the particle numbers were higher in summer versus winter, particularly for particles in the 0.37 to approx. 50 µm size classes (Fig 3.2). This difference was also reflected in the particle volumes, with particles in the 5 – 50 µm size range having the largest contribution to total particle volume for October 2016 – April 2017 (Fig 3.3). The

particle profiles for the Adelaide secondary effluent samples were generally more stable compared with the Adelaide lagoon effluent, with the exception of a large spike in particle number and volume in October 2016, and smaller spikes in July 2016 and March 2017 (Fig 3.2, Fig 3.3). In general, the particle load exiting the Adelaide lagoon were higher compared to the load entering from the secondary effluent (Fig3.1, Fig 3.2). Aside from differences in total particle load, the secondary effluent had a higher proportion of particles larger than 100 µm when considering particle volume (Fig 3.3).

Even though the two lagoons were separated geographically, and by differences in the types of treatment processes used to treat the WW before input into the lagoons, the pattern of particle size distribution was more similar in the two lagoons (Fig 3.2B and 3.2C) than either were to the Adelaide secondary influent (Fig 3.2A), particularly for particles >10 µm. However, for particles < 5µm, the particle counts / profiles were more similar between the Adelaide secondary effluent and Melbourne lagoon samples (Fig 3.2).

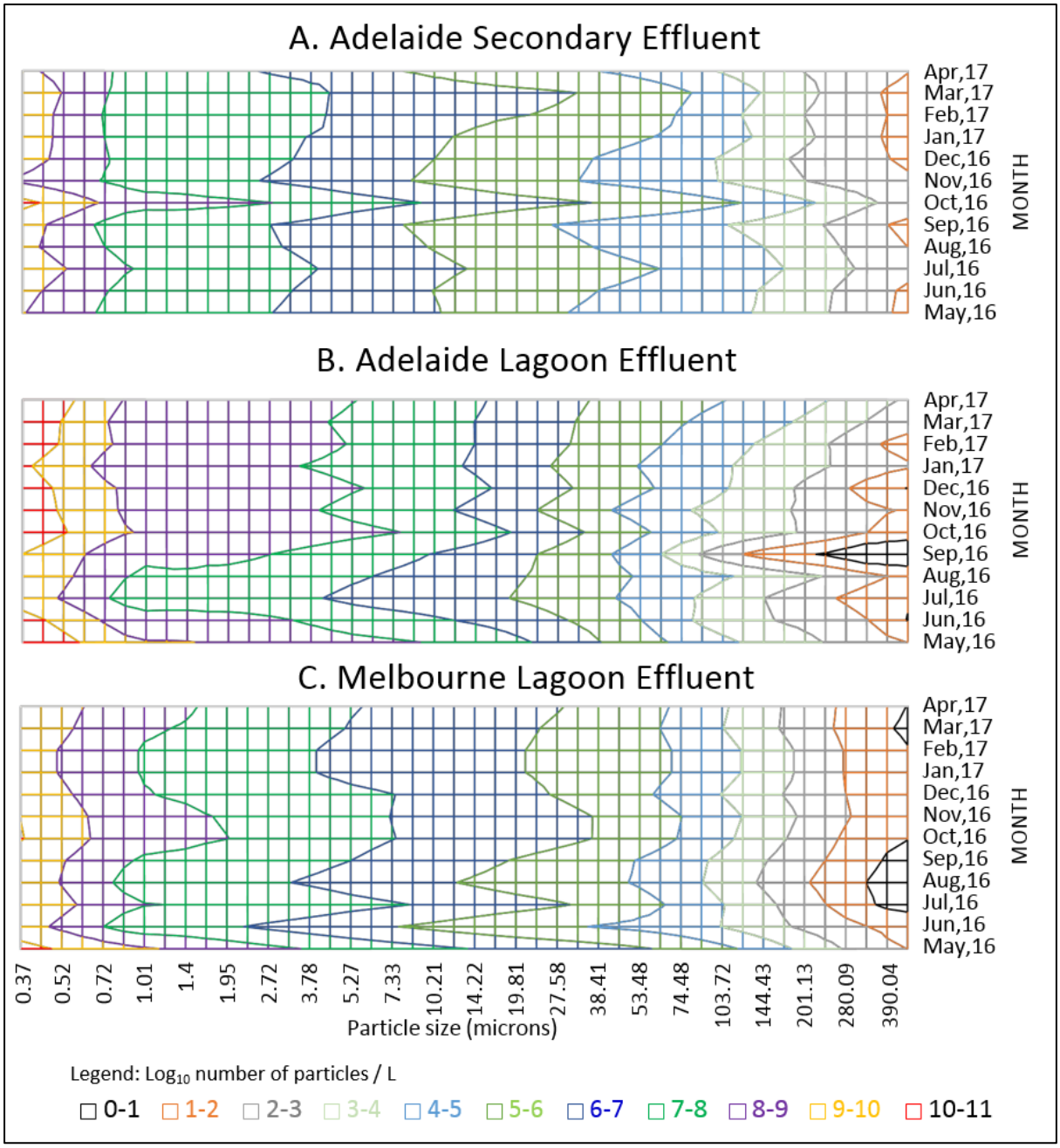

Figure 3.2 Contour plot

Figure 3.2 Contour plot showing the distribution profiles for different particle size classes (x-axis) as a function of time (y-axis) in treated wastewater. Samples (each in three 100 ml subsamples) of a) Adelaide secondary effluent, b) Adelaide lagoon effluent and c) Melbourne lagoon effluent were collected once a month for a year and analysed by Laser *In Situ* Scattering Transmissometry (LISST). The colours indicate the log10 range of the number of particles / L, as shown on the legend.

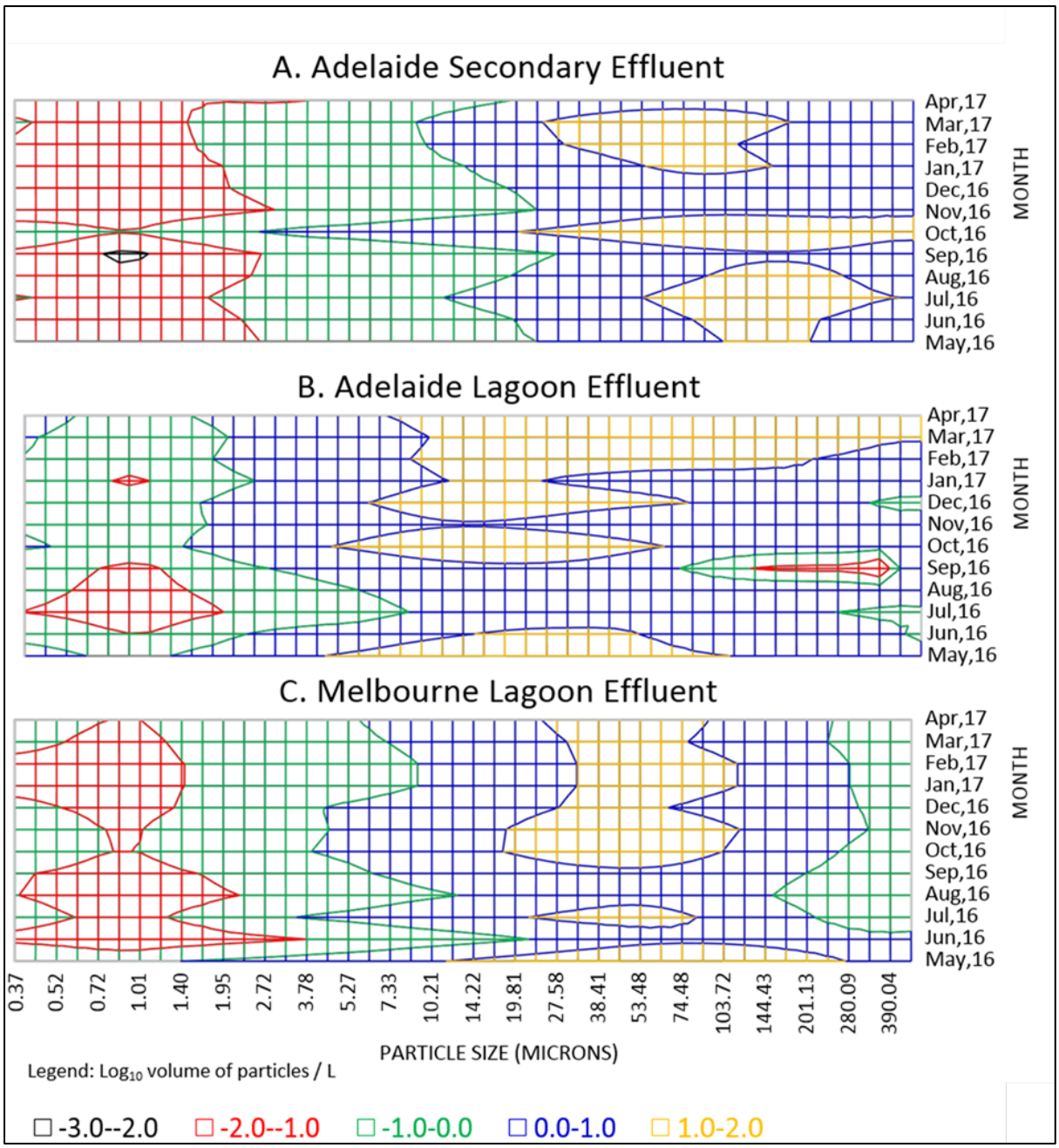

Figure 3.3 Contour plot

Figure 3.3 Contour plot showing the distribution profiles for different particle size classes (x-axis) as a function of time (y-axis) in treated wastewater. Samples (each in three 100 ml subsamples) of a) Adelaide secondary effluent, b) Adelaide lagoon effluent and c) Melbourne lagoon effluent were collected once a month for a year and analysed by Laser In Situ Scattering Transmissometry (LISST). The colours in the graphs indicate the log10 of the volume of particles (µL / L), as shown in the legend.

The TSS and VS in Adelaide secondary effluent were lower than in the Adelaide lagoon effluent in all months except July (Fig 3.4A). There was no apparent correlation between TSS or VS with the total number of particles > 2μm or turbidity (Fig 3.4). However, the particle and turbidity data were in general agreement (Fig 3.4C, 3.4D). A strong relationship was determined between turbidity and particle size for Adelaide secondary effluent (R=0.79) and Adelaide lagoon effluent (0.70) but a weak relationship was found for Melbourne lagoon effluent (R=0.28). The highest TSS values at the two Adelaide locations were recorded in October, which corresponded to the second highest numbers of particles detected using LISST or turbidity (Fig 3.4). There were 3.6 times more particles detected by LISST in the Adelaide lagoon effluent than in Melbourne lagoon effluent, but differences in TSS values were much smaller, TSS was only 1.4 times higher in the Adelaide lagoon than in the Melbourne lagoon. Both TSS and VS were significantly different ($p<0.05$) between the samples for each month and mean solids concentration changed significantly ($p<0.05$) every month for all the samples.

The highest NO_3^- concentration in Adelaide secondary effluent in Oct (Fig 3.5A) reflected high values for TSS, particle numbers and turbidity (Fig 3.4). There was no obvious relationship between particle numbers (Figs 3.1, 3.2, 3.3, 3.5) and nutrients (Fig 3.5), but there appeared to be a similar trend for changes in NO_3^- and TSS (Fig 3.4, Fig 3.5A) in the secondary effluent. The Adelaide lagoon effluent tended to have higher phosphorous levels and lower NO_3^- levels than the inflowing secondary effluent. The pH ranges were also significantly different ($p<0.05$), with Adelaide secondary effluent pH ranging from 6.4 – 8.2, while the Adelaide lagoon effluent pH ranged from 7.1 – 9.1. The October spike in particle counts in the Adelaide lagoon effluent appeared to follow a spike in the phosphorous concentration in September. In contrast with the Adelaide effluent samples, the Melbourne lagoon effluent samples exhibited little seasonal variation for nutrients or pH, with the pH ranging from 7.6 – 8.0. A distinguishing feature of the Melbourne lagoon effluent was that it had much higher

levels of NH_3 (~x60) and total P (~x2) than either of the Adelaide sites throughout the entire period of sampling (Fig 3.5). All the nutrient (means) including NH_3, NO_3^- and total P were significantly different ($p<0.05$) between the samples for each month except NO_2^- which was not significantly different.

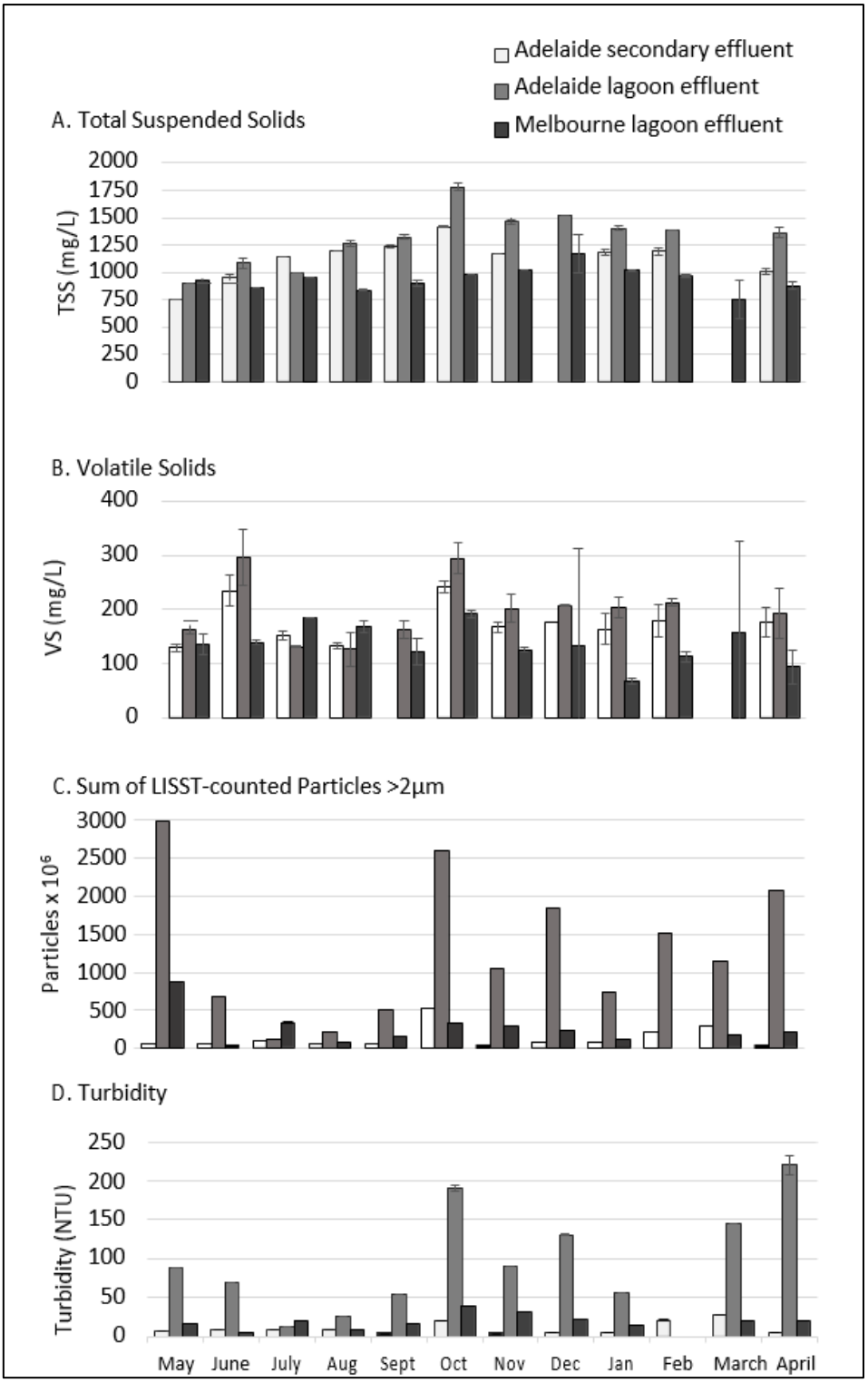

Figure 3.4 Physical characteristics of treated wastewater samples

Figure 3.4 Physical characteristics of treated wastewater samples. Grab samples (10L) of Adelaide secondary effluent (white bars), Adelaide lagoon effluent (grey bars) and Melbourne lagoon (black bars) were collected once a month for a year and duplicate sub- samples were analysed using a HACH 2100 N turbidimeter to measure turbidity or standard methods (A.P.H.A., 1998) for measuring Total suspended solids (TSS) and volatile solids (VS). Triplicate sub-samples were analysed by Laser *In Situ* Scattering Transmissometry (LISST).

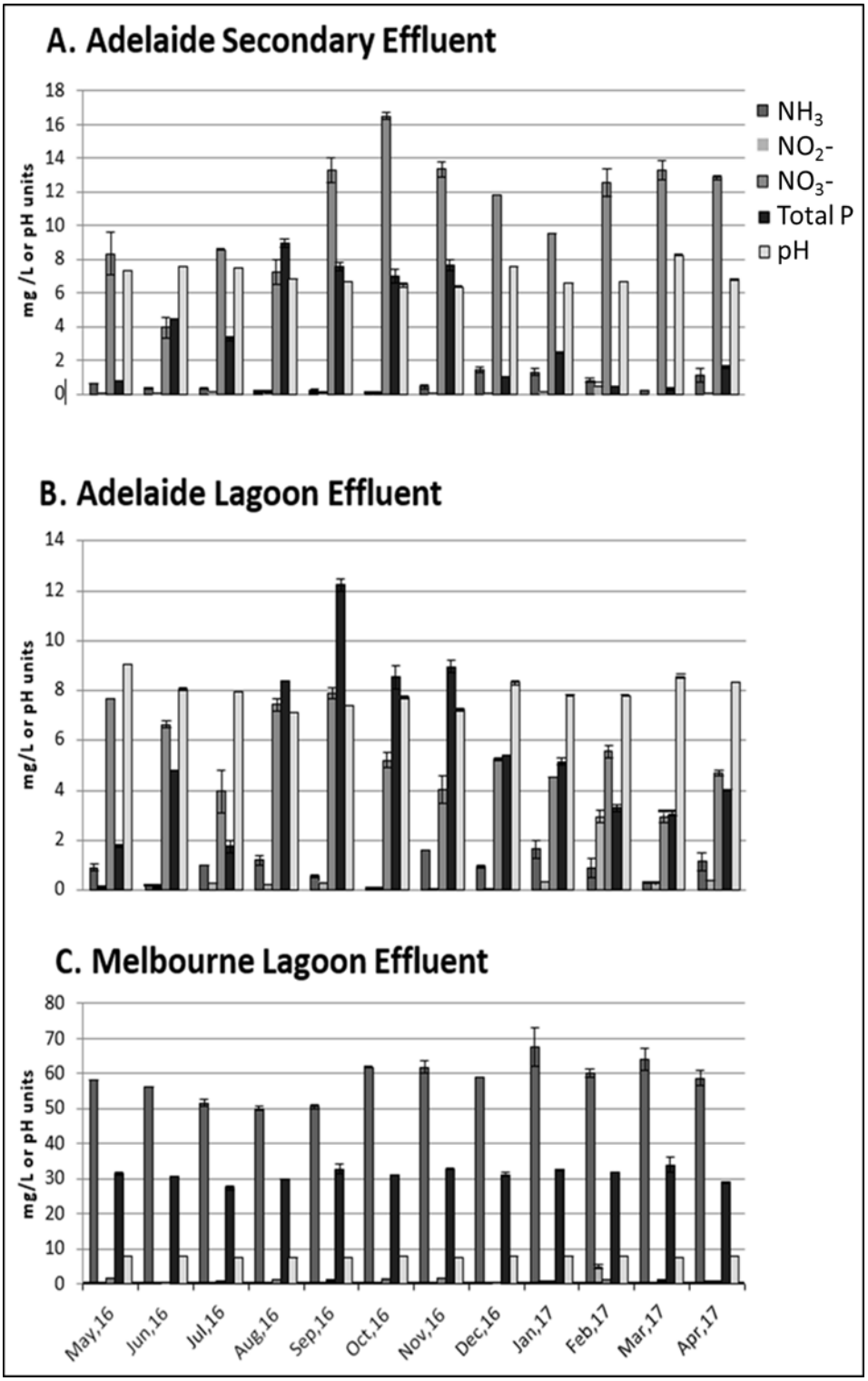

Figure 3.5 Nutrients and pH in WW

Figure 3.5 Nutrients and pH in WW. Grab samples (10L) of Adelaide secondary effluent (A), Adelaide lagoon effluent (B) and Melbourne lagoon (C) were collected once a month for a year and duplicate sub- samples were analysed using a Eutech pH700 pH meter or Hach colorimetric kits for NH_3, NO_2^-, NO_3^- and total phosphorous (P).

Scanning electron microscopy showed that the Adelaide secondary effluent was dominated (seen on all filters) by protozoans including *Arcella* (Fig 3.6C) and *Euglypha* (Fig 3.6A), ciliates, dinoflagellates (Fig 3.6D), and cyanobacteria such as *Microcystis* (Fig 3.6B), with testate amoeba and dinoflagellates occurring frequently and in large numbers. Adelaide lagoon effluent was dominated (seen on all filters) by cyanobacteria (*Microcystis*, Fig 3.6G), algae, and diatoms (Fig 3.6F, 3.6H) such as *Bacillariophyceae.* Melbourne lagoon effluent was dominated by algal species, with *Chrysophytes* (golden algae) the most abundant (observed on all the filters) (Fig 3.6I). Bacteria (Fig 3.6K) were often observed in Melbourne but not Adelaide lagoon effluent. Other less common organisms observed in Melbourne lagoon effluent were euglenoids (Fig 3.6J), testate amoeba and diatoms. Adelaide secondary effluent and Adelaide lagoon effluent each contained similar microorganisms throughout the three months, but there were higher numbers of euglenoids in Melbourne lagoon effluent in July compared with Adelaide effluent samples from May or June.

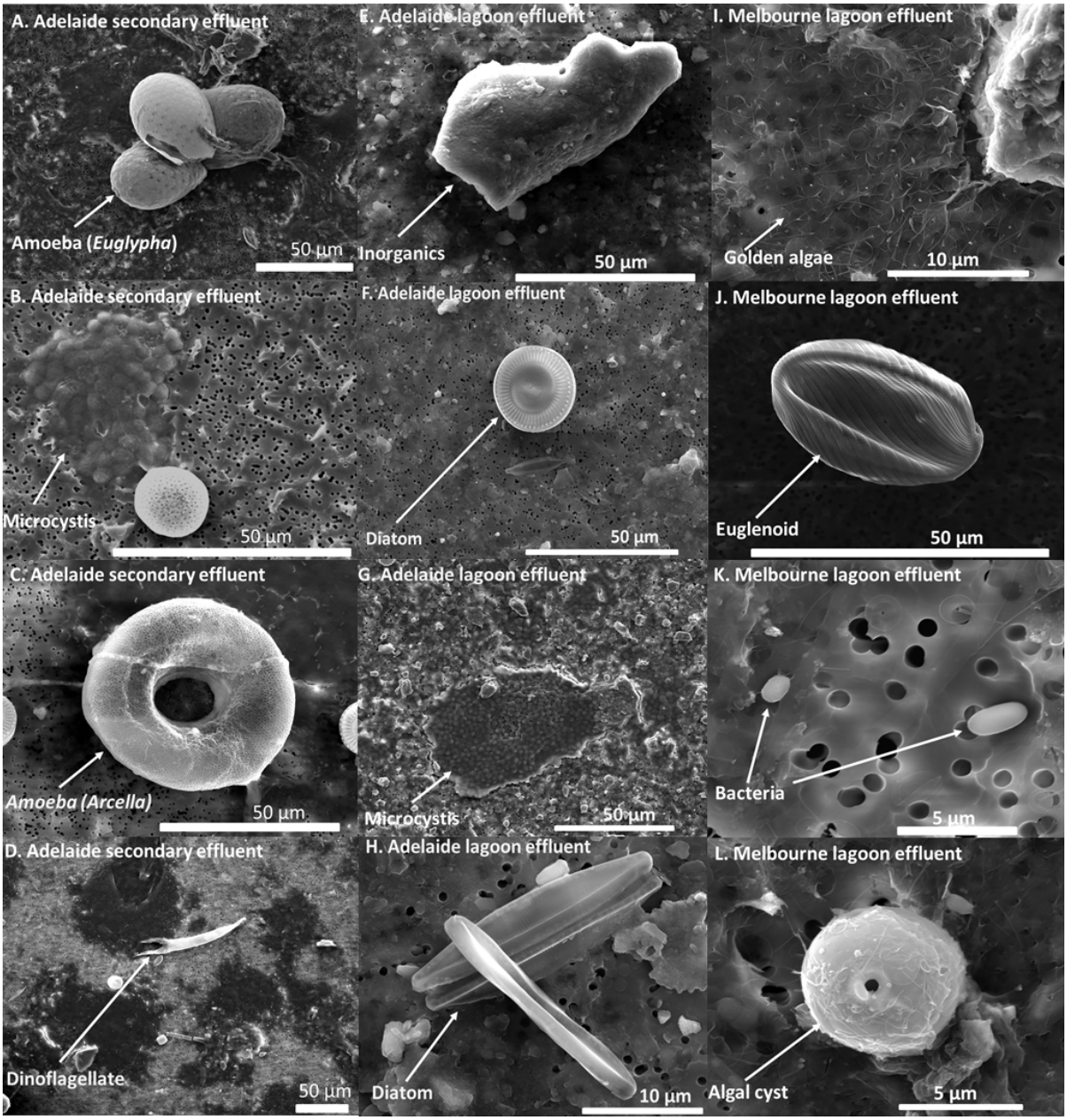

Figure 3.6 Scanning electron microscopy

Figure 3.6 Scanning electron microscopy images of commonly occurring particles observed in Adelaide secondary effluent (a to d), Adelaide lagoon effluent (e to h) and Melbourne lagoon effluent (I to L), viewed under variable magnifications as shown on the scale bars.

The EDX elemental analysis indicated that Control 'empty' filters (Fig 3.7A) were composed of C and O, which corresponded to the composition of the polycarbonate membranes. The ratio of C and O peaks in other sample images needs to be considered to determine if the presence of these elements is real or due to background from the membrane. Objects visible on the SEM images with the appearance of inorganic particles (Fig 3.7C and D) had more complex elemental analysis profiles than the biological particles (Fig 3.7B, E, F, and Fig 3.8B). Inorganic particles usually contained Na, Mg, Si and Al, often accompanied by K, S and Ca. The diatoms contained Si and small amounts of Al and P (Fig 3.8B), whereas other microorganisms tended to have less or no Si, but instead contained P, S, Ca or Na. As might be expected, microorganisms (Fig 3.7B, E, and F) had similar ratios for C and O peaks compared with each other and the polycarbonate membrane.

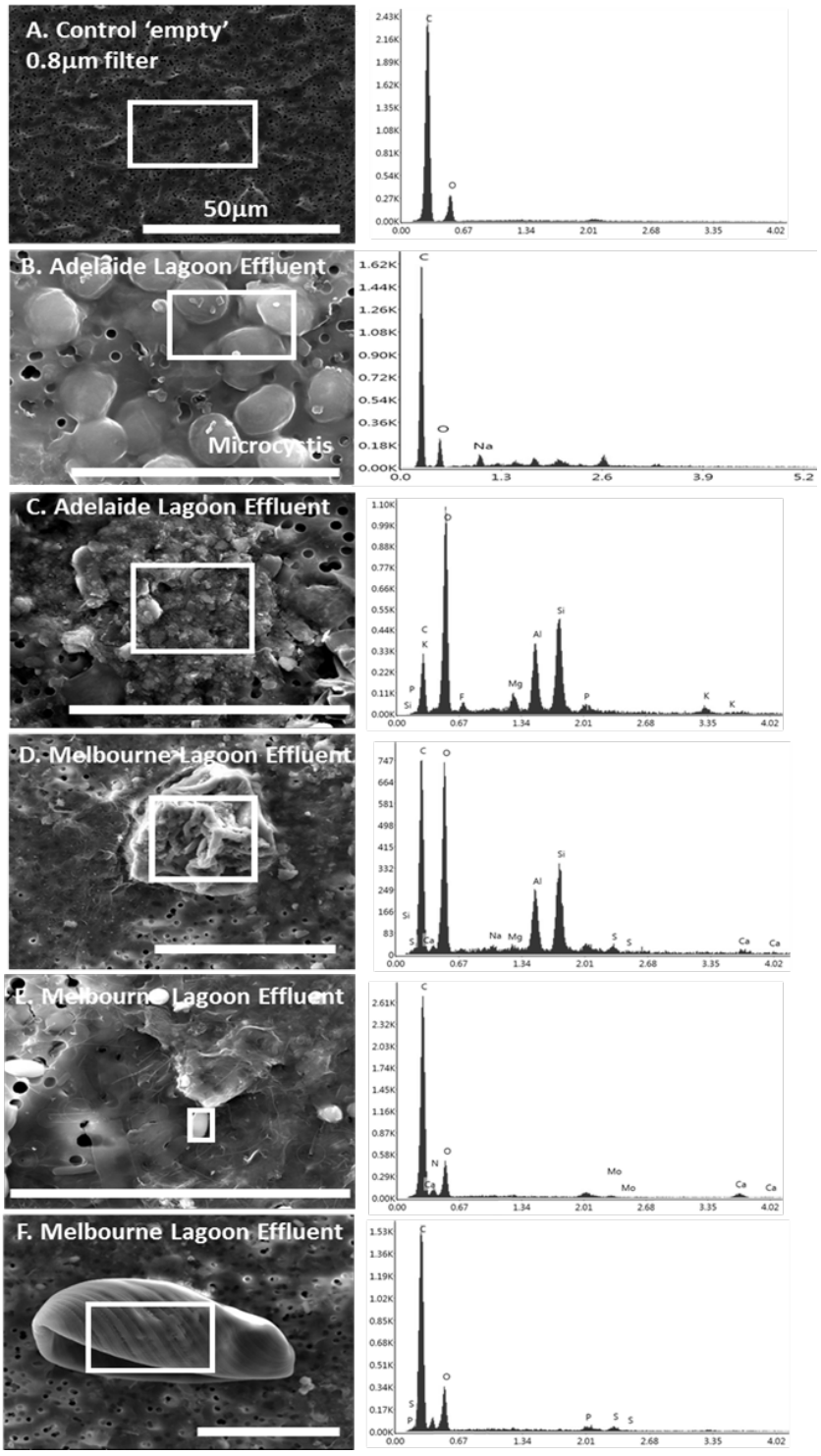

Figure 3.7 Elemental analysis

Figure 3.7 Elemental analysis: Scanning Electron Microscopy (SEM) and elemental analysis of wastewater samples. A) Blank filter membrane control. (B,C) Adelaide lagoon effluent and (D,E,F) Melbourne lagoon effluent samples were collected in winter and filtered through 0.8 µm pore size polycarbonate filters then coated with a 5nm layer of platinum. Images were obtained using an Inspect FEI F50 scanning electron microscope combined with TEAMTM software. Graphs to the right of images show the elemental composition of particles in the indicated areas (white rectangles) determined using an EDAX energy dispersive X-ray (EDX) micro-analyser coupled to the SEM. Y-axis shows proportion of each element in that sample and includes background matrix. Scale bars 20 µm.

The distinctive elemental analysis profiles and the visual and morphological appearance of individual particles were used to designate particles in SEM images as being either 'inorganic' or 'biological' in origin. The percentage of area occupied by 'inorganic' or 'biological' particles in SEM images of both lagoon effluents was quantified, with an example of the grid used to analyse images shown in Fig 3.8A and the results of the analysis presented in Fig 3.8C. It was found that the Adelaide lagoon effluent contained more inorganic material (35±15% of SEM image area) than biological particles (14±6%) in terms of total area, whereas the Melbourne lagoon effluent had equal numbers of biological (24±16%) and inorganic particles (24±12%) (of total membrane area, Fig 3.8C). Normalisation of the data to determine particle composition (ignoring empty space on the membranes) showed that the Adelaide lagoon effluent consisted of approximately 30% biological particles and 70% inorganic particles, whereas in the Melbourne lagoon effluent ratio was approximately 50:50 (Fig 3.9). The ratio of VS (a measure of organic solids) to the inorganic fraction of TSS was 20:80 for Adelaide lagoon effluent and 17:83 for Melbourne lagoon effluent (Fig 3.9). The two different approaches used to measuring biological and inorganic solids were in fair agreement for the Adelaide sample, but not for the Melbourne sample. Aside from sampling variation / differences in sample volume analysed by SEM and VS/TSS, the TSS/VS method only measure particles >2 μm, whereas the SEM allows detection of particles >0.8 μm.

There were more particles larger than 20 μm observed in the Adelaide than in the Melbourne lagoon effluents. In the Adelaide effluent samples, there was an average of 0.07 objects >20 μm per 1000μm^2 of filter, and 83% of these were biological. In contrast, the Melbourne lagoon effluent had an average of 0.02 objects >20 μm per 1000μm^2 of filter, and of these, 43% were biological. These were approximations, for example, in some cases a golden algae mat consisting of several organisms was counted as one biological 'particle'.

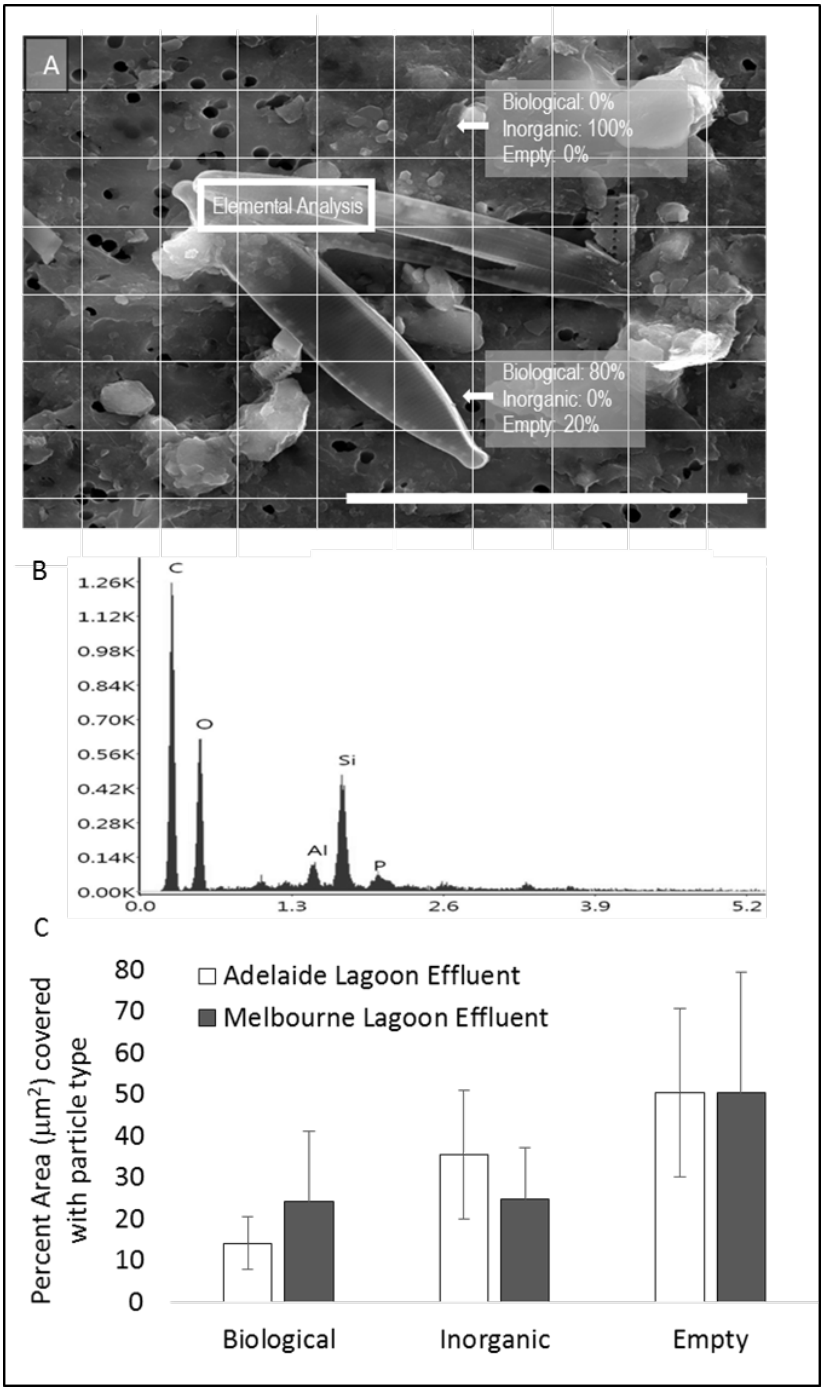

Figure 3.8 Inorganic and Biological Particles in Lagoon Effluent

Figure 3.8 Inorganic and Biological Particles in Lagoon Effluent. A) Adelaide lagoon effluent sample filtered through a 0.8 µm pore size polycarbonate filter before drying and coating with a 5nm layer of platinum. Image was obtained using an Inspect FEI F50 scanning electron microscope combined with TEAMTM software. Image includes area scanned by an EDAX energy dispersive X-ray (EDX) micro-analyser coupled to the SEM to analyse the elemental composition ('elemental analyses). Broad white scale bar = 20 µm. The image was magnified 300x and overlain with a 4µm grid. The percent area of each square occupied by material that was 'biological' or 'inorganic' was estimated; two representative assessments are shown. B) EDAX energy dispersive X-ray analysis of the elemental composition of the area shown in (A). Y-axis shows proportion of each element and includes background matrix. C) Area of SEM images occupied by different types of particles. Total of 40540 µm2 Adelaide and 51492 µm2 Melbourne lagoon effluent SEM images assessed. Mean± stdev images from different filters shown

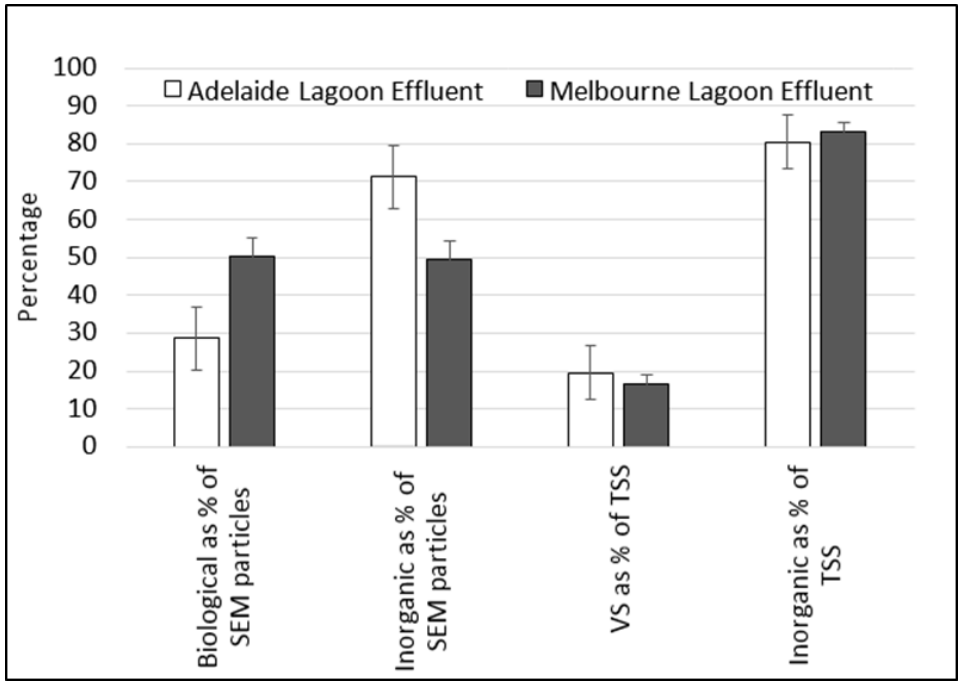

Figure 3.9 Proportions of biological and inorganic particles in lagoon effluent

Figure 3.9 Proportions of biological and inorganic particles in lagoon effluent. Grab samples (10L) of Adelaide (white bars) and Melbourne (black bars) lagoon effluents were collected in each of May, June and July, and filtered through 0.8 µm polycarbonate filters. Images were obtained using an Inspect FEI F50 scanning electron microscope (SEM) and analysed by overlaying with a 4µm grid. The percent area of each square occupied by material that was 'biological', 'inorganic' or 'empty' is presented as an average ± stdev of four different filters for each location. Duplicate sub-samples of each month's grab sample were analysed standard methods (A.P.H.A., 1998) for measuring Total suspended solids (TSS) and volatile solids (VS). Volatile solids or inorganic solids (TSS-VS) values were expressed as a percentage of the TSS for the same month, and the average ± stdev of three months shown.

Particles from all sample locations had negative zeta potential (Fig 3.10). The zeta potentials of particles in the Adelaide effluents appeared to be more variable across different months, particularly for the smaller particles. For example, in the Adelaide secondary effluent, particles with size <0.2 µm ranged from zeta -1.4 to -11 mV, in the particles <1.2 µm ranged from -8.5 to -19 mV and particles in the unfiltered sample zeta potential ranged from -7.7 to -9.8 mV (Fig 3.10A). Zeta potential of the particles in the May Adelaide lagoon effluent were similarly variable, though there was less charge variation for the different size classes in the June and July samples (Fig 3.10B). The charges of the particles in the Melbourne lagoon samples were more homogeneous across the different months (Fig 3.10C). The charges of the particles in the unfiltered (all particles) samples for the Melbourne lagoons samples and the June/July Adelaide

lagoon samples were similar (around -12 mV), but there were differences in some of the size classes, with particles <0.2 μm in size tending to be more consistently negatively charged in the Melbourne lagoon effluent compared with the Adelaide lagoon effluent, whereas the converse was true for particles <0.4 μm. The zeta potential of a solution is affected by pH (Jiang et al., 2009) and the ionic composition of the water. The pHs of the Adelaide secondary effluent and Melbourne effluent samples were relatively consistent for the 3 months, but the May Adelaide lagoon sample had a much higher pH compared with the June or July samples, possibly explaining the charge differences in these samples.

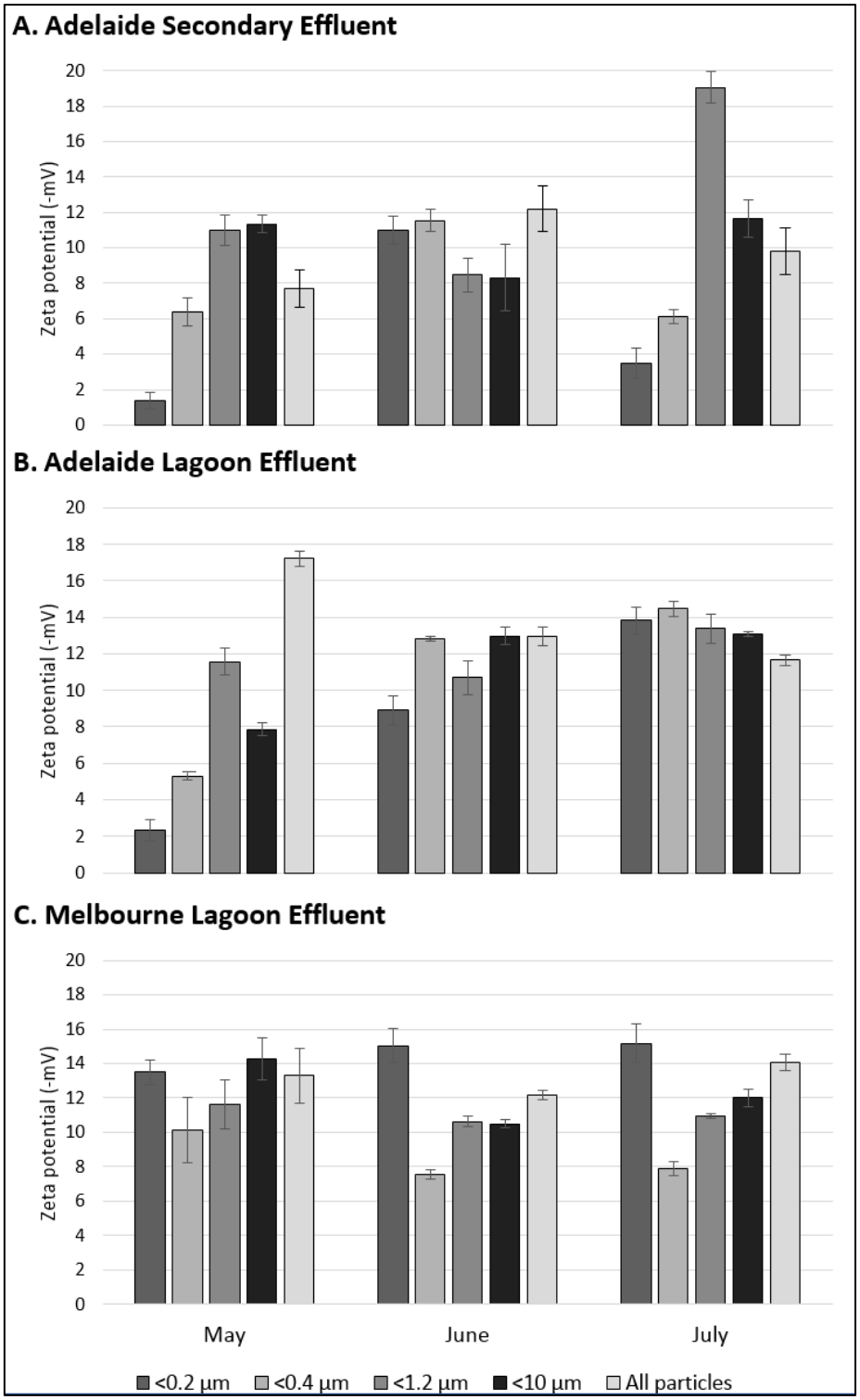

Figure 3.10 Surface charges of wastewater particles

Figure 3.10 Surface charges of wastewater particles. The graphs display the zeta potential (as –mV) of three monthly samples each of a) Adelaide secondary effluent, b) Adelaide lagoon effluent and c) Melbourne lagoon effluent measured using a Malvern Zetasizer WT. The samples were unfiltered (Raw) or serially filtered through 10 µm, 1.2 µm, 0.4 µm and 0.2 µm filters and filtrate from each step was analysed in triplicate (1mL) for zeta potential. Two way analysis of variance was used to compare the mean differences between different samples and months using Tukey's multiple comparison test.

3.4 Discussion:

This study identified different types of particles in wastewater effluents collected from three different processes at two geographically separated sites. There were differences in the numbers, types, sizes and surface charges of particles at two different stages of treatment (effluent following activated sludge treatment and clarification, and effluent following lagoon treatment) in the same treatment plant, and between similar wastewater treatment processes (lagoons) from two different locations.

The highest numbers of particles at all three locations were in the smallest size classes (0.37 – 1.01 μm), with particle numbers decreasing with increasing particle size (i.e., each progressively larger size class had fewer particles than the previous size class). These particles accounted for more than 90% of the total particle count. The converse did not apply when considering the contribution of each size class to the total volume of particles; small particles less than 1 μm in size contributed less than 1% to the total particle volume, but the largest particles did not account for the largest proportion of particle volume. Throughout the year, Adelaide secondary effluent contained an order of magnitude fewer particles than Adelaide lagoon effluent, with the exception of the spring month of October, which had the highest numbers of particles for secondary effluent and was comparable with the particle numbers in the matching Adelaide lagoon effluent sample. The spikes in the particle numbers in the secondary effluent may reflect processes occurring at the Adelaide site. The lagoon effluent is further treated using dissolved air flotation and filtration (DAFF), with the waste streams from this process (backwash water and float water) being returned to the secondary clarifier. These waste streams are likely to have high loads of suspended solids, and some of these are likely to contribute to the particles measured in the secondary effluent. The DAFF is operated to meet demand (with higher production in spring / summer compared with winter), and the contribution of this operation to the secondary effluent will be transient, so may not be evident

in all samples. The spike in particle numbers in October, and possibly in March, may reflect the influence of the DAFF waste streams on the secondary effluent.

The particle size distribution determined by LISST matched the turbidity data more closely than either of them corresponded to TSS (strongly for both the Adelaide samples but weakly for Melbourne sample). Total suspended solids (TSS) are particles >2μm that consist of inorganics such as sediment, silt or sand, and organics such as pathogens and algae. Chemical precipitates can also contribute to TSS. Igniting the dry mass of TSS at 550°C removes volatile solids (VS), which are generally considered to be equivalent to organic materials in the water sample. Therefore, subtraction of VS from TSS gives an approximation of the amount of inorganic material in a sample. Turbidity is measured with a nephelometer, which consists of a light beam and a detector placed to measure light that has been scattered at an angle of 90 degrees from the incident light. High numbers of particles scatter the light more than low numbers, and increased scatter results in more light reaching the detector. Laser In-Situ Scattering and Transmissometery (LISST) passes particles through a light beam and the resulting angles of light scatter are detected using rings of sensors. Turbidity and LISST both measure light scatter caused by particles, and both are affected by the composition, shape and size of particles because these parameters can affect light scatter and reflectivity. Turbidity and TSS are also related, but correlations at different sites vary depending on particle composition. One cause of differences between turbidity and TSS is the presence of coloured dissolved organic matter (DOM) that can reduce turbidity readings but have no effect on TSS. Another is that TSS measures particles >2 μm, whereas turbidity is derived from the scattering effects of all particles in the water sample. The correlation between LISST and turbidity data reflects the similarity in methods. Nevertheless, it is reassuring that there was concordance between these two datasets; the validity and reliability of each dataset was strengthened.

A new protocol for analysing SEM images was developed for this project, and generated data indicating that 29 – 50% of the particles in winter lagoon effluents were biological. When VS values for the same 3 months were expressed as a percentage of TSS, it was concluded that 17 – 20% of the effluents were comprised of organic components. Considering the differences in methodology for SEM and VS/TSS, the results are promising, especially for the Adelaide lagoon effluent samples. Further work is required to determine the cause for the differences between SEM and VS/TSS in the Melbourne samples. Possible causes include differences in the sample volume analysed (sampling effects) and that the inorganic fraction of TSS will also incorporate inorganic components from some organisms (eg diatoms). Furthermore, the density and mass of biological objects is likely to be lower compared with inorganic objects, so the use of surface area in the SEM method is likely to underestimate the load of inorganic material compared with methods that uses weight, such as TSS and VS.

The SEM analysis of samples collected in May, June and July showed that Adelaide secondary effluent was dominated by microorganisms such as testate amoeba, ciliates, dinoflagellates and *Microcystis*, which, with the exception of *Microcystis*, are typical of an activated sludge fauna (Chen et al., 2004, Tyagi et al., 2008, Madoni, 2011). *Bacillariophyceae* is one of the most common algae in stabilisation ponds (Shanthala et al., 2009) and wastewater lagoons (Silva, 1998) and has been reported in Adelaide lagoons previously (Buisine and Oemcke, 2003, Yap et al., 2012). *Microcystis* was also dominant in the Adelaide lagoon effluent and this genus is commonly found in lagoon systems (Vasconcelos and Pereira, 2001). Melbourne lagoon effluent was dominated by algal species *Chrysophytes* which have been reported as being abundant in wastewater stabilisation ponds (Pham et al., 2014). Different nutrient profiles, their concentration and availability affect algal dynamics and growth (Kesaano and Sims, 2014). A key difference between the Adelaide and Melbourne lagoon effluents was the level of nitrogen and total P. The Adelaide lagoon effluent had low NH_3 (generally <1 mg/L), relatively low

NO_3^- (between 2 and 8 mg/L) and variable total P concentrations (between 2 and 12 mg/L). In contrast, the Melbourne lagoon effluent had consistently high NH_3 (between 50 and 65 mg/L), very low NO_3^- (<1 mg/L) and consistently high total P (between 27 and 34 mg/L). Both locations had similar dissolved organic carbon (Adelaide lagoon 35±13 mg/L, Melbourne lagoon 36±22 mg/L). The differences in nutrients likely explain the differences in the dominant microorganisms observed in the SEM analyses. Cyanobacteria are known for their ability to outcompete eukaryotic algae under nutrient limited conditions, with *Microcystis* particularly efficient at capturing and storing nitrogen and phosphorous (Beversdorf et al., 2013).

The changes in the particle counts, in particular for particles >2 μm, are likely due to a combination of seasonal factors (in particular light, temperature, nutrients and wind) which affect the performance of the activated sludge process and lagoon processes. The lagoons are relatively shallow and so will undergo mixing when there are strong winds, potentially resulting in sediment resuspension, so the particles / turbidity observed could represent sediment or a bloom of algae / zooplankton, depending on the conditions. The particle numbers in the Adelaide lagoon effluent were lowest in winter / early spring (June – September), with the highest number recorded in May and the second highest in October. The October spike in particle count followed an increase in total P in both the secondary and lagoon effluents and an increase in NO_3^- in the secondary effluent, however both the parameters were already high before they peaked as described. Nutrients, combined with warmer temperatures, support algal growth (Abdel-Raouf et al., 2012), suggesting that the high particle numbers were due to algal growth. Conversely, the low light intensity, short day length and cold temperatures in winter can inhibit algal growth (Jung et al., 2009), even though total P concentrations were increasing in late winter (August). The dominance of algae at both sites as measured by the SEM analysis tends to support this. Interestingly, total P concentrations in the Adelaide lagoon effluent were

higher than the secondary effluent (which is the influent into the lagoon system), suggesting that phosphorous was being released from the lagoon sludge.

Activated sludge is believed to have a zeta potential in the range of -6 to -12 mV (Bennoit and Schuster, 2001) and accumulated particles obtained from the membranes fouled with secondary clarified effluent had a zeta potential below -20 mV (Xu et al., 2006). The zeta potential of particles in the Adelaide secondary effluent were in the -1 to -19 mV range, similar to these earlier reports. Algal cells have a negative zeta potential (Khoshnevisan and Barkhi, 2001) which typically ranges from -10 to -35 mV (Henderson et al., 2008). Other micro-organisms also tend to be negatively charged (Li and McLandsborough, 1999, Searcy et al., 2006b) which leads to our premise that the higher the negative charge carried by the particles, the higher the repulsion from negatively charged microorganisms, and the lower the likelihood of particle-pathogen association. With the exception of colonial organisms such as *Microcystis*, there was little evidence in the SEM images of microbial interactions with particles.

3.5 Conclusion:

A new protocol for analysing SEM images was established in this study. Adelaide lagoon effluent had higher number of larger particles (>20 μm) which were identified as more inorganic (70%) in nature than Melbourne lagoon effluent (50%). Particle size distribution followed a similar trend in both the lagoon effluents. Adelaide lagoon effluent had the highest monthly average number of particles than the other two samples. Adelaide secondary effluent had higher number of larger (>100 μm) particles than both the other two samples. We concluded that particle size and surface charge are important factors that can determine their association with pathogens. This study indicates that seasonal variations can greatly impact particle counts and nature and nutrient loads in wastewater lagoon systems, and this can be helpful for designing targeted removal strategies. Our study provides the basis for future studies

to analyse specific particle pathogen interactions in wastewater environments which would in turn be useful for determining targeted treatment options.

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Chapter 4: Partitioning behaviour of *E. coli* and FRNA bacteriophage in secondary treated effluents from two Australian wastewater plants

4.1 Introduction:

Microbes in a surface and wastewater columns can either be free in suspension or associated with particles (Jamieson et al., 2005, Characklis et al., 2005b, Fries et al., 2008, Walters et al., 2014, Symonds et al., 2014, Qian et al., 2016). Wastewater contains a mixture of organic and inorganic particles which can be dissolved, colloidal, supracolloidal and settleable (Pallarès et al., 2011, Crittenden et al., 2012). Bacteria interact with a wide range of different sized particles in aquatic environments (Hipsey et al., 2006, Kollu and Örmeci, 2012, Kunkel et al., 2013, Walters et al., 2014), whereas viruses can be associated with smaller non-settleable particles (Templeton et al., 2007, Symonds et al., 2014, Verbyla and Mihelcic, 2015). A variety of factors affect the association between microbes and particles; the nature and size of particles, the surface characteristics of the microbes, and environmental conditions (Madge and Jensen, 2006b, Verbyla and Mihelcic, 2015, Liang, 2015).

Attachment to particles can influence the fate and settling velocity of pathogens (Characklis et al., 2005b, Krometis et al., 2007b) as well as confer additional advantages such as providing nutrition and protection from environmental stresses such as predation (Walters et al., 2014). Particle-pathogen association makes pathogens resistant to disinfection and increases their survival rates (Kollu and Örmeci, 2012, Jin et al., 2013, Liu et al., 2013a), and larger particles offer more protection than smaller particles (Walters et al., 2014). Particle association increases requirements for the amount of disinfectant with consequent increases in cost and increased production of harmful disinfection by-products. This in turn necessitates advanced treatments in order to produce effluent that meets standards and quality guidelines (Blume and Neis, 2004, Liang et al., 2013, Verbyla and Mihelcic, 2015). Hence it is important to characterise the particle fraction that associates with pathogens in order to identify suitable removal mechanisms and produce recycled water economically with minimal by-products.

The association of microbes with particles has been documented in stormwater and surface waters (Characklis et al., 2005b, Krometis et al., 2007b, Kollu and Örmeci, 2012, Qian et al., 2016), but few reports describe pathogen-particle associations in wastewater. The association is highly dependent on the nature of the particles and the aquatic environment. Wastewater environments tend to be very different from surface waters and stormwaters. Three methods; centrifugation, sedimentation and filtration, have been used to separate particle-associated microbes (Garcia-Armisen and Servais, 2009). Centrifugation separates the particles on the basis of both size and density, whereas filtration separates only on the basis of size (and charge, depending on the nature of the filter material). Separation by centrifugation has not been applied to wastewater and few studies have reported particle-pathogen association at different stages of the treatment train. This study was therefore designed to examine samples from two (2) Australian wastewater treatment plants to examine the hypothesis that the different treatment processes would generate different particle profiles. *E. coli* and FRNA bacteriophage were chosen to represent enteric bacteria and viruses respectively. Centrifugation and serial filtration were applied to examine particle-microbe associations. The specific aim of this study therefore were to assess the association of FRNA bacteriophage and *E. coli* with particles within different wastewater environments and to determine if there was preferential attachment to a particular type or size of particle.

4.2 Methods

4.2.1 Sample collection- Grab samples (10 L) of secondary-treated clarified effluent or final lagoon effluent were collected from BWWTP but only final lagoon effluent was collected from WWWTP between May and July 2015.

4.2.2 Physical analysis- Three sub-samples (Fig 4.1) were taken from each 10 L parent sample and these aliquots were examined for concentrations of suspended solids (SS), total suspended solids (TSS) and volatile solids (VS), NH_3, NO_2^-, NO_3^-, total P, TOC and DOC as described in

section 2.3.2 (chapter 2). The results for each parameter were compared between different locations by performing one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Triplicate 100 mL subsamples of each sample were also analysed for particle size distribution using laser *in situ* scattering transmissometry (LISST-XR, Sequoia Instruments) as described in section 2.3.3 (Chapter 2). The particle count of each group was $\log_{10}$ transformed for analysis. The means were compared with Tukey's multiple comparison test using Graphpad Prism (version 7.03) and two-way analysis of variance was performed by setting the interaction between sample and month for each size group.

4.2.3 Partition techniques

4.2.3.1 Partitioning by centrifugation- Samples were removed from refrigeration and inverted gently to resuspend the settled particles. Duplicate 900 mL aliquots of each sample were centrifuged for separation of particle-associated microbes as described in section 2.3.4.1 (Chapter 2) (Fig 4.1). Mean microbial counts of supernatant and pellet were compared to the mean microbial counts of corresponding raw (untreated) sample. Additionally, One-way analysis of variance was performed to analyse the statistical differences between the controls and treatments by comparing the means using Bonferroni and Tukey's multiple comparison tests. For the calculation of percent *E. coli* in the pellet, the estimated number of *E. coli* in the pellet was divided by the total number of *E. coli* in the sample (estimated from the number of *E. coli* in 900mL of supernatant + the number of *E. coli* in the pellet). Percent removal data were Arcsin transformed before comparison to the control by one-way ANOVA and Tukey's test with significance assigned at $p < 0.05$.

4.2.3.2 Partitioning by Serial filtration- One aliquot of each sample was serially filtered using 10 µm, 1.2 µm, 0.4 µm and 0.2 µm (Fig 4.1) polycarbonate filter membranes as described in

section 2.3.4.2 (chapter 2). Raw (unfiltered sample), filtrate and filter membrane retentate were then analysed in triplicate to determine the partitioning of both *E. coli* and FRNA bacteriophage. Mean microbial count of each filtrate was then comparted to the corresponding raw (untreated) sample. Samples were compared to the control in the same way as described earlier. Additionally, the reduction in microbial counts after successive filtration was expressed as percent removal (percentage of microbe that partitioned in each filtrate) and analysed the same way as described earlier.

4.2.3.3 Partitioning Controls-

Sample preparation for analyses included making sample dilutions in phosphate buffered saline (PBS) to obtain counts within a measurable range (20 – 200 colony or plaque forming units per plate). Pure cultures of *E. coli* (ATCC700891) or the FRNA bacteriophage MS-2 (ATCC 15597-B1) were suspended in PBS at a final concentration of 10^2/mL and used as controls. These controls were partitioned (centrifuged or serially filtered) to analyse the natural behaviour of indicators under these conditions in the absence of any particulate matter.

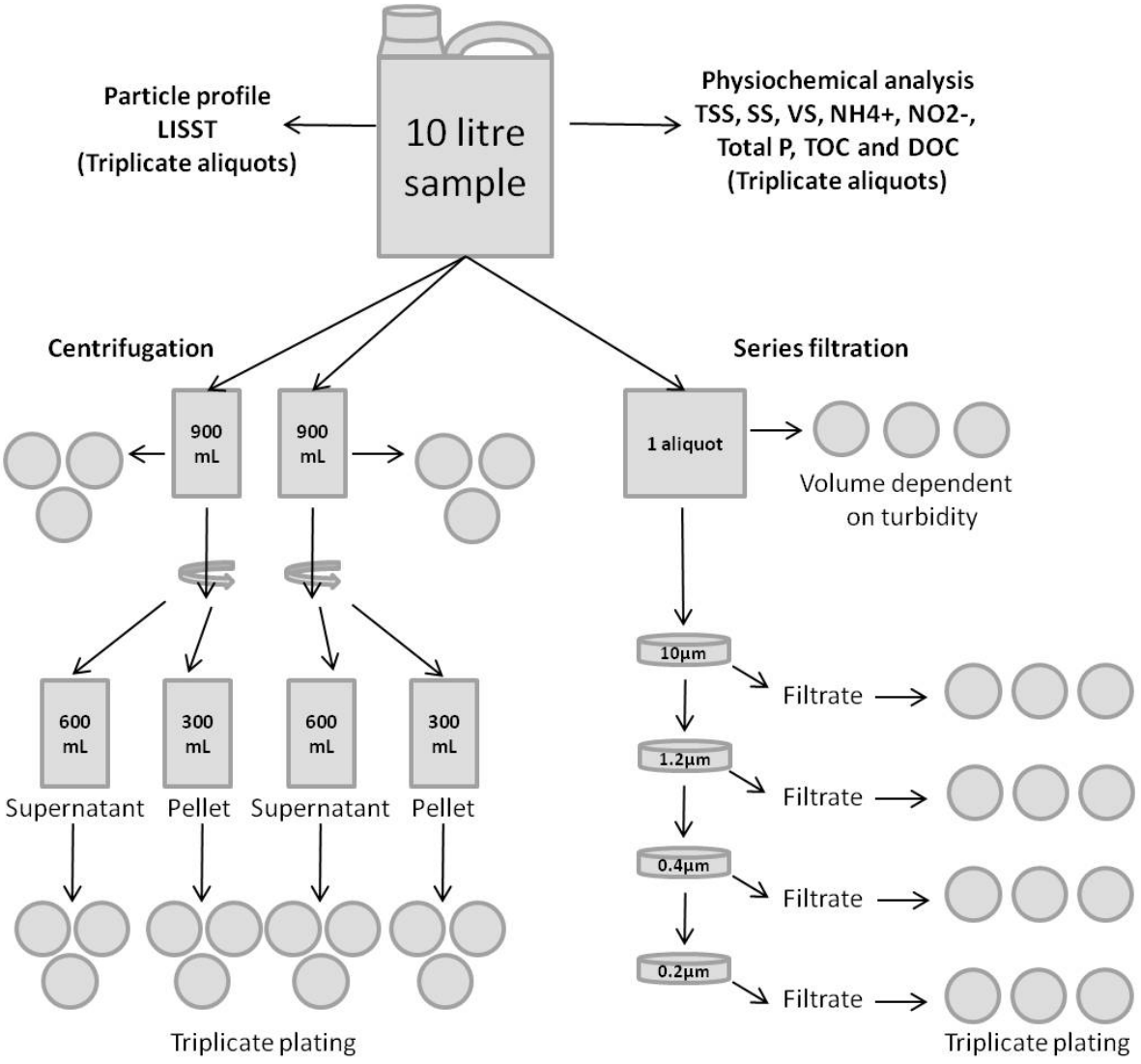

Figure 4.1 Schematic of the two partitioning methods used

Figure 4.1 Schematic of the two partitioning methods used - centrifugation and series filtration. One 10 L sample was collected each month in a 20% thiosulphate dosed jar. Two aliquots of 900 mL each and 1 aliquot (volume determined based on initial turbidity) were partitioned using centrifugation and series filtration respectively. Samples were centrifuged at 931xg for 10 minutes, then 600 mL supernatant, and 300 mL 'pellet' were collected and analysed for indicator organisms in triplicate. Samples were serially filtered through 10µm, 1.2 µm, 0.4 µm and 0.2 µm polycarbonate filter membranes. Equal volumes of filtrate were collected at each filtration step and the filtrate was analysed for indicator organisms in triplicate.

4.2.4 Microbial analysis- *E.coli* and FRNA bacteriophage were selected because they are accepted surrogates for pathogenic bacteria and enteric viruses, respectively, in wastewater systems (van den Akker et al., 2014, Young et al., 2016). *E. coli* and FRNA bacteriophage were measured in raw (untreated) and treated samples, supernatants and pellets after centrifugation, and filtrate and filter membrane retentate after filtration as described in section 2.3.5 (Chapter 2). The numbers of *E. coli* and FRNA bacteriophage detected before and after each treatment were used to calculate the total microbial load in the working volumes, which were plotted in the graphs.

4.3 Results

4.3.1 Wastewater characteristics: Adelaide secondary and lagoon effluents both had low levels of NH_3 and NO_2^- but higher levels of NO_3^- (Fig 4.2). The Melbourne lagoon effluent had low levels of NO_2^- and NO_3^-, but significantly higher levels of NH_3 than the Adelaide samples ($p<0.0001$). Total P content differed in all three samples, with Melbourne lagoon effluent having a significantly higher total P concentration ($p<0.0001$) than the two Adelaide sites. Turbidity and solids also varied at the three different sites; Adelaide lagoon effluent had the highest turbidity ($p<0.0001$) and volatile solids ($p<0.05$), whereas Melbourne lagoon had the highest suspended solids ($p<0.01$) but the lowest total suspended solids ($p<0.01$). The Adelaide secondary effluent had lower levels of TOC, DOC and SS than the two lagoon effluents. Although the physiochemical profiles were characteristic of the three locations, the samples from each location did not change significantly during the three-month sampling period. An exception was DOC in the Adelaide secondary effluent, which doubled in concentration in July (21 mg/L) compared with May and June (11 and 10 mg/L) (data not shown).

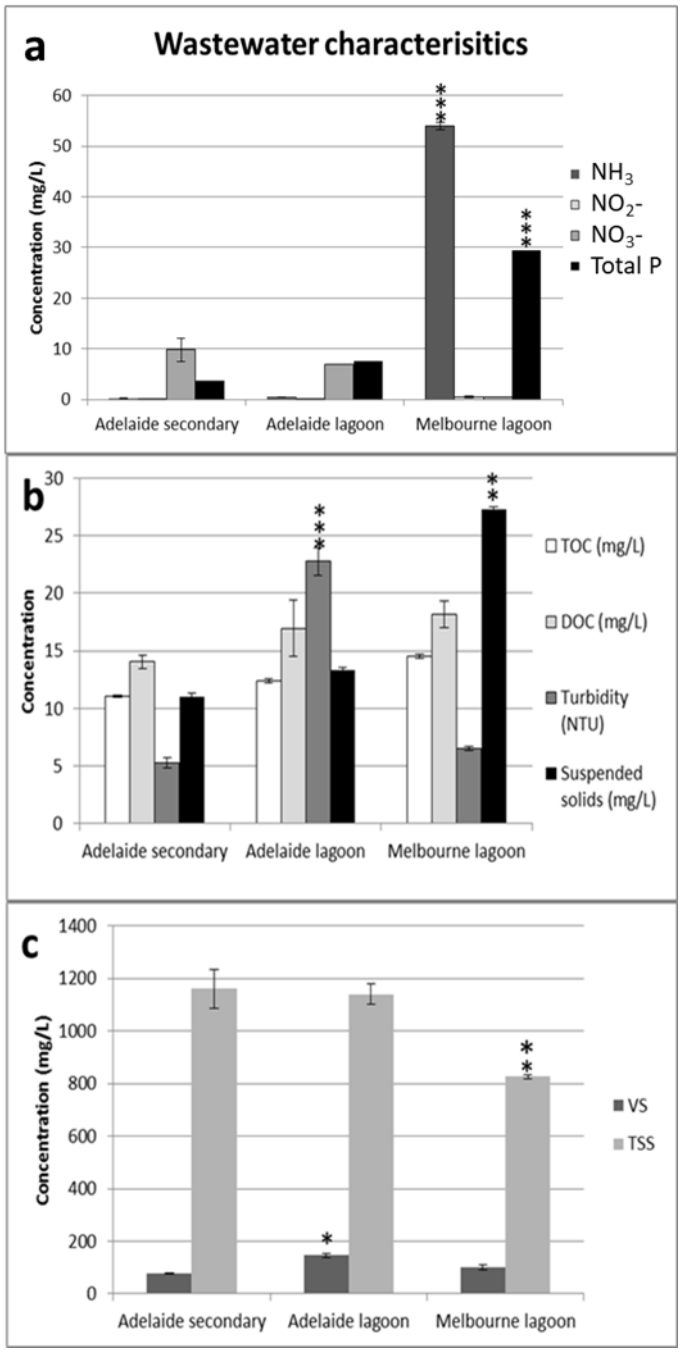

Figure 4.2 Physiochemical and nutrient analysis

Figure 4.2 Physiochemical and nutrient analysis of wastewater samples collected from Adelaide and Melbourne wastewater treatment plants. Analyses were performed in triplicate for each monthly sample and each bar represents average (± standard error of mean) values for the three months. The nutrient analyses were performed using various calorimetric kits and the characterisation of solids was performed using standard methods for water and wastewater analysis. One way ANOVA with Tukey post-hoc test was used to determine statistically significant differences between locations for the same analyte, * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

TOC – total organic carbon, DOC- dissolved organic carbon, NTU- nephelometric turbidity units, VS- volatile solids, TSS- total suspended solids

4.3.2 LISST Particle profiling: All the samples were similar in that there were higher numbers of smaller particles (<1.2 µm) and lower numbers of larger particles (Fig 4.3). Adelaide secondary effluent had higher numbers of large particles (>100 µm) in all three months compared to the other two samples ($p < 0.001$). The Adelaide lagoon effluent had significantly higher numbers of middle-sized particles (1.2-10 µm and 10-100 µm) than either Adelaide secondary effluent ($p < 0.0001$) or Melbourne lagoon effluent ($p < 0.001$) during June and July, but significantly fewer middle-sized particles than Melbourne lagoon effluent in May ($p < 0.001$). Comparison of the individual particle sizes (Fig 4.4) between 1 – 10 µm from the Adelaide lagoon effluent samples showed that there were fewer 1.2 -5 µm particles in May than in June and July.

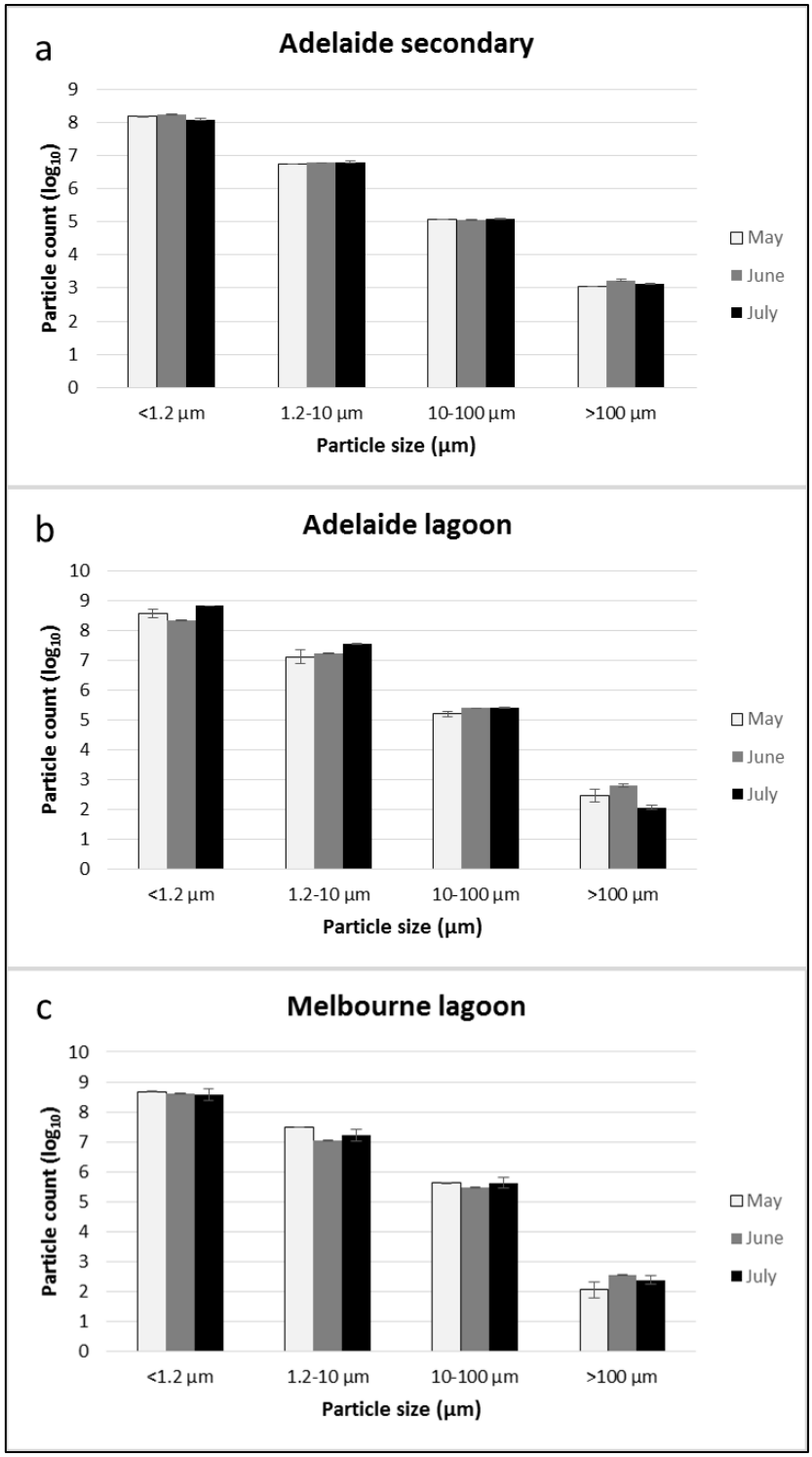

Figure 4.3 Particle density profiles

Figure 4.3 Particle density profiles a) Adelaide secondary effluent, b) Adelaide lagoon effluent and c) Melbourne lagoon effluent obtained by laser in situ scattering (LISST).Wastewater samples collected each month (100 ml) were analysed in triplicate. The particle data obtained was grouped into different size classes (< 1.2 µm, 1.2-100 µm, 10-100 µm and > 100 µm) and the mean ± SE number of particles in each class presented. Two way ANOVA (month and location) with Tukey post-hoc test was used to compare particle numbers in the same size class at different locations..

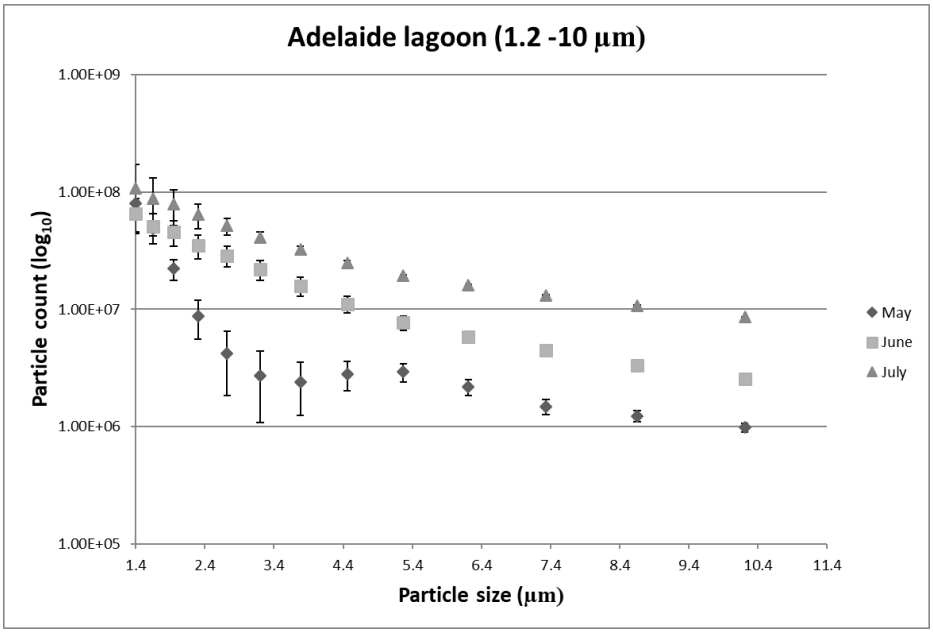

Figure 4.4 Particle density profile of 1.2-10 µm sized particles

Figure 4.4 Particle density profile of 1.2-10 µm sized particles in Adelaide lagoon effluent obtained by laser in-situ scattering. Wastewater samples collected each month (100 ml) were analysed in triplicate. The particle data obtained was grouped into different size classes and the mean ± SE number of particles in each class presented.

4.3.3 Partitioning by Centrifugation: MS-2 phage, a member of the FRNA bacteriophages, was used as a control to study the natural behaviour of phage under centrifugation in the absence of particles (Fig 4.5d). Centrifugation did not remove any MS-2 phage from the particle-free PBS control. The numbers of FRNA bacteriophage in the Adelaide secondary effluent increased from May to June (Fig 4.5a), whereas FRNA bacteriophage numbers decreased in the Melbourne lagoon sample (Fig 4.5b). FRNA bacteriophage were below detection limit (<1 PFU/mL) in the Adelaide lagoon effluent. Centrifugation did not remove FRNA bacteriophage from the Adelaide secondary effluent or the Melbourne lagoon effluent, and these samples were comparable to the PBS control.

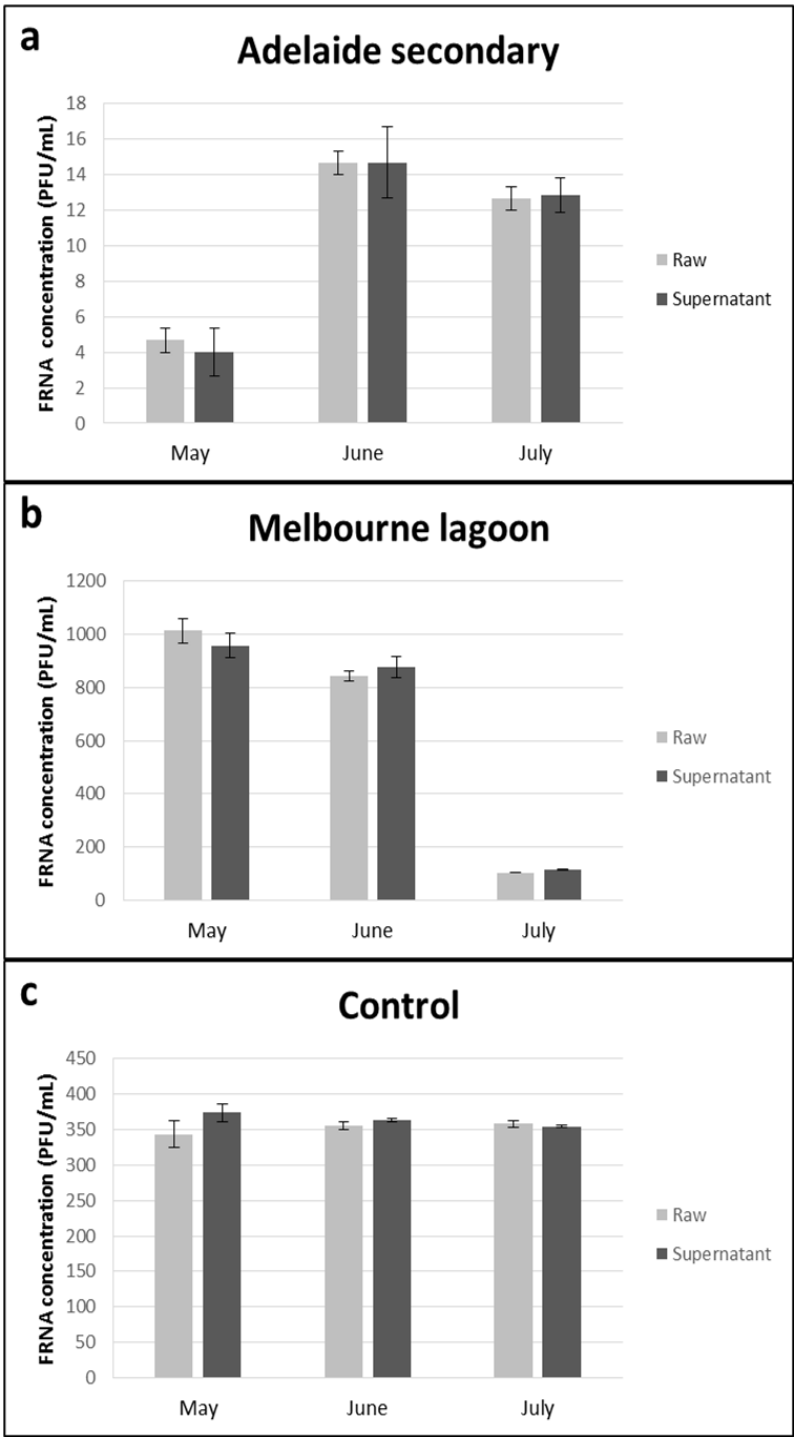

Figure 4.5 Effect of centrifugation on FRNA bacteriophage partitioning

Figure 4.5 Effect of centrifugation on FRNA bacteriophage partitioning. Ten litre grab samples were collected monthly from May 16 to July 16 from A. Adelaide activated sludge clarified effluent (secondary effluent) and B Melbourne lagoon effluent. The samples were subjected to slow speed centrifugation and the number of FRNA bacteriophage in supernatants and pellets were enumerated in triplicate using the double layer agar plaque assay on tryptic soy agar. Graphs a and b show the average FRNA bacteriophage count (expressed as plaque forming unit (PFU)) before and after centrifugation of samples. Graph c shows the partitioning of FRNA bacteriophage control suspended in phosphate buffer saline and centrifuged under the same conditions.

The particle-free PBS spiked with cultured *E. coli* was used as a control to analyse the natural partitioning behaviour of *E. coli;* centrifugation resulted in the partitioning of 29-31 % of *E. coli* into the pellet (Fig 4.6 d). Adelaide secondary effluent and Melbourne lagoon effluent had higher *E. coli* concentrations than the Adelaide lagoon effluent (Fig 4.6). As with the FRNA bacteriophage, the numbers of *E. coli* in the Adelaide secondary effluent increased as the winter progressed (10,000 – 25,000 CFU/100mL, Fig 4.6a). The concentration of *E. coli* in the Adelaide lagoon effluent was relatively consistent for the 3 months (Fig 4.6b). Melbourne lagoon effluent had a pronounced decrease in *E. coli* concentration in June compared to the other months (5,000 CFU/100mL compared with 24,000 CFU/100mL, Fig 4.6c). The amounts of *E. coli* removed by centrifugation in both Adelaide locations were similar to each other (17 - 18% for secondary effluent, 17-20% for lagoon effluent), and significantly lower than the control. In contrast, centrifugation removed 50-54% *E. coli* from the Melbourne lagoon effluent (Fig 4.6e); significantly higher removals than the controls or the other samples ($p<0.05$).

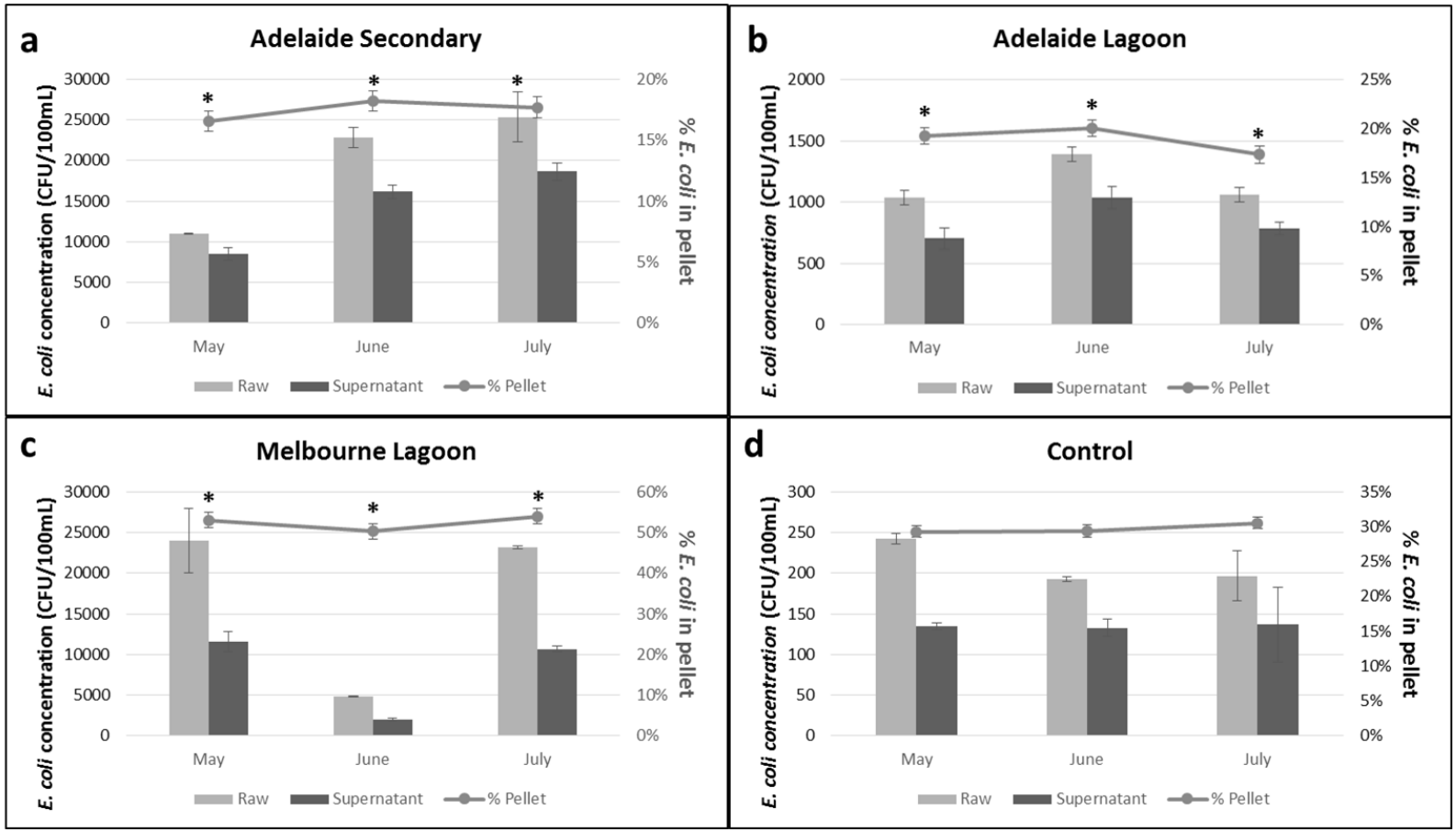

Figure 4.6 Effect of centrifugation on E. coli partitioning

Figure 4.6 Effect of centrifugation on *E. coli* partitioning. A ten litre grab sample was collected each month from May to July from A. Adelaide activated sludge clarified effluent (secondary effluent), B. Adelaide stabilisation lagoon effluent and C. Melbourne lagoon effluent. The samples were subjected to centrifugation (931 X g for 10 minutes) and the number of *E. coli* colonies enumerated in triplicate using membrane filtration on MI agar. Graphs A to C show the average *E. coli* count (expressed as colony forming units) before and after centrifugation of samples. Graph D shows the partitioning of cultured *E. coli* suspended in phosphate buffer saline (PBS) and centrifuged under the same conditions. The secondary vertical axis on each graph shows the percentage of *E. coli* that partitioned into the pellet after centrifugation. Percent removal data were Arcsin transformed before analysis by one-way ANOVA and Tukey's test. Significant difference from the PBS control * $p<0.05$.

4.3.4 Partitioning by Serial Filtration: The particle-free PBS solution spiked with MS-2 phage was serially filtered to examine the natural partitioning behaviour. In these control experiments, there was <10 % removal at each filtration step (Fig 4.7e and f) and most of the MS-2 phage were recovered from the filtrate that passed through the smallest 0.2 µm pore size filter. The Adelaide secondary effluent, which had low concentrations of FRNA bacteriophage (<6 PFU/mL), behaved differently from the control: in May, all the FRNA bacteriophage were removed by filtration, and approximately 20% in June and 60% in July (Fig 4.7b). In the case of the July sample, most of the removal occurred following filtration through the 1.2 µm membrane. FRNA bacteriophage numbers in the Adelaide lagoon effluent was below the detection limit for all three months and hence no data are presented. Filtration of the Melbourne lagoon effluent, however, had similar results to the control in that most of the FRNA bacteriophage were recovered in the 0.2 µm filtrate. There was < 8 % removal at each filtration step for any of the Melbourne lagoon effluent samples (Fig 4.7d).

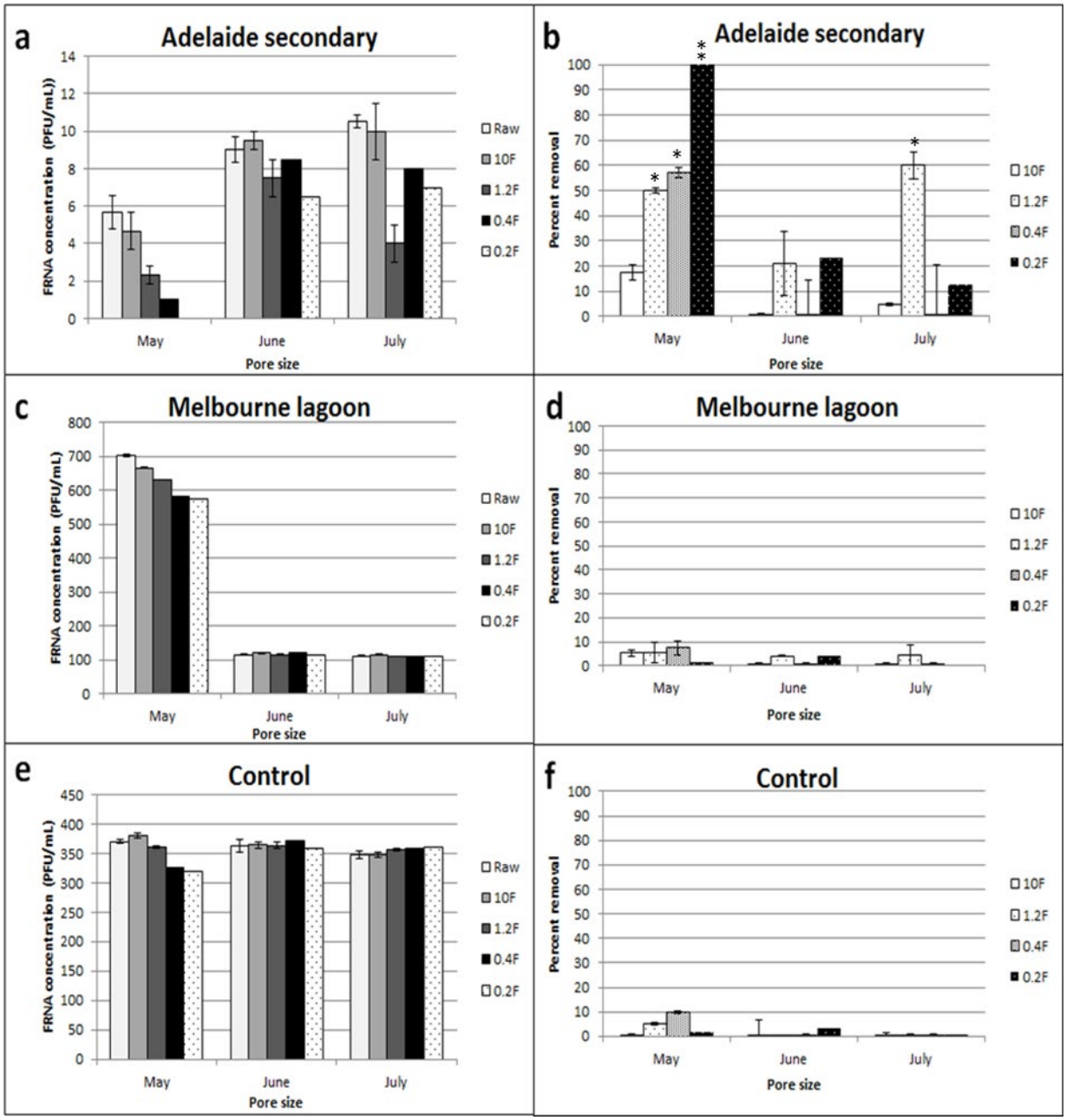

Figure 4.7 Effect of serial filtration on FRNA bacteriophage partitioning

Figure 4.7 Effect of serial filtration on FRNA bacteriophage partitioning. A ten litre grab sample was collected monthly from May to July from Adelaide activated sludge clarified effluent (secondary effluent), Adelaide stabilisation lagoon effluent and Melbourne lagoon effluent. The samples were subjected to series filtration and the FRNA bacteriophage colonies were enumerated in triplicate using a double layer agar plaque assay on Tryptic soy agar before and after each filtration step. Graphs a and c show the average FRNA bacteriophage count (expressed as plaque forming unit (PFU)) in each filtered sample. Graph e shows the partitioning of FRNA bacteriophage control suspended in phosphate buffer saline (PBS) and filtered similarly to the samples. Graphs b, d and f compare the average percent removal of FRNA bacteriophage at each filtration step and significant difference between the percent removals (Arcsin transformed) of samples and control was analysed by one-way ANOVA and Tukey's multiple comparison tests with significance assigned at * $p<0.05$ and ** $p<0.01$

The particle-free PBS control was spiked with *E. coli* and serially filtered to examine its natural size-based partitioning behaviour (Fig 4.8g). In general, the *E. coli,* which are typically 0.5-1 µm wide and 1-2 µm long, passed through the 10 µm and 1.2 µm filters but were completely retained on the 0.4 µm filter. An exception was the May control, which had 24% *E. coli* removed by the 10 µm filter. Except for the Adelaide lagoon May sample (Fig 4.8d), the *E. coli* in the samples were not significantly removed by 10 µm filtration. There were differences between sample locations for *E. coli* removal on the 1.2 µm filters. The Adelaide secondary effluent had consistent and significantly higher removal by the 1.2 µm filter (average of 20 % throughout the sampling period) compared to the control (Fig 4.8b and h). In the case of the Adelaide lagoon effluent, there was no apparent removal of *E. coli* by 1.2 µm filtration for the May sample, but significant ($p \leq 0.05$) removal (60-70%) for the other months (Fig 4.8d). The Melbourne lagoon effluent samples had significant ($p \leq 0.05$) removal of *E. coli* (70-80%) by 1.2 µm filtration (Fig 4.8f). Filtration through the 0.4 µm filter resulted in the removal of any remaining *E coli* from the samples.

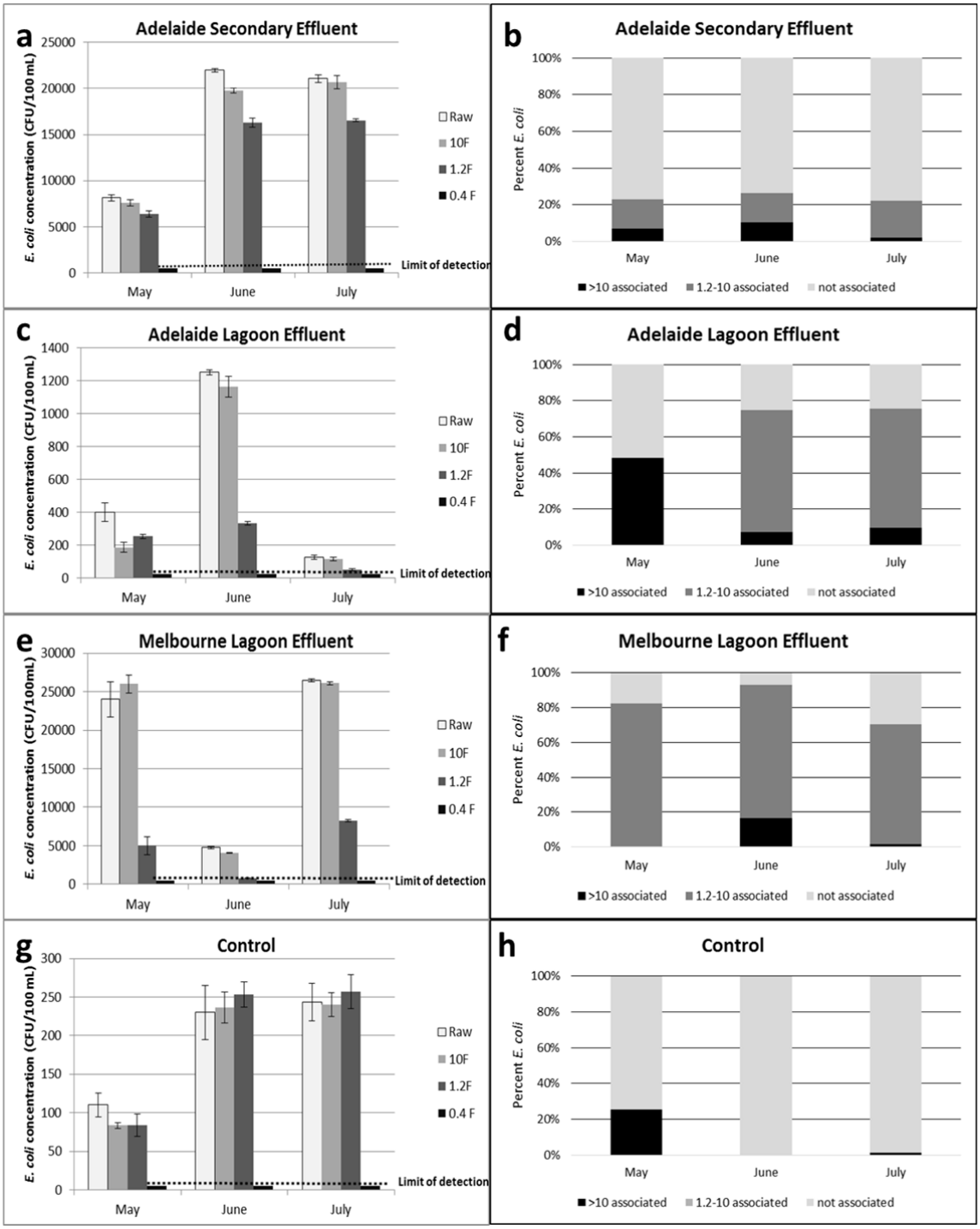

Figure 4.8 Effect of series filtration on *E. coli* partitioning

Figure 4.8 Effect of series filtration on *E. coli* partitioning. A ten litre grab samples were collected from May to July from; a and b- Adelaide activated sludge clarified effluent (secondary effluent), c and d- Adelaide stabilisation lagoon effluent and e and f- Melbourne lagoon effluent. The samples were subjected to series filtration and the *E. coli* colonies enumerated in triplicate using membrane filtration on MI agar before and after each filtration step. Graphs a to c show the average *E. coli* count (expressed as colony forming units) before (raw) and after filtration. Graph g and h shows the partitioning of *E. coli* control suspended in phosphate buffer saline (PBS) and filtered similarly. The *E. coli* count after each filtration was examined by one-way ANOVA to analyse any differences between filtration steps. Graphs b, d, f and h compare the average percent removal of *E. coli* in samples and control after 10 µm and 1.2 µm filtration. Percent removal data were Arcsin transformed then subjected to one-way ANOVA and Tukey's multiple comparison tests to analyse differences from particle free control shown as $p<0.05$.

4.4 Discussion

This study examined the occurrence of two pathogen surrogates, MS-2 phage for enteric viruses and *E coli* for enteric bacteria, in wastewater effluents from two geographically separated treatment plants with different treatment processes. The removal of the surrogates by centrifugation or filtration was compared to the particle profiles and physicochemical parameters of the wastewater effluents collected from three different sites.

4.4.1 Geographical and Temporal Distribution of MS-2 phage

The loads of FRNA bacteriophage increased during winter in Adelaide secondary effluent, but they were removed by the treatment train to the extent that they were not detected in the Adelaide lagoon effluent; phage levels in the Adelaide lagoon effluents were generally below the limit of detection. In contrast, the loads of FRNA bacteriophage in the Melbourne lagoon samples decreased as winter progressed (sample collected at the end of lagoons). It has been reported that secondary treatment is associated with up to 2.5 $\log_{10}$ removal of phage whereas lagoon treatment can achieve up to 4 $\log_{10}$ removal of phage (AWGR, 2006, AWGR, 2008), and the Adelaide WWTP data therefore confirm previous reports regarding the phage removal efficacy of lagoon storage and treatment (Monis, 2015). Enteric virus numbers in different treatment plants are affected by a number of factors, including the input of sewage (domestic, industrial or both), stormwater intrusion (effectively causing dilution of the sewage), incidence of disease within community and the treatment processes (Keegan et al., 2012b). In the current study, the treatment plants represent different geographic locations and have different treatment processes, and these factors are likely to be sufficient to account for the differences in phage load at the Adelaide and Melbourne WWTPs (Keegan et al., 2012b). Hence environmental factors and different kinds of stabilisation ponds can account for differences in phage concentrations between the samples.

4.4.2 Removal of Virus Surrogate MS-2 Phage

The PBS buffer controls spiked with MS-2 phage showed that neither centrifugation nor filtration caused a reduction in phage numbers, suggesting that adherence to the surfaces used in the partitioning experiments (e.g. centrifuge tube wall or filter membrane material) is unlikely to be a cause of phage loss, at least for the pH and ionic conditions for this buffer.

Centrifugation did not remove FRNA bacteriophage from any of the effluent samples, suggesting that there was no attachment of phage to settleable particles. Similarly, filtration did not remove FRNA bacteriophage from the Melbourne lagoon effluent but removed FRNA bacteriophage from some of the Adelaide secondary effluents. Adelaide secondary effluent differed from the lagoon samples in that it contained significantly higher numbers of large (>100 μm) particles, but if FRNA bacteriophage had adhered to these particles then they should have been completely retained by the 10μm pore size membranes. Furthermore, there was no evidence of FRNA bacteriophage association with settleable particles in the centrifugation experiments and the three secondary effluent samples behaved differently to each other but had the same particle profiles. The low FRNA bacteriophage counts (6-10 PFU/mL) in the secondary effluent samples may have contributed to the high and variable filtration results, since the loss of as few as 1 PFU/mL during sample processing could cause a high percent removal, whereas a similar loss in the Melbourne lagoon or Control sample would cause a much lower percent removal to be calculated. This could be the reason for any apparent large differences in removal for some samples. The Adelaide secondary effluent also had lower levels of DOC and SS than the Melbourne lagoon effluent, suggesting that a complex interaction between different physicochemical factors resulted in the removal of phage by filtration (but not centrifugation) in the Adelaide secondary effluent.

Although previous filtration studies found particle-associated viruses in wastewater treatment ponds (Hejkal et al., 1981a, Templeton et al., 2005), virus particle association has been reported

to be largely dependent on the nature and type of particles and the type and strain of virus (Templeton et al., 2008). Different virus strains have characteristic affinities for various types of particles (Moore et al., 1975, Gerba et al., 1980, Meschke and Sobsey, 1998), and the nature of the particles plays a role in FRNA bacteriophage attachment that is independent of the surface properties of the virus (Hébrant et al., 2014, Fauvel et al., 2017). Others have reported minimal association of viruses with settleable particles (Characklis et al., 2005b, Templeton et al., 2008, Silva et al., 2008) or minimal to low association of viruses with settleable particles in wastewater treatment ponds (Symonds et al., 2014). The present study also found minimal association of viruses with settleable particles in secondary treated and lagoon wastewater effluents.

4.4.3 Removal of Bacteria

The control filtration experiments using cultured *E. coli* spiked into PBS demonstrated that *E. coli* cells passed freely through 10 and 1.2 µm filters, with no evidence of cells binding to the membranes (Fig 8H). An exception was the May experiment, where there was 24% removal on the 10 µm filter. The cause for this is unclear, but cell aggregation in this particular culture preparation is a possible explanation. Centrifugation experiments using the same control samples showed that approximately 30% of the *E. coli* partitioned into the pellet (Fig 6D).

The partitioning behaviours following centrifugation of *E. coli* in effluent samples (Fig 6A-C) was different to that of *E. coli* in the PBS control (Fig 6D). In the case of the Adelaide secondary effluent and lagoon effluent samples, fewer *E. coli* were recovered from the pellets compared to the control (17-21% for effluent samples versus 30% for the control). In contrast, significantly higher numbers of *E. coli* partitioned into the pellets of the Melbourne lagoon effluent samples (approximately double compared to the Adelaide samples). The differences in partitioning could be due to particle association, due to differences in particle density, or due

to the differences in the cell density of the cultured *E. coli* compared with the faecally-derived *E. coli*, or a combination of these.

The filtration results suggest that size of particles associated with *E. coli* is not a good predictor of the settleability of *E. coli*. The Adelaide secondary effluent samples had up to 30% of *E coli* captured on either the 10 or 1.2 µm filters (Fig 8b), significantly higher than the PBS controls (Fig 8h) but had a lower percentage of *E. coli* partition into the pellet following centrifugation compared to the controls (Fig 6). Samples from both of the lagoon sites had higher numbers of particles in the 1.2-10 µm and 10-100 µm size ranges compared with the Adelaide secondary effluent samples. The Adelaide lagoon effluent sample had a much higher association of *E. coli* with large particles compared with Adelaide secondary effluent, inferred by higher removal on the 10 or 1.2 µm filters (Fig 8D), but similar settleability of *E. coli* in response to centrifugation. The Melbourne lagoon effluent sample had high removals of *E. coli* on the 1.2 µm filters, comparable with or higher than the Adelaide lagoon samples, but the *E. coli* in the Melbourne samples had more than twice the amount of removal following centrifugation. These results suggest that there are differences in the nature of the particles at the different sites, in particular the particle density, causing differences in the settleability of particle-associated *E. coli*. Some organisms, such as cyanobacteria, can be buoyant, and association of *E. coli* with such cells could explain the differences in settleability. Differences in nutrient concentrations between the samples, especially nitrogen and phosphorous, can also impact the presence of different types of particles as described earlier in Chapter 3. Different particles would exhibit different characteristics such as surface charge or density, which would highly impact the association behaviour of organisms. We conclude that *E. coli* were associated with lighter suspended particles in Adelaide effluents and heavier settleable particles with a different composition in Melbourne lagoon effluent.

Other studies have reported the association of *E. coli* with settleable particles > 8 µm in stormwater, ≤ 12 µm in river water and also to soil particles > 2 µm (Characklis et al., 2005b, Oliver et al., 2007, Soupir et al., 2008, Kunkel et al., 2013). Faecal coliform bacteria attach to particles ranging from >5 to >10µm (Qualls et al., 1985, Schillinger and Gannon, 1985, Emerick et al., 2000), hence our particle profile and *E coli* association study is in broad agreement with the previous reports, and also extends the observations made by others to include secondary wastewater and lagoon effluents.

The nature (organic or inorganic) and size distribution of particles along with other characteristics of the aquatic environment, affect bacterial attachment or association. The characteristics of particles in any particular wastewater reflect the different types of particles in the aquatic systems (sewage, river and storm water) that feed the WWTP (Templeton and Butler, 2011a, Abdel-Raouf et al., 2012). Soupir et al (2010) noted that higher TSS concentrations may provide more attachment sites for pathogens, and other factors such as pH, cell hydrophobicity and particle morphology are also known to affect the affinity of *E. coli* for inorganic particles (Hipsey et al., 2006). The association of *E coli* with particles was different in the Melbourne and the Adelaide effluents, probably because of a number of interacting factors, rather than any single parameter. The different geophysical characteristics of the two sites probably caused differences in the composition and nature of the particles. The different treatment trains in the two WWTPs utilise different physical and biological processes, which would also affect particle composition and size profiles (Neis and Tiehm, 1997a, Garcia-Mesa et al., 2012). The Melbourne lagoon had significantly lower TSS than either of the two Adelaide sample sites, and this supports our conclusion that physicochemical and particle size distribution differences between the two sites caused differences in removal of bacteria by filtration and centrifugation.

4.6 Conclusion

The association of *E. coli* with particles can impact transport within water systems and removal by sedimentation (Characklis et al., 2005b, Krometis et al., 2007a). Understanding pathogen-particle association and removal is important when designing targeted removal mechanisms with high treatment efficiencies. This study indicates that sedimentation is unlikely to be an effective strategy for removing pathogens from Adelaide wastewater effluents during the winter, but that it may have application in the Melbourne WWTP. Our data support further studies to characterise pathogen-particle associations and removal during an extended sampling period throughout the year, and the characterisation of the particle size cohort (1.2-10 µm) primarily associated with pathogens points the way to designing effective filtration methods for pathogen removal.

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Chapter 5: Partitioning behaviour of *C. parvum* oocysts in secondary treated effluents as compared to anaerobic bacterial spores

5.1 Introduction

Cryptosporidium is a major public health concern because the oocysts are robust, stable in the environment (particularly at temperatures below 15 °C), resistant to chlorine, and have a low infectious dose (King et al., 2005, Nasser, 2016). Oocysts are primarily transmitted by direct faecal-oral transfer or ingestion of contaminated food or water, with the latter two routes of infection causing outbreaks of the gastrointestinal illness cryptosporidiosis (Fayer et al., 2000). As a consequence of human infections, *Cryptosporidium* oocysts are commonly detected in wastewaters, with numbers fluctuating depending on the level of community-wide illness (King et al., 2016). Due to its public health significance, *Cryptosporidium* must be inactivated or removed from treated wastewater prior to discharge to the environment or reuse (King et al., 2017). The removal efficiency of *Cryptosporidium* oocysts by wastewater treatment processes is dependent upon wastewater and environmental conditions, and the type of process applied (Bonadonna et al., 2002, King et al., 2016). Some disinfection methods, such as exposure to ultraviolet radiation, are known to be highly effective at inactivating *Cryptosporidium*, but can be adversely impacted by turbidity and excess organic matter (Morita et al., 2002, Nasser, 2016). Different microbes can exist either free in suspension or associated with particles in waters and wastewaters, with the free phase microbes existing as discrete particles or as aggregated groups (as reviewed by (Chahal et al., 2016)). It has been established that particle-associated microbes are less susceptible to inactivation due to various modes of protection offered by the particles, making wastewater with high numbers of particles more difficult to disinfect (Emerick et al., 1999, Li et al., 2009, Kollu and Örmeci, 2012).

Although the surface properties of *Cryptosporidium* oocysts have been characterised, little is known about their interactions with particles. The extent of oocyst association with particles in wastewater is governed by various factors such as pH, the presence of organics, ionic conditions and the surface characteristics of the particles (Searcy et al., 2005), in under typical

environmental conditions, oocysts have a negative surface charge, which might cause repulsion and hinder association with similarly charged particles, such as clays and sand (Considine et al., 2002, Kuznar and Elimelech, 2004, Brookes et al., 2006). Both particle-associated and free oocysts have been isolated from wastewater (Tsuchihashi et al., 2003, Clancy et al., 2004). However, direct observation of particle-associated *Cryptosporidium* is limited by their relatively low numbers in wastewater, and relatively high costs of methods required to concentrate and purify oocysts (Li et al., 2009). Therefore, majority of experiments investigating the particle association of oocysts have used seeding of *Cryptosporidium* into environmental or artificial samples or have utilised a surrogate organism or particle to study behaviour under the selected conditions (Dai and Hozalski, 2003, Monis et al., 2017). The use of cost-effective surrogates would help determine the conditions under which more focussed experiments using oocysts can be used. Anaerobic spore forming bacterial (*Clostridium* spp.) spores have been previously used as a surrogate for *Cryptosporidium* spp. in particle association studies (Characklis et al., 2005b, Cizek et al., 2008b).

Previous *Cryptosporidium* particle-association studies have mainly focussed on stormwater and not much has been reported for wastewater. Previous studies have assessed particle association using three main techniques: centrifugation, filtration and homogenisation. Centrifugation has been applied to analyse the association of *Cryptosporidium* with settleable particles in stormwater (Characklis et al., 2005b, Cizek et al., 2008b, Krometis et al., 2010), while filtration and homogenisation have been used in wastewater to separate particle-associated microbes (Örmeci and Linden, 2002a, Madge and Jensen, 2006a). As yet, these three techniques together have not been used for assessing the partitioning of *Cryptosporidium* in wastewater.

Understanding the particle-association behaviour of oocysts could lead to improved modelling predictions of pathogen fate and identify new pathogen removal mechanisms, potentially

leading to the development of new cost-effective approaches for removal of oocysts and production of safer recycled water. This study was designed to analyse the partitioning behaviour of anaerobic bacterial spores and *Cryptosporidium* oocysts, and to evaluate the performance of anaerobic spores as a surrogate for *Cryptosporidium* partitioning.

5.2 Methods

5.2.1 Sample Collection: Monthly 10 litre grab samples were obtained from Adelaide (secondary-treated clarified effluent and final lagoon effluent) and Melbourne (55E lagoon effluent) treatment plants between June and August 2016.

5.2.2 Physical analysis: The concentration and size distribution of particles in samples before and after homogenisation were measured using a Sequoia laser in situ scattering and transmissometry (LISST portable| XR) as described in section 2.3.3 (Chapter 2). The profiles were aggregated into 4 size classes: <1.2 µm; 1.2-10 µm; 10-100 µm; and >100 µm; and the mean of each size class was then determined. Concentrations of NH_3, NO_3^-, NO_2^- and total P were measured as described earlier in section 2.3.2 (chapter 2).

5.2.3 Partitioning analysis: Each sample jar was taken out of the fridge and inverted several times to resuspend the settled particles. Samples were centrifuged as described earlier in section 2.3.4.1 (Chapter 2). This method was selected to separate free-phase anaerobic spores from anaerobic spores associated with heavy or settleable particles (Characklis et al., 2005b, Cizek et al., 2008b). Both the raw (parent) and supernatant were then analysed for native anaerobic bacterial spores. A particle free phosphate buffered saline (PBS – 900mL x 3) control was spiked with cultured *C. perfringens* spores (AWQC in house method) at a concentration of 10^3/mL and treated the same way. Briefly, culturing *C. perfringens* spores utilises a bioball of *C. perfringens*, which was first cultured onto Columbia Horse blood agar plates anaerobically.The cultured colonies were inoculated into cooked meat broth. The cells were then stressed for conversion into spores and recovered by centrifugation. The reduction in spore

count after centrifugation was expressed as percent removal, which was calculated by using the spore count before and after centrifugation.

One aliquot of each sample was serially filtered through 10 µm, 1.2 µm, 0.4 µm (Millipore and 0.2 µm filter membranes as described in section 2.3.4.2 (Chapter 2). Following each filtration step, aliquots of equal volume were collected from each filtrate to ensure sufficient volume was obtained for microbial analysis. The raw (unfiltered sample) was also analysed to evaluate the initial spore concentration. A particle free PBS (200mL x 3) control was spiked with cultured *C. perfringens* spores at a concentration of 10^3/mL and treated the same way. The removal of spores after each filtration step was expressed as a percentage, which was calculated by using the spore count before and after each filtration step.

Triplicate samples were homogenised using both of the homogenisation techniques as described in section 2.3.4.3 (Chapter 2). Samples were also homogenised without the presence of any buffer (Control A and B, matching Buffer A and Buffer B homogenisation speeds respectively) at the two speeds to evaluate the effect of speed alone on dispersion. Raw (non-homogenised) and homogenised samples with and without buffers were then analysed for anaerobic bacterial spores and particle analysis by LISST as described in section 2.3.3 (Chapter 2).

5.2.4 Spore enumeration: Spores of anaerobic sulphite reducing clostridia were enumerated in triplicate using the standard method AS/NZ 4276.17:2000 (Australian/New Zealand Standard Method, 2000). 100 mL samples were collected in sterile plastic jars and heat treated at 75^o C for 20 minutes by immersing in a water bath. Dilutions were prepared as required using Phosphate buffered saline and 100 mL of each dilution was filtered through 0.45 µm cellulose nitrate membrane. The filter membranes were then carefully placed onto Tryptose sulphite cycloserine (TSC CM0857) plates containing perfringens selective supplement (SR0088) and

incubated at 35° C for 48 hours in 2.5 L anaerobic jars with anaerobic generator sachets (Oxoid Anaerogen AN0025A) and Oxoid anaerobic indicators (BR0055). After incubation, straw yellow to dark brown colony forming units (CFU) were counted as CFU/100 mL.

5.2.5 *Cryptosporidium parvum* analysis: The gamma irradiated *C. parvum* oocysts used in this study were of cattle origin (strain lowa) and purchased from BTF (Sydney, Australia) at a stock concentration of 10^8 per mL in phosphate buffered saline. The Adelaide secondary effluent and PBS controls were spiked with non-viable *C. parvum* oocyst stock solution to a final concentration of 10^2 per mL and mixed overnight at room temperature (~22 - 23°C) to represent natural mixing and allow association with particles. The same treatment was also applied to lagoon effluent samples, however, the higher turbidity of these samples interfered with direct enumeration of oocysts by fluorescence microscopy. Financial constraints precluded the use of high performance purification methods such as immunomagnetic purification, so these samples were excluded from further study. The spiked samples were centrifuged and serially filtered in the same way as described above for partitioning analysis. For centrifugation, 3 x 900 mL aliquots of each sample or control were centrifuged at 931 x g for 10 min. This resulted in a significantly higher % removal compared with spores, so two lower speeds, 465 x g and 232 x g, were also used to further evaluate the impact of centrifugation speed on partitioning behaviour. In the case of filtration, 3 x 100 mL aliquots of sample or control were processed. Based on the filtration results, insufficient numbers of oocysts were associated with particles to allow experiments to assess the effect of homogenisation on oocyst-particle association.

5.2.6 Oocyst enumeration The oocysts were enumerated as previously described (King et al., 2015b) to observe removal and association behaviour. Briefly, treated and untreated raw samples (one mL aliquots in triplicate) were stained with 30 µL Easystain and incubated in the dark for a minimum of 1 hour at room temperature. Samples were then filtered onto 13 mm

diameter 0.8 µm pore size polycarbonate filter membranes (Rowe scientific), mounted on microscope slides using mounting oil and sealed with a coverslip. The slides were then scanned using a fluorescent microscope (Olympus BX40) under blue light. Green fluorescent oocysts in the 4 – 5 µm size range were counted and reported as average oocysts per mL ± standard error of mean.

5.2.7 Statistical analysis: All microbial analyses were conducted in triplicate and the results reported as mean ± standard error of mean CFU per 100 mL. Spore counts after centrifugation and filtration (samples and control) were converted to percent removal and then percentages were transformed to Arcsin values. The Arcsin removal values of centrifugation and each filtration step were then compared between samples and the control using Origin 8. For the homogenisation experiments, the increase in the number of spore counts (log_{10} transformed) was compared before and after treatment. The means of the sample and controls were analysed by Tukey's multiple comparison test and any significant differences between the control and treatment were analysed using one-way analysis of variance (ANOVA). *C. parvum* counts before and after centrifugation and filtration were also represented as percent removal and analysed the same way as the spores. Particle profiles of all the samples were normalised by log_{10} transformation and then compared to each other using Graph pad Prism 7.03. Two-way analysis of variance was performed and the means were compared using the Tukey's multiple comparison tests setting the interaction between sample and month for each size class and size class and month for each sample.

5.3 Results

The Adelaide secondary and lagoon effluents had lower levels of NH_3 and higher levels of NO_3^- whereas the Melbourne lagoon effluent had low levels of NO_3^- and significantly higher levels of NH_3 than the other two samples (Fig 5.1). The Adelaide lagoon effluent had significantly higher levels of NH_3 than the Adelaide secondary effluent in July and August ($p<0.001$). Total P content decreased significantly in July and increased significantly in August ($p<0.001$) in the Adelaide samples but was similar in all the three months in the Melbourne lagoon effluent (Fig 5.1). The Melbourne lagoon had significantly higher total P content than the other two samples during all months of the year ($p<0.001$). Melbourne lagoon effluent had the most consistent pH during all the months of the study whereas pH decreased significantly ($p<0.05$) in Adelaide samples in August compared with June and July.

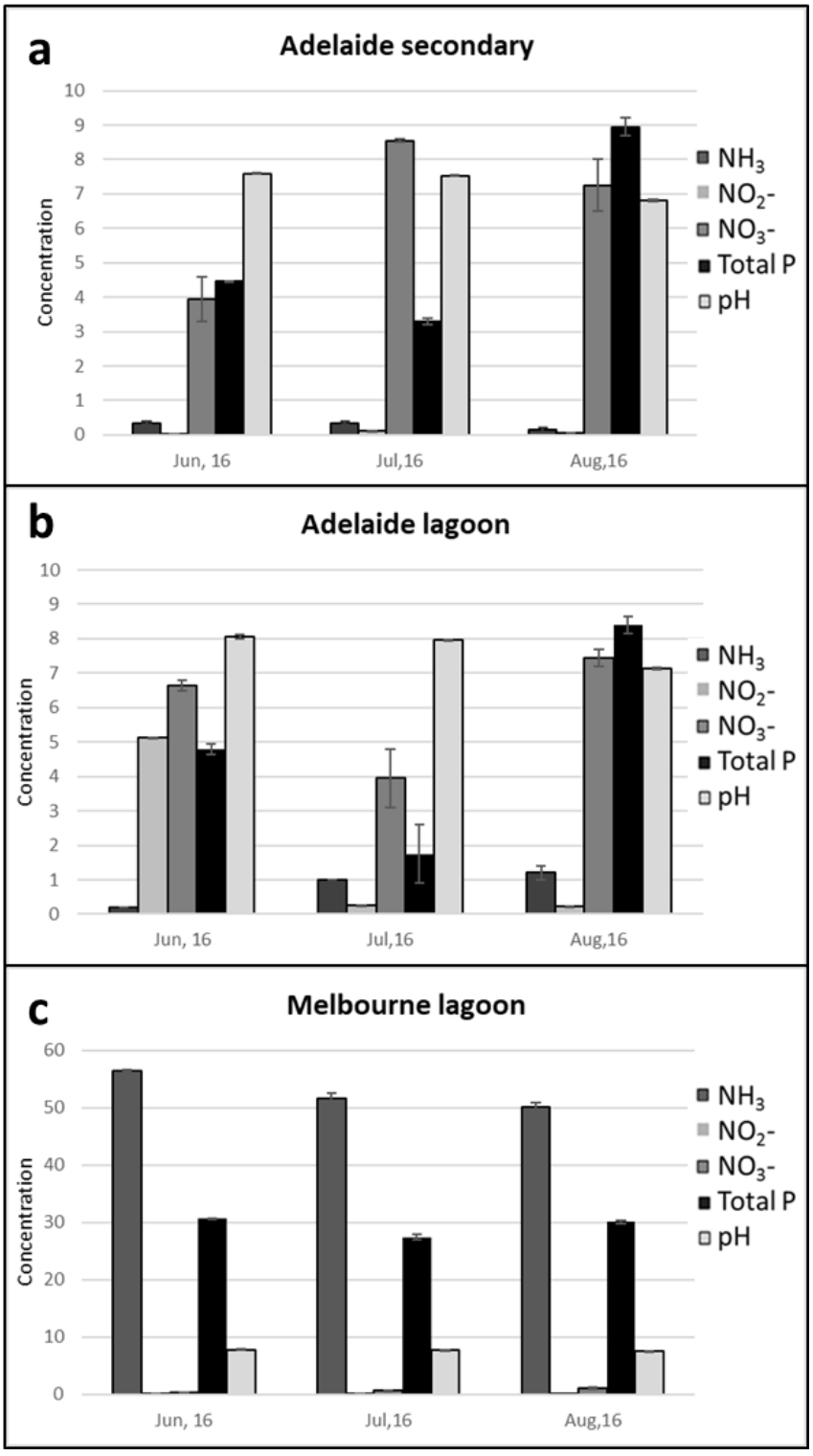

Figure 5.1 Physiochemical properties of the three wastewater samples

Figure 5.1 Physiochemical properties of the three wastewater samples. The concentration of various nutrients in a) Adelaide secondary effluent, b) Adelaide lagoon effluent and c) Melbourne lagoon effluent was measured in triplicate each month using HACH colorimetric kits. The pH of each monthly sample was recorded in triplicate using Eutech ph700 pH meter. Two-way analysis of variance was used to analyse the differences between the samples and within months for each sample.

The particle profile in Adelaide secondary effluent was similar in each of the three months and there were more than two-fold larger particles (> 100 μm) than in the other 2 samples (Fig 5.2a). In June, Adelaide lagoon effluent had significantly higher numbers of small particles in (< 1.2 -100 μm) than the other two months (Fig 5.2b). Melbourne lagoon effluent had the most variable profile over the three months and all the different sized particles changed significantly in each month except the larger particles (>100 μm) for the first two months ($p<0.05$). Homogenisation with both buffers A and B significantly decreased the number of larger particles (>100 μm) and increased the number of mid-sized particles (10 -100 μm) in Adelaide secondary effluent for all the three months ($p<0.05$). There was no significant difference in the smaller particles (<1.2-10 μm) in Adelaide lagoon effluent. The 10-100 μm particle size class in Adelaide lagoon effluent showed an increase in particle counts following homogenisation but this was only significant for buffer B in July. Both the buffers significantly decreased the count in size classes <1.2 μm, 10-100 and >100 μm in Melbourne lagoon effluent ($p<0.05$). Buffer A showed the largest reduction in larger particles (>100 μm) for all the samples and the largest reduction in all the size classes in Melbourne lagoon effluent ($p<0.05$).

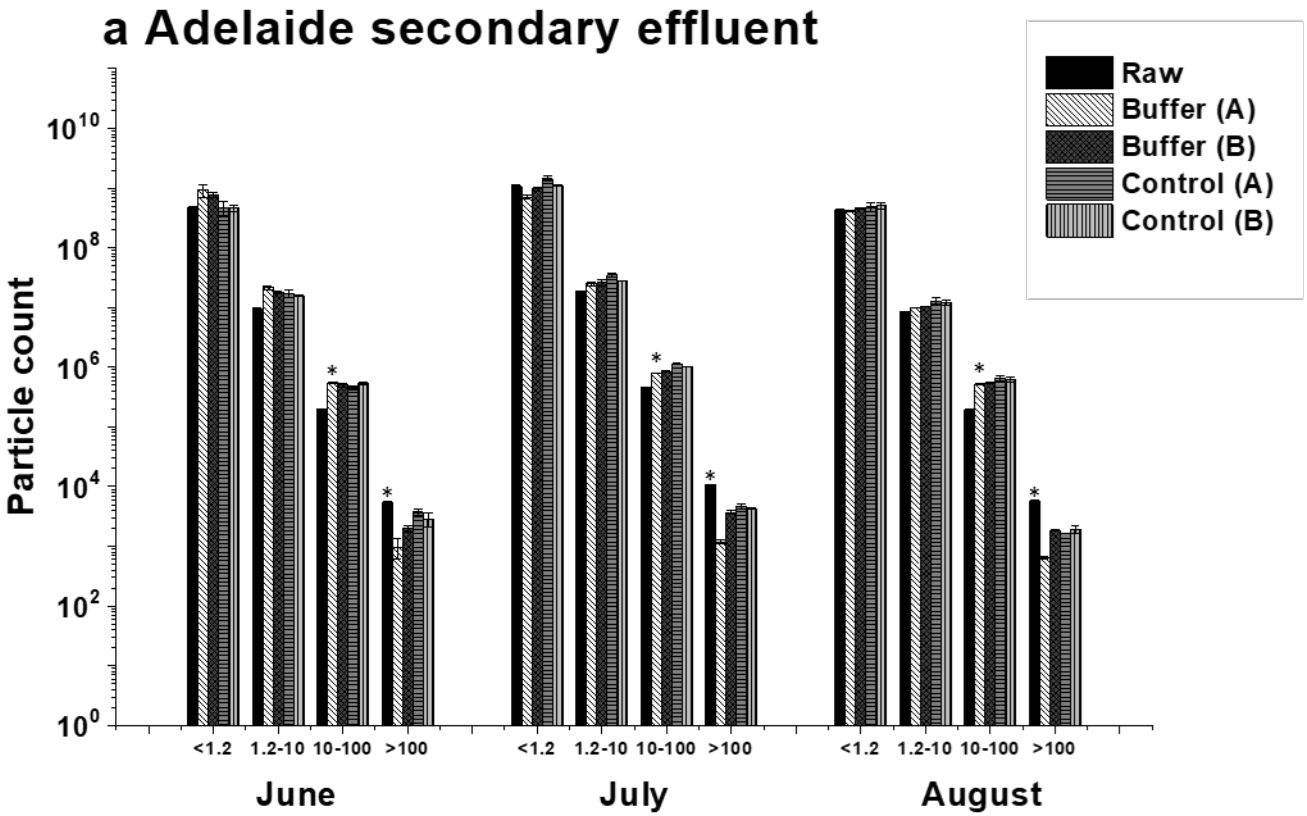

Figure 5.2 Particle size distribution before and after particle dispersion

Figure 5.2a Particle size distribution before and after particle dispersion in the three wastewater samples. Triplicate monthly samples (collected in June, July and August) were homogenised in the presence of two 10 % buffers (Buffer A and B) or without any buffer (Control A and B, retrospectively) under the same homogenisation conditions. Particle size distribution was measured in triplicate by laser *in situ* transmissometry (LIIST|XR) for the raw (untreated) water and samples after homogenisation. The differences between treatment groups were analysed by two-way analysis of variance ($p<0.05$). The x-axis indicates the particle size ranges (in microns)

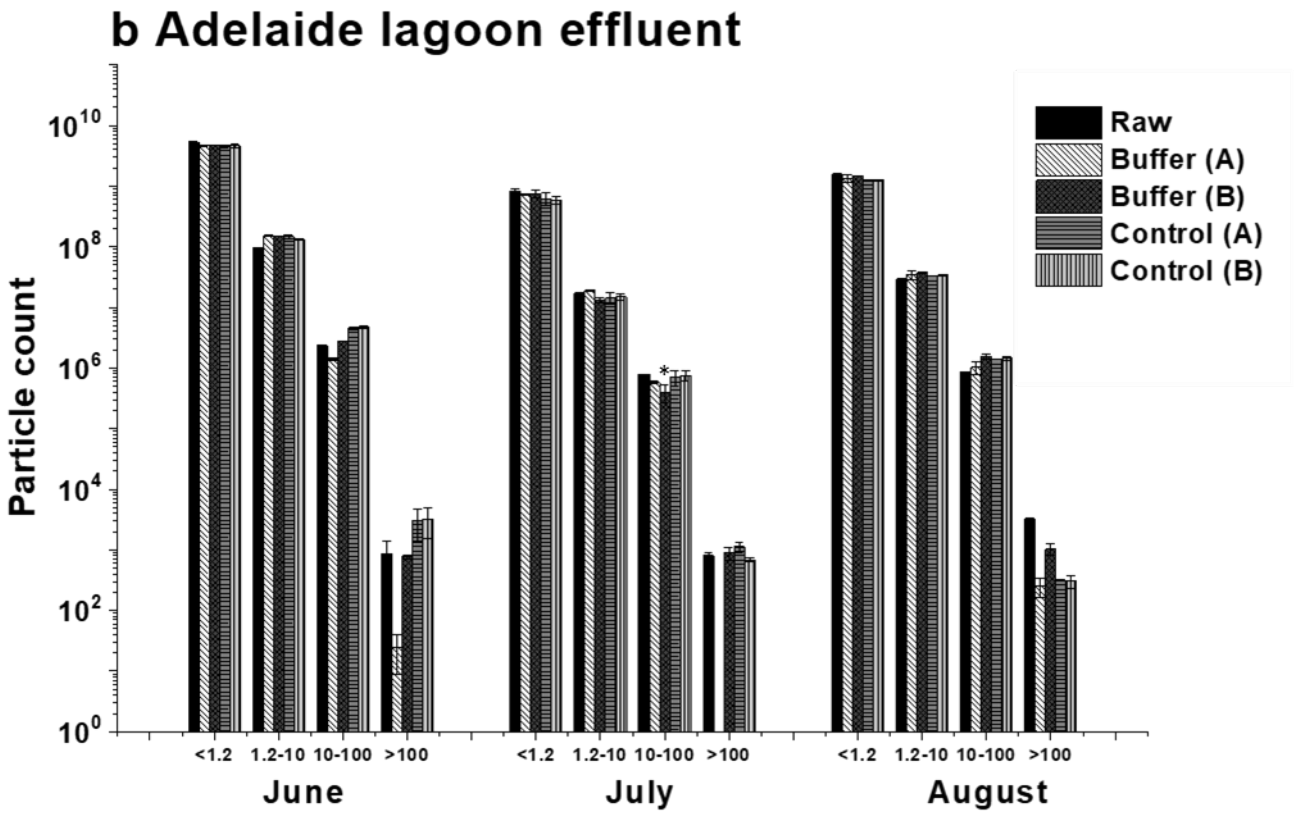

Figure 5.2b Particle size distribution before and after particle dispersion Fig 5.2b: Particle size distribution before and after particle dispersion in the three wastewater samples. Triplicate monthly samples (collected in June, July and August) were homogenised in the presence of two 10 % buffers (Buffer A and B) or without any buffer (Control A and B, retrospectively) under the same homogenisation conditions. Particle size distribution was measured in triplicate by laser *in situ* transmissometry (LIIST|XR) for the raw (untreated) water and samples after homogenisation. The differences between treatment groups were analysed by two-way analysis of variance ($p<0.05$) The x-axis indicates the particle size ranges (in microns).

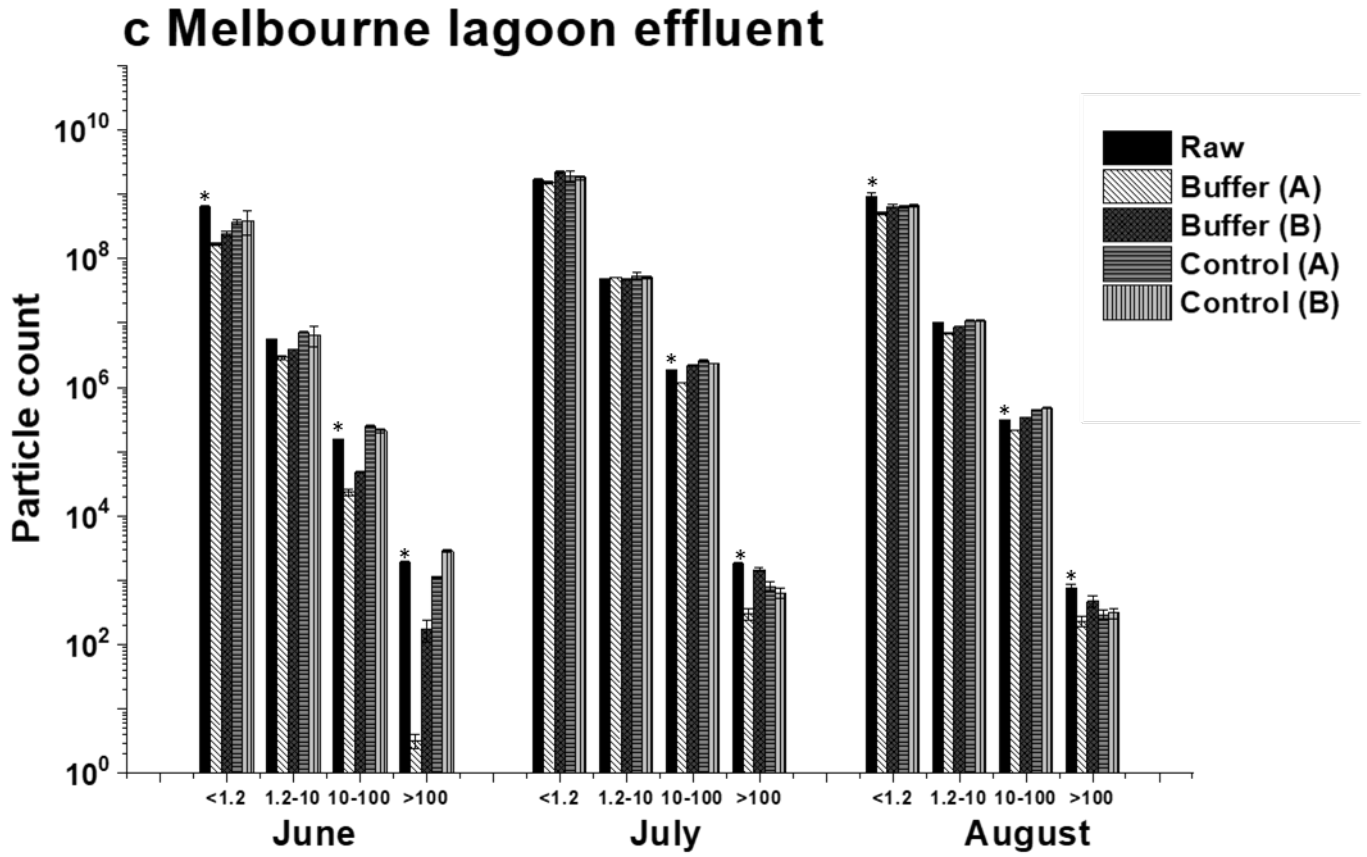

Figure 5.2c Particle size distribution before and after particle dispersion in the three wastewater samples. Triplicate monthly samples (collected in June, July and August) were homogenised in the presence of two 10 % buffers (Buffer A and B) or without any buffer (Control A and B, retrospectively) under the same homogenisation conditions. Particle size distribution was measured in triplicate by laser *in situ* transmissometry (LIIST|XR) for the raw (untreated) water and samples after homogenisation. The differences between treatment groups were analysed by two-way analysis of variance ($p<0.05$). The x-axis indicates the particle size ranges (in microns).

Anaerobic bacterial spores were detected as dark cream to brownish black colonies on TSC agar before and after the treatments. Adelaide secondary effluent and Melbourne lagoon effluent had similar patterns in that the spore count increased in July and decreased in August, whereas in Adelaide lagoon effluent, the counts decreased in both July and August. The spore count decreased after centrifugation in all the three samples in all three months (Fig 5.3). The percent removal was significantly different from the particle-free control in all three samples, except for Melbourne lagoon effluent in August (Fig 5.3). Adelaide secondary effluent had the highest removal of spores (90 %) in July and August ($p \leq 0.001$) and the lowest removal of spores during June (65%), which was statistically significant ($p < 0.05$). Adelaide lagoon effluent showed the most consistent removal of 83% ($p \leq 0.001$). Melbourne lagoon effluent had the higher rates of removal of 75 % and 80 %, respectively, during June and July ($p < 0.05$).

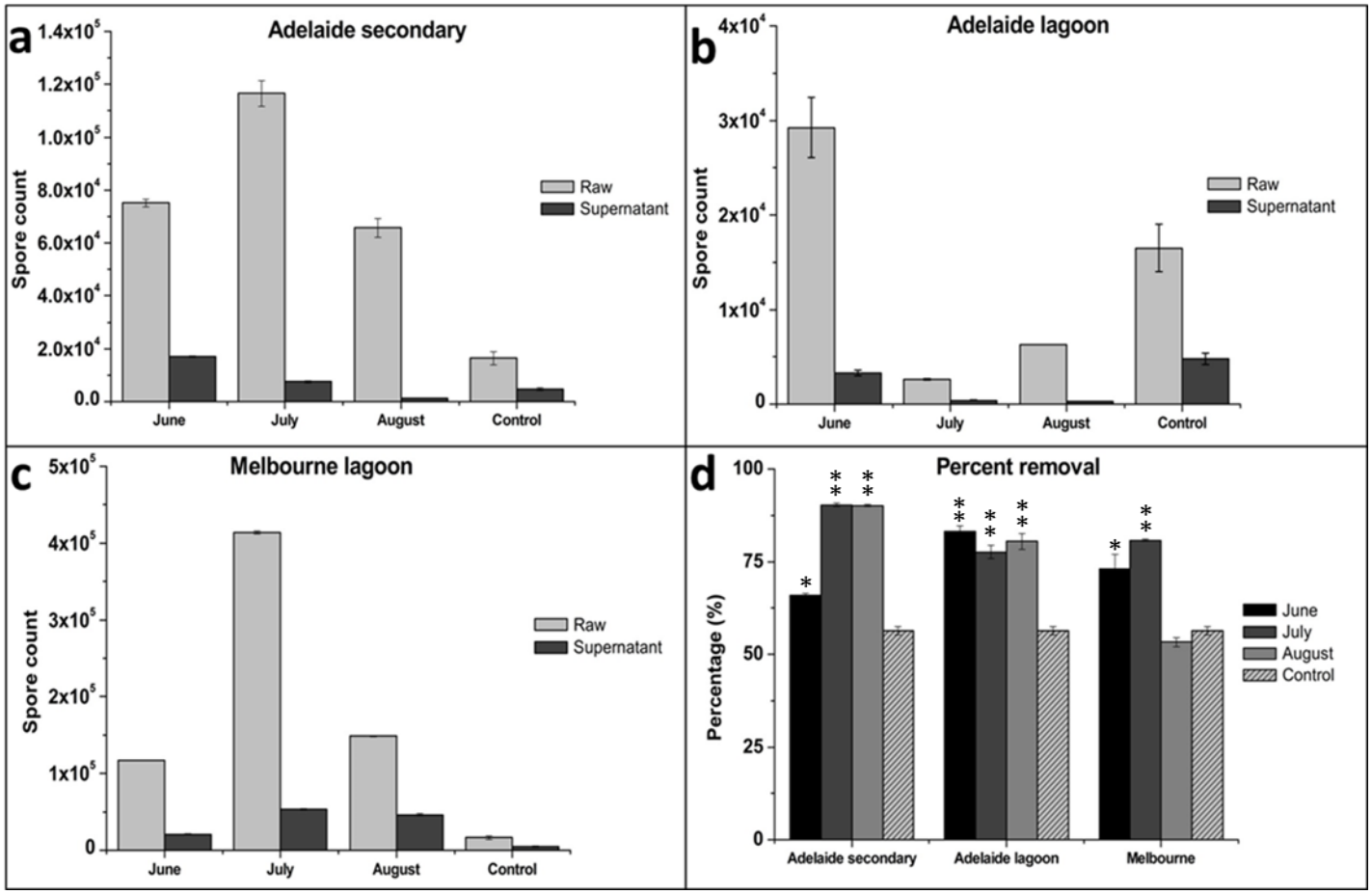

Figure 5.3 Effect of centrifugation on native anaerobic bacterial spores

Figure 5.3 Effect of centrifugation on native anaerobic bacterial spores in the three wastewater samples. Samples (900 ml x 1 aliquot for each month) were centrifuged at 931 x g for 10 minutes at 4°C. Following centrifugation, 600 ml supernatant was carefully removed and the concentration of spores was measured in both supernatant and the parent (raw) sample. The decrease in spore count after centrifugation was converted to percent removal using the counts before and after centrifugation. A no particle Phosphate buffered saline control was spiked with cultured *C. perfringens* spores and treated the same way. One way analysis of variance was used to analyse the differences between the samples and control *- $p<0.05$ and ** - $p<0.001$.

Spore counts were measured after each filtration step and the percent removal was calculated (Fig 5.4d). The spores were completely removed by filtration through a 1.2 µm filter membrane. Therefore, the removal percent was reported only for 10 µm filtration and was compared to the PBS control. Adelaide secondary effluent showed the most consistent removal, with a maximum of 90% in July (Fig 5.4). Adelaide lagoon effluent had a variable removal of 60-80 % across the months, with the highest removal of 80 % in June. Melbourne lagoon effluent had the lowest removal of 25-47% of the three sites ($p < 0.05$) (Fig 5.4).

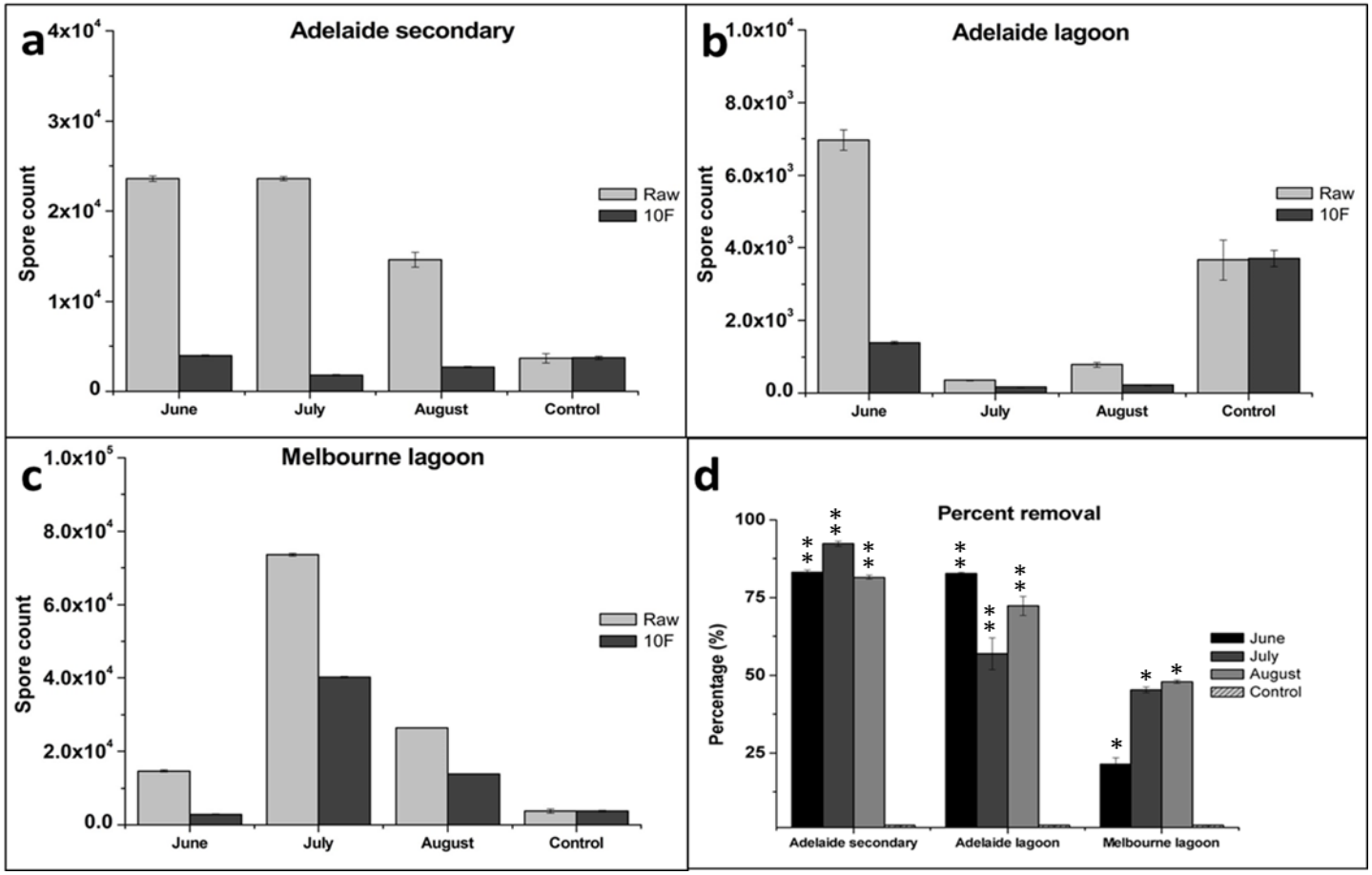

Figure 5.4 Effect of serial filtration on native anaerobic bacterial spores

Figure 5.4 Effect of serial filtration on native anaerobic bacterial spores in the three wastewater samples. Samples were serially filtered through 10 µm, 1.2 µm, 0.4 µm and 0.2 µm polycarbonate filter membranes. Following each filtration step, the concentration of spores was measured in both filtrate and the parent (raw) sample. The decrease in spore count after filtration was converted to percent removal using the counts before and after filtration. A no particle Phosphate buffered saline control was spiked with cultured *C. perfringens* spores and treated the same way. One way analysis of variance was used to analyse the differences between the samples and control (**- $p<0.001$ and * - $p<0.05$). Here 10F – 10 µm filtrate. The spores were completely removed at 1.2µm filtration and hence no counts are presented for any other filtrate except 10 F.

The spores were enumerated after homogenisation to detect any increase in number caused by dispersion or detachment from particles. Homogenisation increased the spore count in all the samples. The spore count was significantly higher after homogenisation in Buffer A than in raw ($p<0.05$), Buffer B ($p<0.05$) or control ($p<0.05$) in secondary effluent, in each of the three months (Fig 5.5). Similar results were obtained from Adelaide lagoon effluent in that Buffer A and B had significantly higher ($p<0.05$) spore counts for all the months. Buffer B worked better in July and August but was not significantly different from Buffer A. Only Buffer A caused a significant increase in the spore count in Melbourne lagoon effluent in July ($p<0.05$). Buffer B did not increase the spore count significantly for all the months.

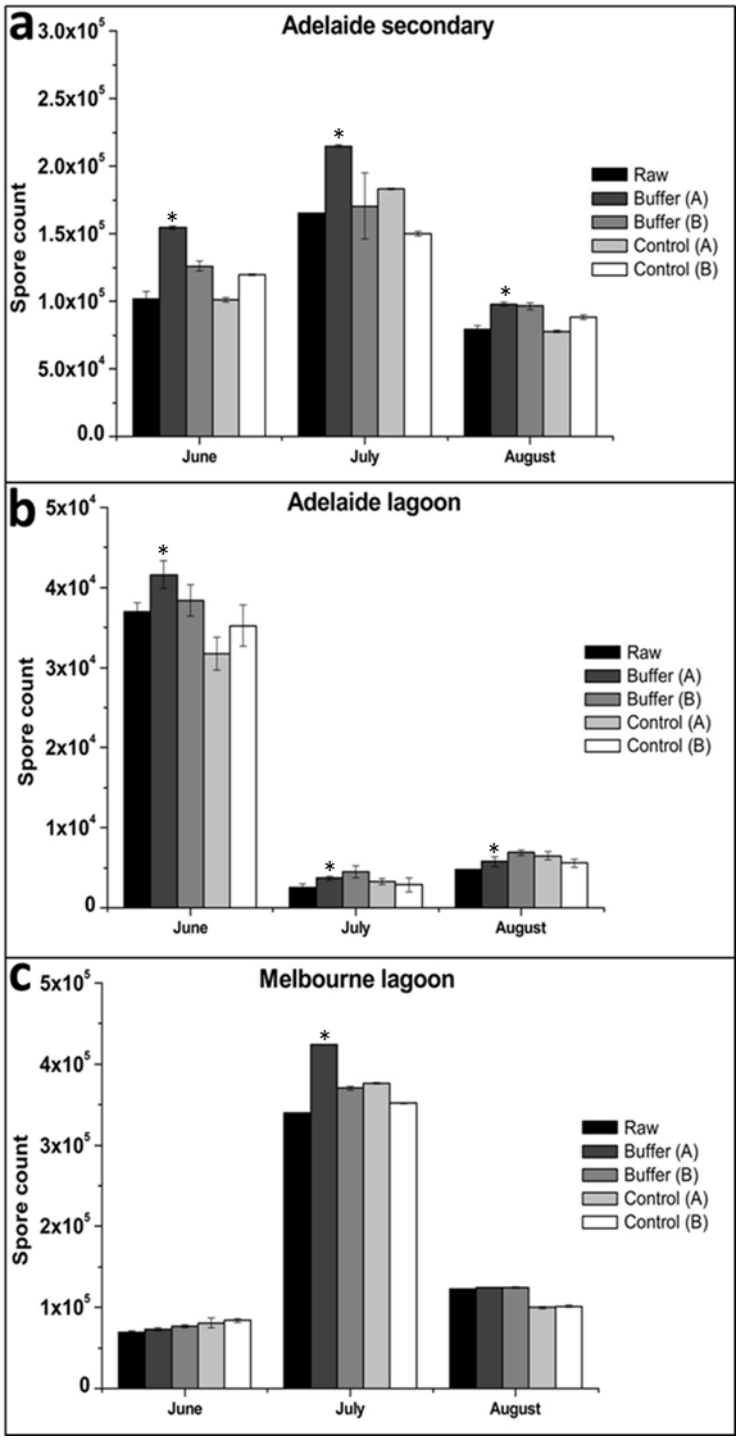

Figure 5.5 Effect of particle dispersion on the spore count

Figure 5.5 Effect of particle dispersion on the spore count. Samples (90 mL x 3) were homogenised in the presence of two buffers (A and B) and 100 mL x 3 each sample without any buffer at the same conditions (Control A and B). The spore count was detected before and after homogenisation. The differences between the samples and within the months for each sample were analysed by two way analysis of variance. The counts were log transformed before analysis.

Green fluorescent circular-shaped *C. parvum* oocysts were counted before and after centrifugation and filtration in Adelaide secondary effluent only. The number of oocysts in supernatant was significantly lower than in the raw wastewater sample, and in the particle free control (Fig 5.6). The percent removal ranged from 60 -90 % in the three monthly samples and 75-85% in the control for centrifugation at 931 x g (Fig 5.6). The percent removal for Adelaide secondary effluent decreased as the centrifugation speed decreased and ranged between 40-60% at 465 x g and 30-40 % at 232 x g. The percent removal for the control also decreased from 55% at 465 x g to 45 % at 232 x g. The percent removals of samples were not significantly different from the control when different speeds were compared Filtration removed oocysts in all the three monthly samples and was significantly higher than in the particle-free control (Fig 5.7). The oocysts were completely removed by 1.2 μm filter owing to their bigger size and hence no results were presented for any other filtrates except 10 F. The removal percent varied from 10 to 35 % for the samples and 1 to 1.5 % for the control (Fig 5.7). June sample showed the maximum removal, with an average of 22 % and was significantly different from control at $p < 0.05$.

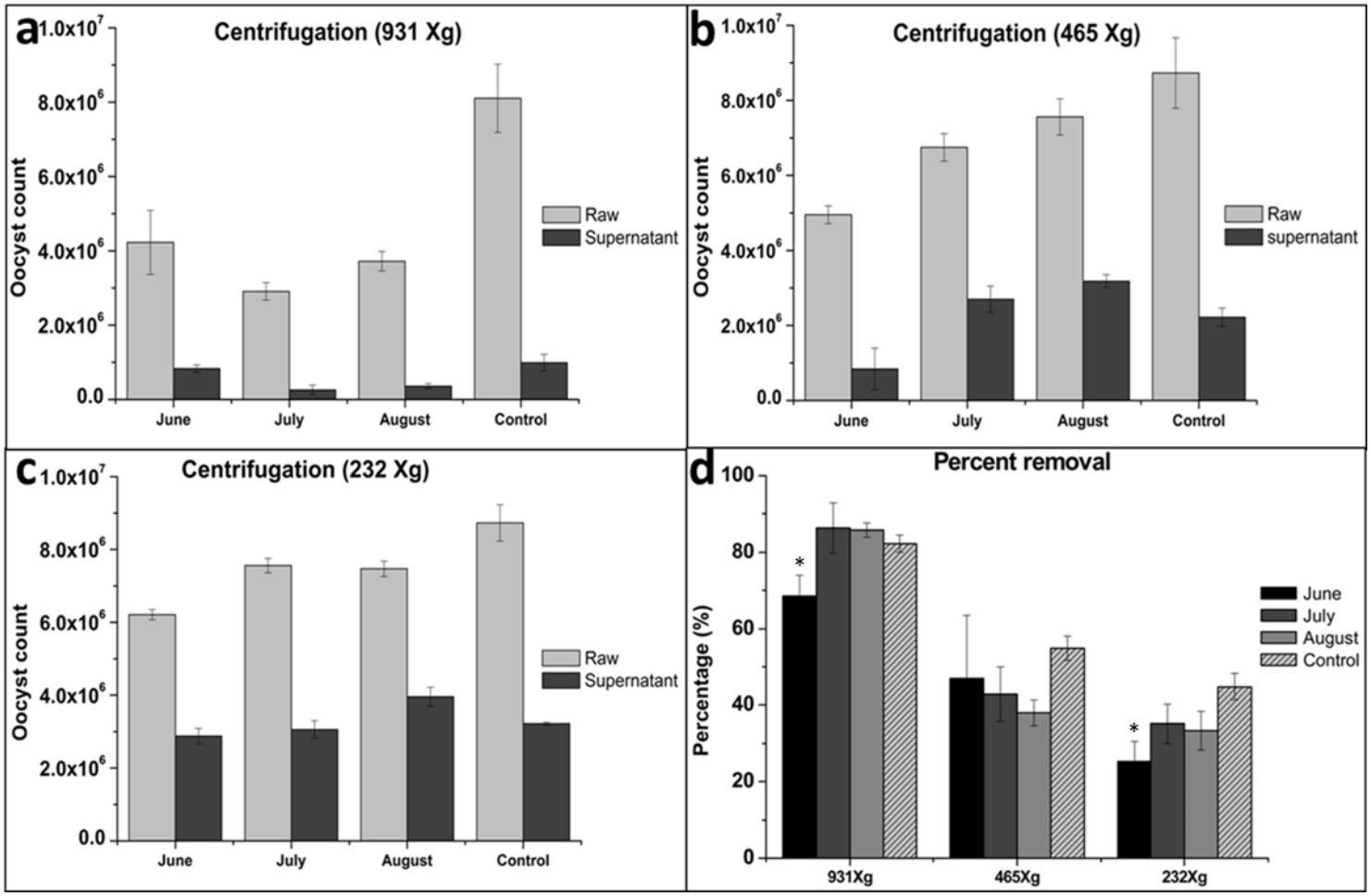

Figure 5.6 Partitioning of *C. parvum* oocysts by centrifugation

Figure 5.6 Partitioning of *C. parvum* oocysts by centrifugation in Adelaide secondary effluent. *C. parvum* oocysts were spiked (10^2/mL) in secondary effluent and 900 ml x 3 aliquots were centrifuged at 961 x g, 465 x g and 232 x g. The supernatant (600 mL) was carefully collected and the oocysts were counted in triplicate before and after centrifugation by fluorescence microscopy. The counts are plotted in $\log_{10}$ scale and the differences in counts after centrifugation was converted into percent removal. A no particle phosphate buffered saline control was also spiked and treated the same way. The percentage was converted into arcsin values and compared with Tukey's multiple comparison test by performing one-way ANOVA using Graphpad Prism 7.03 ($p<0.05$).

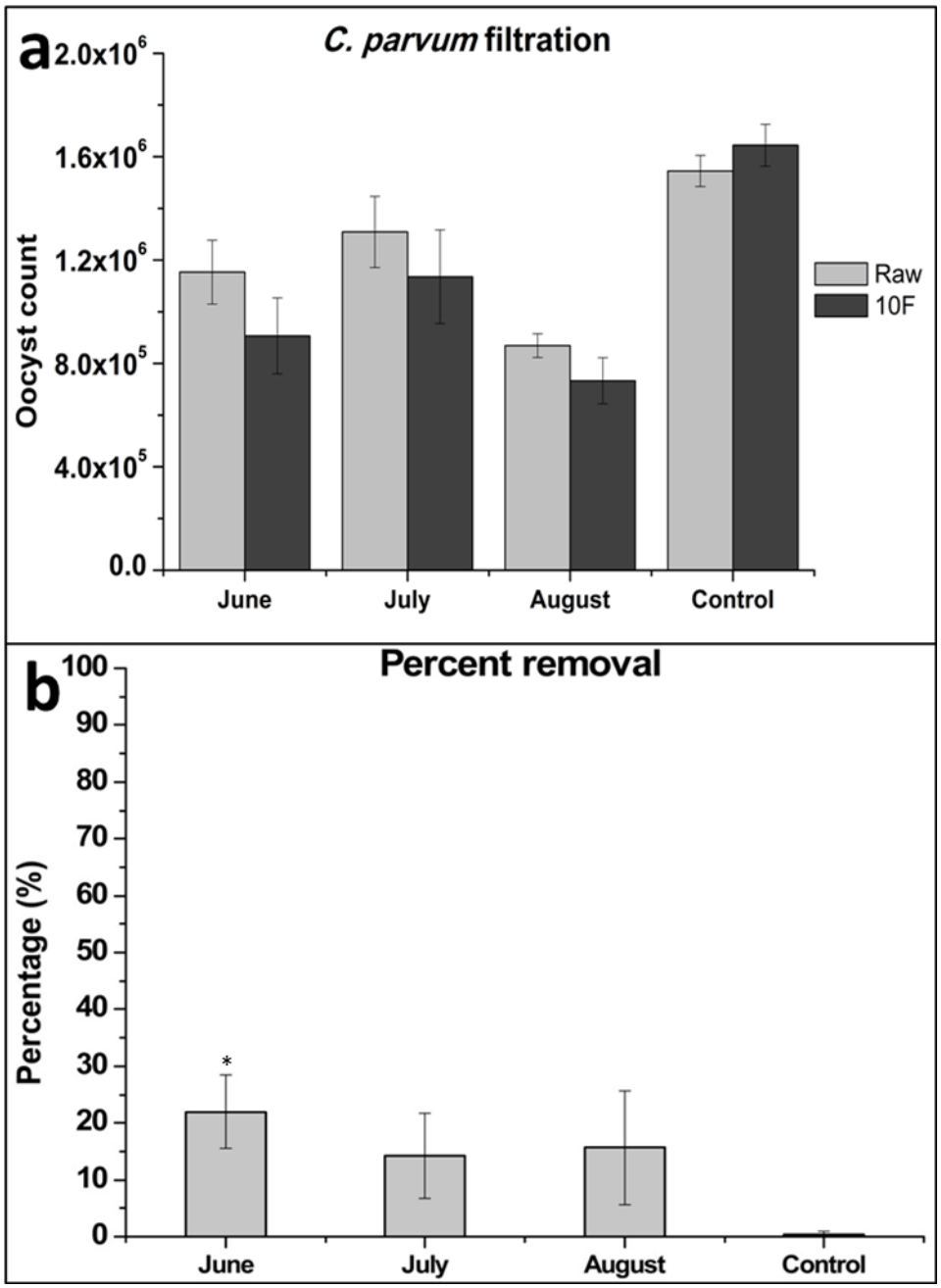

Figure 5.7 Partitioning of *C. parvum* oocysts by series filtration

Figure 5.7 Partitioning of *C. parvum* oocysts by series filtration in Adelaide secondary effluent. *C. parvum* oocysts were spiked (10^2/ml) in secondary effluent and 200 ml x 3 aliquots were filtered through 10 µm, 1.2 µm, 0.4 µm and 0.2 µm polycarbonate filter membranes. The oocysts were counted in triplicate by fluorescence microscopy before and after filtration. The counts are plotted in $\log_{10}$ scale and the differences in counts after filtration were converted into percent removal. A no particle phosphate buffered saline control was also spiked and treated the same way.
Here 10F – 10 µm filtrate. The oocysts were completely removed by 1.2 µm filtration and hence no counts are presented after 10 µm filtration.

5.4 Discussion

This study characterised the partitioning behaviour of anaerobic spore forming bacteria using three different techniques and compared it to the partitioning behaviour of *C. parvum* oocysts.

The decrease in the numbers of larger particles and increase in smaller particles after homogenisation suggest that larger particles were disaggregated to produce smaller particles. This is supported by the observation that there was a higher reduction in large particle numbers by homogenisation in the presence of buffers than in the respective buffer-free homogenisation controls. The significantly higher reduction by all the buffers and controls in the August Adelaide lagoon effluent was supported by the particle profile of the sample before homogenisation, which revealed significantly higher number of large particles in August than both the other months. Buffers decreased the total number of particles (the sum of all the size classes) in Melbourne lagoon effluent after homogenisation than the raw parent sample, but there was no increase in the numbers of smaller particles. It could be that the buffers reduced the particle to sizes below the detectable limit of the analyser i.e. < 0.3 microns. The differences in homogenisation patterns were supported by the differences in the particle profiles of the raw samples. Buffer A was found to be the most effective in breaking down the particles in effluents from all of the sample locations, as supported by the highest reduction of larger particles (>100 µm) in all the samples compared with the Buffer B and control treatments. The results are also supported by the previous studies, which reported the breakdown of larger particles in the presence of dispersion buffers (Parker and Darby, 1995, Emerick et al., 1999, Caron et al., 2007a, Li et al., 2009).

The significant removal of spores by centrifugation suggests their association (>50%) to denser, settleable particles, which has been reported previously (Characklis et al., 2005b, Krometis et al., 2007b, Krometis et al., 2010). The significant removal of spores by particles 10 µm or larger was further confirmation that spores are associated with larger and denser

particles. However, there were differences in the percent removal in certain months. In the Melbourne lagoon effluent, in August there was no significant removal of spores by centrifugation but there was significant removal by filtration. A variety of factors, such as the availability of particles, availability of more sites for attachment, surface properties of the spores and environmental factors such as pH and ionic strength of the water can play an important role in attachment (Characklis et al., 2005b, Krometis et al., 2010). In Melbourne lagoon effluent, the numbers of larger particles > 100µm were significantly lower in August than in the other months, therefore reduced availability of particles could be one reason for lower removal. However, the filtration removal was the highest for the same month. This suggests that the type of particles; their structure and site availability could play a part in attachment. In this case and also for Adelaide secondary effluent in June, pathogens might be attached to buoyant particles, which did not sediment during centrifugation but were readily removed by filtration.

Homogenisation increased the spore counts significantly in Adelaide secondary effluent and Adelaide lagoon effluent, suggesting the association of more than one spore to each particle. This also suggests that spores might not be very tightly bound or associated with particles and strong turbulence can disrupt any association. The increase in spore counts in Adelaide samples by buffer A coincided with the significant breakdown of larger particles in the same samples. The spore count did not increase significantly, but the particle breakdown was significant in Melbourne lagoon effluent. This suggests that the numbers of spores in Melbourne were lower than in the two Adelaide locations. This conclusion was supported by a comparable spore count in the context of lower percent removal by filtration that the spores settled at the same velocity as the particles.

C. parvum oocysts were not significantly removed by any of the centrifugation speeds and were only significantly removed for one of the three months for filtration. However, while not

significant, the trend for oocyst removal by centrifugation tended to be lower in the spiked secondary effluent compared with the particle-free buffer control (i.e., more were removed by centrifugation in the control), and the trend for removal by filtration on the 10 µm filter tended to be higher in the spiked samples compared with the control. This suggests that *C. parvum* may be associated with particles in Adelaide secondary effluent, but additional experiments are required to demonstrate particle attachment (such as by direct microscopic observation using a stain). Lower removal by centrifugation in the samples suggests that oocysts might be associated with less dense particles such as colonies of cyanobacteria. Association with denser particles would result in higher removal by centrifugation, and thus a decrease in removal suggests association with less dense particles. During the microscopic counting used in this study, oocysts were visualised on or within different particles, however, the method used is not effective to prove that they were associated because vacuum filtration was used to concentrate the entire sample onto a filter membrane instead of observing the oocysts / particles in a freely suspended sample. Attachment of oocysts to particles has been reported in previous spiking studies (Medema et al., 1998b), as has oocyst interaction with a variety of particles, such as clay, and water and wastewater biofilms (Dai and Boll, 2003a, Searcy et al., 2005, Helmi et al., 2008a). The association between spores and particles is influenced by factors such as surface charge and hydrophobicity (Dai and Boll, 2003a, Characklis et al., 2005b). Also oocyst attachment is reversible and factors such as pH and ionic strength of the water can control it (Drozd and Schwartzbrod, 1996).

Anaerobic bacterial spores have previously been considered as important surrogates for oocyst studies. The data presented herein suggest that, while not statistically significant, there is some evidence of oocyst-particle association, but the degree of association is much lower than that for spores. This is exemplified in the 10µm filtration results, where approx. 75% of spores in secondary effluent were removed by filtration, compared with 0% of spores in particle-free

buffer. In contrast, at most 20% of spiked oocysts were removed by filtration from secondary effluent, compared to 0% in the buffer control. Although spores and oocysts have a similar surface charge and hydrophobicity (Drozd and Schwartzbrod, 1996), oocysts (~4 μm) are bigger than spores (~1 μm) (Redunker et al., 1985, Novak et al., 2003) and therefore, it might be harder for larger oocysts to associate with particles as compared to smaller spores. Other differences between the spores and oocysts are that the spores are denser and have external appendages (Hijnen, 2010). Therefore, the external appendages might favour attachment and high density might favour settling. Additionally, the oocysts were spiked, and although they were mixed overnight with the effluent water, it might still not represent natural mixing. Another possibility is that for this secondary effluent the associations might not have been strong enough and oocysts might have dissociated more quickly than spores.

The results clearly indicate strong association of anaerobic spores with particles but poorer particle-association for *C. parvum* oocysts. The findings suggest that spores are not a very good surrogate for studying the particle association of oocysts. Although the association between particles and oocysts was not as clear cut, larger particles can still shield oocysts from disinfection. Also, greater association of native oocysts with particles may negatively affect their removal and disinfection. Therefore, it is important to monitor water quality at different stages of treatment to identify individual wastewater characteristics such as particle size, as described in Chapter 3, and then determine the size to which pathogens attach. This would help to implement removal strategies such as selective filtration (micro filtration) that could be applied before disinfection. Removal of particles greater than 10 μm could be greatly beneficial in improving *C. parvum* disinfection, however, might not be beneficial in removing particle associated *E. coli*, which are associated with much smaller particles as discussed in Chapter 4. Therefore these results are central in understanding the nature and size of particles with which

the pathogens are associating with implications for designing targeted removal mechanisms (such as filtration pore size) at appropriate treatment stages.

5.5 Conclusion:

We conclude that native anaerobic spores associated with larger settleable particles (>10 µm) in all three wastewater samples and more than one spore was associated with a single particle. We suggest that chemical combined with physical disaggregation is a better option for the enumeration of particle-associated pathogens in order to provide a more accurate assessment of risk. We also suggest that alternative methods should be used to confirm the particle association of *C. parvum* oocysts.

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Chapter 6: Effect of particle-pathogen interactions on disinfection kinetics of pathogens in treated wastewater effluents.

6.1 Introduction

Ultraviolet (UV) radiation and chlorination are widely used to disinfect WW, but the association of microbes with particles can reduce the efficacy of disinfection (Emerick et al., 1999, Templeton et al., 2005, Templeton et al., 2008). Different-sized particles provide various modes of protection to pathogens against disinfection. Particles >10 µm have been shown to offer greater protection to bacteria from disinfection (Qualls et al., 1985, Emerick et al., 1999, Madge and Jensen, 2006a), whilst smaller particles have been shown to protect associated viruses (Templeton et al., 2005). The larger size of protozoan parasites suggests a requirement for much larger (> 25 µm) particles before they can be protected from disinfection (Amoah et al., 2005). The particle sizes that *E. coli* and *C. parvum* oocysts were associated with were determined to be 1.2 to 10 µm and >10 µm respectively in Chapters 5 and 6. In the case of UV radiation, particles can attenuate or scatter UV light and also shield any pathogens embedded in them (Madge and Jensen, 2006a). In the case of chlorination, protection can be linked to the chlorine demand of the matrix, incomplete penetration of chlorine into particles and the presence of extracellular material surrounding the particle (Templeton et al., 2005, Dietrich et al., 2007).

In the absence of any interference, the inactivation of pathogens by disinfection has been modelled using first order kinetics. Deviation from the usual first order disinfection kinetics has been observed previously in WW effluents at relatively high disinfectant doses (Loge et al., 2002). This deviation is characterised by a plateau in the dose response curve and is defined as the tailing (Tan et al., 2017, Torres-Palma et al., 2017). Tailing has been linked to the association of microbes with particles (Loge et al., 2002). A consequence of tailing is that high doses of disinfectants are required to meet safety targets, increasing the cost of production of recycled water (either through increased energy or chemical costs). A common approach to reduce tailing is to remove particles by techniques such as filtration, which further increases

both the capital and operational costs of producing recycled water. Another potential solution is the disaggregation of particles in order to release associated pathogens and increase disinfection efficiency. Previous studies have investigated physical and chemical disruption techniques to improve disinfection (Winward et al., 2008, Torres-Palma et al., 2017). Therefore, it is important to understand the impact of particles on the disinfection processes and to investigate various particle removal mechanisms to devise cost-effective targeted treatment options and achieve optimal disinfection. Since little is known about the impact of particles on the disinfection kinetics of *Cryptosporidium parvum* oocysts, further characterisation is required. However, the number of enteric protozoa (including *Cryptosporidium* oocysts) in wastewater is low and direct observation is hampered by the detection limit of available methods (Li et al., 2009). Consequently, studies mostly rely on artificially seeding of oocysts into wastewater samples or the use of surrogates. This study was designed to analyse the impact of naturally occurring wastewater particles on the chlorine and UV disinfection kinetics of *E. coli*, FRNA bacteriophage and *C. parvum* oocysts in lagoon effluents from two different treatment plants in Australia. A further objective was to analyse the impact of filtration and disaggregation techniques on the disinfection kinetics of these organisms. Knowledge of the ways in which particles govern disinfection kinetics will help to identify appropriate treatment technologies to ensure adequate and cost-effective disinfection for water reuse.

6.2 Methods

6.2.1 Sample collection

Wastewater lagoon effluents were collected from Bolivar wastewater treatment plant (BWWTP) in Adelaide, South Australia and Western wastewater treatment plant (WWWTP) in Melbourne, Victoria. Lagoon effluents were collected from points downstream of Bolivar wastewater lagoons and 55E Melbourne wastewater lagoons in July 2017. Grab samples (30L)

were collected in 20% thiosulphate dosed 10 L jerry cans, transported at 4°C and refrigerated on arrival.

6.2.2 Wastewater characterisation

The concentrations of SS, TSS and VS, NH_3, NO_3^-, NO_2^-, total P were measured in triplicate as described in section 2.3.2 (Chapter 2) Turbidity and pH were recorded in triplicate using a HACH 2100 N turbidimeter and an Eutech pH700 pH meter respectively. The concentrations of total and dissolved organic carbon (TOC and DOC) were measured in triplicate using a Sievers Innox OX laboratory TOC analyser. The samples were diluted in ultra-pure water to meet standard ranges and to avoid any blockage or damage of the analyser tubing. The particle size distributions of each sample were analysed by laser *in situ* scattering and counting using a LISST portable|XR (Sequoia instruments) in triplicate as described in section 2.3.3 (Chapter 2).

6.2.3 Preparation of test and control samples

30L grab sample of lagoon effluent from each site were collected in July 2017. Triplicate 1L subsamples were analysed using different methods (Fig 6.1).

FRNA bacteriophage or *C. parvum* oocysts were not detected in raw untreated (parent) WW samples (lagoon effluents), therefore samples were spiked with the FRNA bacteriophage MS-2 (10^5/mL) and live *C. parvum* oocysts (10^4/mL). The MS-2 phage was ATCC strain 15597-B1 and *C. parvum* oocysts were the lowa strain purchased from BTF (Sydney) at a concentration of 10^8/mL in phosphate buffered saline (PBS). Previous study (Chapter 5) showed that FRNA bacteriophage were not associated with particles, hence only untreated spiked raw lagoon effluents were used for the MS-2 phage disinfection experiments. *C. parvum* oocysts were not subjected to chlorine disinfection because they are resistant to chlorine (Nasser, 2017, Adeyemo et al., 2019).

The working volumes of samples in the disinfection experiments were adjusted to optimise the microbial detection assays, each of which had different limits of detection or sensitivity parameters.

Particle free controls were set for each organism by suspending cultured *E. coli* (10^2/mL), MS-2 phage (10^5/mL) and *C. parvum* oocysts (10^4/mL) in PBS for UV disinfection and demand free water (DFW) for chlorine disinfection, both at a pH of 7.4.

The effects of particles on disinfection were examined using two separate techniques; filtration and homogenisation.

6.2.4 Filtration Experiment

Previous studies (Chapters 4 and 5) exploring the particle association behaviour of microorganisms informed the selection of a 10µm polycarbonate filter membrane for removing larger particles which could impact disinfection of *C. parvum* and 1.2 µm polycarbonate filter membrane for removing particle associated *E. coli* . Samples were filtered through selected pore sizes and the filtrates were disinfected to analyse disinfection kinetics.

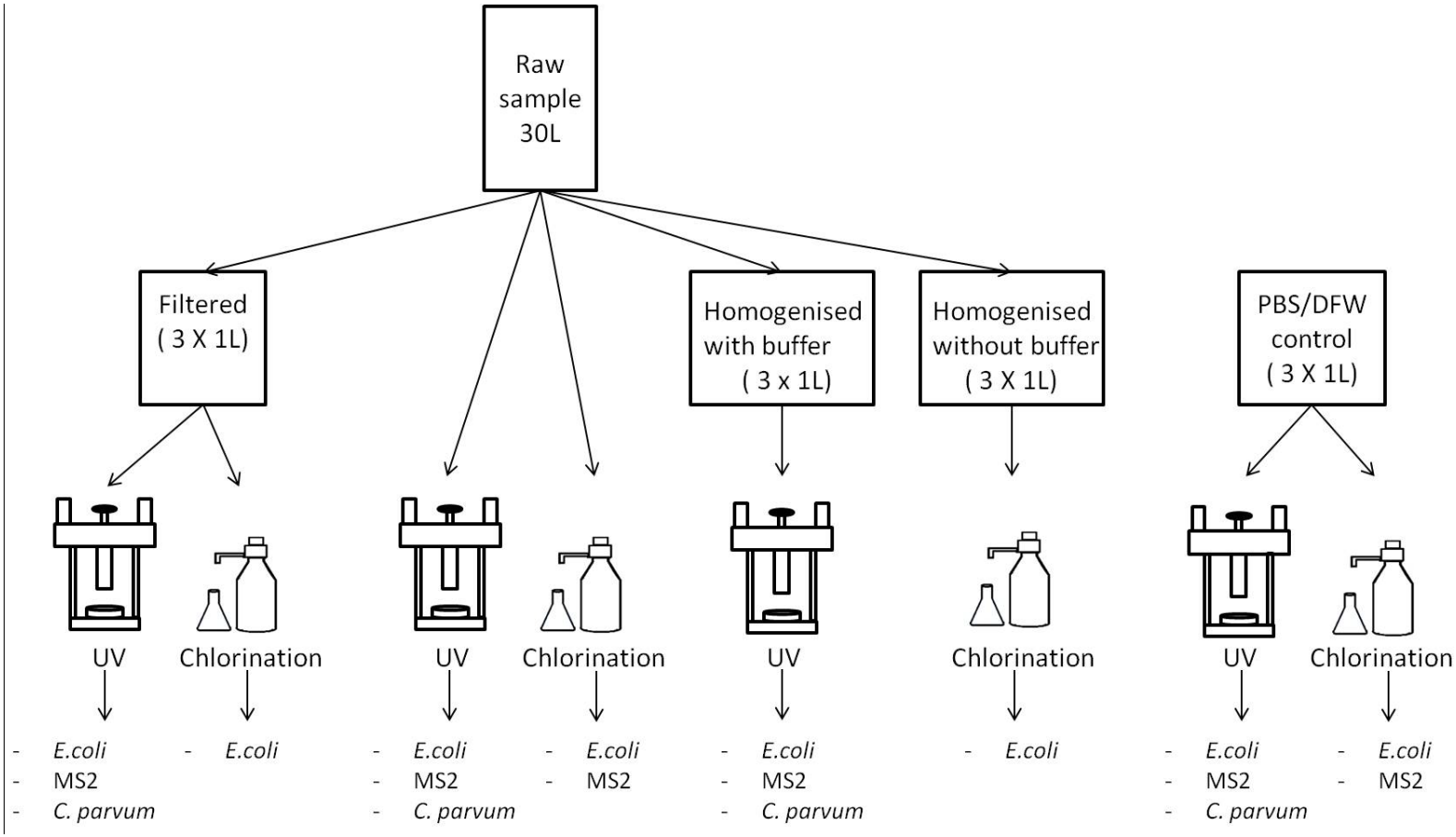

Figure 6.1 Schematic for disinfection experiments.

Figure 6.1 Schematic for disinfection experiments. One 30 litre sample of lagoon effluent was collected into thiosulphate-dosed jars at each location. A. Three 1L aliquots were filtered through 10 µm *(C. parvum* oocysts) or 1.2 µm filter membranes (*E. coli)* to assess the impact of particles on UV or chlorination disinfection of microorganisms. B. Three 1 L aliquots were homogenised in the presence or absence of dispersion buffer before UV or chlorination respectively. C. Controls were particle-free phosphate buffered saline (PBS) or demand free water (DFW) which were disinfected using UV and chlorination respectively at pH 7.4. Three indicator micro-organisms were detected and enumerated; bacteria *E. coli*, MS-2 phage for FRNA bacteriophage, and *C. parvum* to represent pathogenic protozoa.

6.2.5 Homogenisation Experiment

Effluent samples were homogenised (blended) to break and disaggregate particles at 13,500 rpm using a WiseTis Digital homogeniser (HG-15D) in the presence or absence of dispersion buffer (Buffer A as used earlier Chapter 5) (10%) which consisted of 10^{-6} M Zwittergent 3-12 detergent, 10^{-3} M EGTA, 0.1 M Tris buffer.

Samples destined for UV disinfection were homogenised with dispersion buffer, whereas the dispersion buffer was not used before chlorination because it increased chlorine demand.

6.2.6 Ultra Violet (UV) disinfection

A bench scale collimated beam apparatus (Trojan Technologies, Ontario, Canada) equipped with a low pressure mercury UV lamp was used to irradiate the samples. The measurement of UV irradiance across a sample's surface area, along with the other parameters such as sample UV absorbance and sample depth, were used to calculate the exposure time (using a calculation sheet based on these parameters to calculate times) required to deliver predetermined UV doses (Bolton and Linden, 2003). This assisted in delivering different test doses to the samples. The times required for delivering set UV doses ranged between 20 seconds to 90 minutes for each sample based on volume, surface area UV absorbance and turbidity. Different doses were selected for different organisms as per their sensitivity which was determined in test experiments before the analysis. Different sample volumes without any dilution were used for each microbial disinfection experiments due to differences in the initial microbial concentration.

The UV light intensity was recorded with a ILT1400 radiometer (International Light Technologies, MA, USA) and a Genesys 6 UV-visible spectrophotometer (Thermo Fischer scientific TM) was used to measure the sample UV absorbance at 254 nm.

E. coli: A sample volume of 160 mL was irradiated in polypropylene disposable cups (Sarstedt) with dimensions 150 X 67 mm and 500 mL volume. Each time *E. coli* was UV irradiated the sample was agitated with a magnetic stirrer throughout the disinfection. The raw untreated sample, 1.2 µm filtered sample, homogenised sample (with and without dispersion buffer) and PBS control were irradiated in triplicate using UV doses ranging from 0-20 mJ/cm^2.

FRNA bacteriophage: A sample volume of 4 mL was irradiated in 35mm X 10mm sterile polystryrene petri dishes (Sarstedt). Each time MS-2 phage was UV irradiated the sample was agitated with a magnetic stirrer throughout the disinfection experiments. The raw untreated (spiked) and particle free PBS controls were UV irradiated at UV doses of 10, 20, 20, 40, 50 and 60 mJ/cm^2.

C. parvum oocysts: A sample volume of 4 mL was irradiated in 35mm X 10mm sterile polystryrol petri dishes (Sarstedt) by continuously stirring the samples with micro stir bars (10 mm X 3 mm) for adequate mixing without interfering with irradiance. Each time *C. parvum* samples were UV irradiated the samples were agitated with a magnetic stirrer throughout the disinfection. The raw untreated (spiked), 10 µm filtered and PBS control samples were UV irradiated using 0, 1, 3 and 5 mJ/cm^2 doses in triplicate.

6.2.7 Chlorination

Chlorine gas was generated by mixing trichloroisocyanuric acid (TCCA) and concentrated hydrochloric acid (HCl). A chlorine stock was prepared by bubbling chlorine gas into ultra-pure water until it reached saturation. The stock concentration was measured every day by dosing 100 mL of Ultrapure (Milli-Q) water with 100 µL of the stock solution. The resulting chlorine concentration was measured using the N,N Diethyl-P-Phenylenediamine-ferrous ammonium sulphate (DPD-FAS) colourimetric method (APHA-AWWA-WEF, 1998). All the glassware used for chlorination experiments was washed with 5% nitric acid overnight, rinsed

three times in distilled water and three times in ultra-pure water and autoclaved. All the samples and the control were maintained at a temperature of 25 ±0.5 °C.

E. coli: 1 L Adelaide and Melbourne lagoon samples were dosed in triplicate with the volume of stock chlorine solution required to achieve an initial dosing concentration of 3 and 2 mg/L chlorine respectively. The choice of dose was based on the preliminary experiments and initial sample turbidity. Aliquots of 90 mL were withdrawn at time intervals of 0, 1, 3, 5 and 10 minutes and quenched in 10% (10 mL) sodium thiosulphate solution at a stock concentration of 20% weight/volume. Aliquots of 20 mL were withdrawn each time to determine free available chlorine and other residual chlorine species (chloramines). Monochloramine was measured by titrating 3 drops of 20% potassium iodide solution (DPD –FAS method). Subsequent addition of potassium iodide causes the monochloramine containing solution to change colour due to a catalytic reaction. Total residual chlorine was measured by adding a pinch of powdered potassium iodide and titrating using the same method. A particle-free control was dosed with a chlorine concentration of 1mg/L. Aliquots of the particle-free control were withdrawn at time intervals of 0, 0.5, 1, 1.5 and 2 minutes. Particle free control did not pose any chlorine demand because of absence of any particles, therefore lower time intervals were sufficient to achieve determined disinfection.

FRNA bacteriophage: 500 mL of Bolivar and Melbourne lagoon effluents were chlorinated in triplicate at a dosing concentration of 10mg/L and 5 mg/L respectively. Aliquots of each sample (4.5 mL and 20 mL) were collected at time intervals of 0,1,3,5,10,15 and 20 minutes for FRNA bacteriophage analysis and residual chlorine titrations respectively. The 4.5 mL aliqouts for phage analysis were quenched each time in 10 % (500 µL) sodium thiosulphate solution at a stock concentration of 20% weight/volume. Particle-free control samples were spiked with a chlorine dose of 1mg/L. Aliquots were withdrawn at 0, 1, 2, 3, 5 and 10 minutes for analysis and residual chlorine (free, monochloramine and total residual) concentrations.

6.2.8 Microbial analysis

The concentration of *E. coli* and FRNA bacteriophage in the samples was determined by microbial anylis techniques described in section 2.3.5 (chapter 2). The infectivity of oocysts before and after disinfection was determined using a previously described cell culture infectivity assay (King et al., 2011). Briefly, a modified excystation technique was used which involved incubating the oocysts for 20 minutes at 30°C followed by centrifugation at 1800 x g for 10 minutes. A re-suspension in 1 mL supplemented RPMI 1640 medium at room temperature was used for cell culture infections. Pre excystation-treated oocysts were then applied to HCT-8 (ATCC CCL-244; human ileocecal colorectal adenocarcinoma) cell monolayers in 48 well plates for up to 48 hours. The incubated wells were then fixed with methanol, stained with Sporo-Glo (polyclonal antibody), washed with PBS and counted under blue light at 40X magnification using a Nikon Eclipse 80i fluorescence microscope.

6.2.9 Inactivation kinetics

The log inactivation of microbes after UV disinfection was calculated as log N/N_o, where N_o was the mean concentration of microbes without any exposure and N was the mean concentration of microbes after each exposure. For chlorine inactivation, the important parameters were concentration of the free available chlorine, log inactivation and the time over which the organisms were exposed to chlorine. Firstly, $\log_{10}$ inactivation was plotted against the exposure time to estimate the exposure time required for a particular log inactivation value. The free available chlorine was then plotted against exposure time and the resulting chlorine decay curve was used for determining the chlorine contact time (CT) (chorine concentration x time) required to achieve the selected log inactivation value. Monochloramine and total residual chlorine were measured but they were not used for CT calculation. The calculation of a CT value for a particular $\log_{10}$ inactivation of *E. coli* and FRNA bacteriophage was determined by estimating the area under the curve of chlorine concentration vs. time (Ho et al., 2006). The curves obtained in the study were not linear and the decay rates were not constant.

The complexity of these tailed curves was avoided by considering the CT values before the tailing region only and using linear regression. The empirical approach used here considered the integral between time 0 and the time taken for inactivation of a respective log_{10} value, directly from the chlorine decay graphs and no rate constant was considered.

6.2.10 Statistical analysis

The average number of particles and concentration of each nutrient was log_{10} transformed and the means were compared by Tukey's multiple comparison test by performing two way analysis of variance (ANOVA) using Graphpad prism 7.03. Two way ANOVA was performed using Graphpad Prism 7.03 and the means of log inactivation at each dose were compared using Tukey's multiple comparison test by setting the interactions between treatment and dose for each sample.

6.3 Results

6.3.1 Raw water characteristics: Wastewater characteristics were measured and represented as an average (± standard error of mean) for both the samples (Table 6.1). Adelaide lagoon effluent had higher turbidity and solids (SS, TSS and VS) compared to Melbourne lagoon effluent. Adelaide lagoon effluent also had a higher pH compared to Melbourne lagoon effluent. Both the lagoon effluents had minimal to no detectable NH_3 and different concentrations of NO_3^-. The particle profiles of both the lagoon effluents were significantly different ($p<0.05$) from each other for all the size classes of particles (Fig 6.2). Both the lagoon effluents had similar particle proportions in that there were higher number of smaller particles and the count decreased as the size increased. Melbourne lagoon effluent had proportionally higher 10-100 µm particles as compared to Adelaide lagoon effluent.

Table 6.1 Water quality chemistry

Table 6.1 Water quality chemistry of Adelaide and Melbourne lagoon effluents. The solids were measured in triplicate using standard methods for water and wastewater analysis. The concentration of various nutrients (NH_3, NO_3^-, NO_2^- and Total P) were measured colorimetrically using the appropriate HACH nutrient analysis kits. Turbidity and pH were measured using a HACH 2100 N turbidimeter and a Eutech pH700 pH meter respectively. All the values are reported as averages ± standard error.

	Adelaide lagoon	**Melbourne lagoon**
Turbidity (NTU)	63.9±1.4	11.9±0.2
TOC (mg/L)	15.9±0.1	10.6±0.1
DOC (mg/L)	11.8±0.2	8.9±0.9
SS (mg/L)	12.2±2.9	8.2±0.3
TSS (mg/L)	1356.2±12.5	1039.9±21.7
VS (mg/L)	212.9±3.2	157.7±0.5
pH	8.9±0.2	7.7±0.1
NH_3(mg/L)	0.0045±0.0005	0
NO_3^- (mg/L)	13.4±0.5	16.7±0.7
NO_2^- (mg/L)	0.012±0.001	0.026±0.001
Total P (mg/L)	6.1±0.02	26.1±0.8

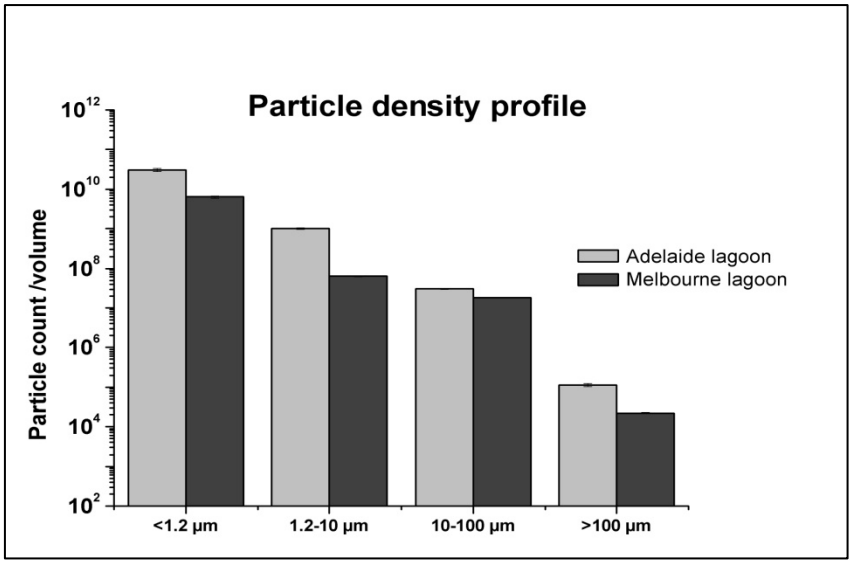

Figure 6.2 Particle density profiles

Figure 6.2 Particle density profiles of Adelaide and Melbourne lagoon effluents obtained by laser in-situ scattering (LISST). Raw samples were analysed in triplicate and the average particle volume / sample volume for each particle size class was converted into particle counts per volume (using the volume of a sphere for each size class). The particle counts were grouped into 1.2-100 µm, 10-100 µm and > 100 µm based on their size.

6.3.2 UV disinfection:

E. coli: The concentration of culturable *E. coli* was measured after exposure to UV doses delivered after 0, 5, 10, 15 and 20 mJ/cm^2 for both the Adelaide and Melbourne lagoon samples. The average log inactivation (N/N_o) was plotted as a function of UV dose for the raw, homogenised, 1.2 µm filtered samples and particle-free PBS controls (data not shown). In some experiments, complete inactivation was observed for particular UV doses. These values were excluded from the calculation of log inactivation. Tailing was evident in the raw Adelaide lagoon effluent for doses higher than 10 mJ/cm^2, with a maximum $\log_{10}$ inactivation of approximately 1.7 (Table 6.2). Melbourne raw lagoon effluent showed a higher inactivation (up to 2.5 log), with tailing apparent after 5 mJ/cm^2. At doses above 5 mJ/cm^2, both the filtered and homogenised lagoon (Adelaide and Melbourne) samples and the control resulted in inactivation below the detection limit of the culture technique, resulting in > 2 $\log_{10}$ inactivation. Inactivation in homogenised Melbourne lagoon effluent after exposure to 5

mJ/cm^2 was 1.7 log$_{10}$ inactivation, significantly higher than in the equivalent particle-free PBS control ($p<0.05$).

It appeared that there was less inactivation in the filtered and homogenised samples than in raw parent samples that contained more particles.. In the case of the Adelaide and Melbourne raw samples, the starting numbers of *E. coli* were similar, but the log inactivation at 5 mJ/cm^2 in the Melbourne sample was double that in the Adelaide sample (Table 6.2).

Table 6.2 Ultraviolet disinfection of *E. coli*

Table 6.2 Ultraviolet disinfection of *E. coli* in wastewater effluents and particle-free PBS (phosphate buffered saline) control. The table displays the $\log_{10}$ inactivation of the samples at various UV doses. At certain doses, complete inactivation was observed in some of the samples (expressed as greater than (>) values).

	Adelaide lagoon				**Melbourne lagoon**				**PBS control**			
Dose (mJ/cm^2)	5	10	15	20	5	10	15	20	5	10	15	20
Raw	1.1± 0.05	1.7± 0.12	1.8± 0.08	1.7± 0.06	2.2±0 .07	>2	>2	>2.5	1.1± 0.03	>4	>4	>4
1.2 µm filtered	0.8± 0.09	>2	>2	>2	1.2±0 .05	>2.5	>2.5	>2.5	NA			
Homogenised	0.8± 0.07	1.2± 0.09	>1.0	>3	1.7±0 .07	>1.9	>3	>3	NA			

NA- not applicable- PBS control was not treated (filtered or homogenised)

C. parvum: Oocysts of *C. parvum* were spiked into raw lagoon water samples, mixed to allow for interaction with particles, and then exposed to UV doses of 1, 3 and 5 mJ/cm^2 (Fig 6.3). The samples from both locations (raw and 10 μm filtered) and the PBS controls had similar inactivation up to the dose of 3 mJ/cm^2. At the UV dose of 5 mJ/cm^2, the raw Adelaide and Melbourne lagoon samples showed an average $\log_{10}$ inactivation of approximately 3.5, whereas the filtered samples and the PBS controls did not yield any infectious oocysts, showing > 3 $\log_{10}$ inactivation (Fig 6. 3).In Adelaide lagoon effluent, raw and filtered samples displayed similar inactivation curves to the control. However, for Melbourne lagoon the inactivation curve for the PBS control was different from the raw and filtered samples. The log inactivation at each dose for every sample was significantly different ($p>0.05$) from log inactivation at other doses i.e. log inactivation at 1 mJ/ cm^2 was significantly different from log inactivation at 3 mJ/cm^2.

FRNA bacteriophage: The number of FRNA bacteriophage in the raw samples were too low to conduct inactivation experiments, so cultured FRNA (MS-2 phage) were spiked (10^5 /mL final concentration) into the samples and inactivation was measured for UV doses ranging from 10-60 mJ/cm^2. Both the samples and the particle free control behaved similarly at the applied doses (Fig 6.3). The phage displayed a linear inactivation with increasing dose and a maximum $\log_{10}$ inactivation of approximately 2.5 ± 0.5 was observed in all the samples including the control at the highest dose of 60 mJ/cm^2. The bacteriophage were less inactivated in Melbourne lagoon effluent than in the PBS control or Adelaide lagoon effluent, specifically for doses >30 mJ/cm^2.

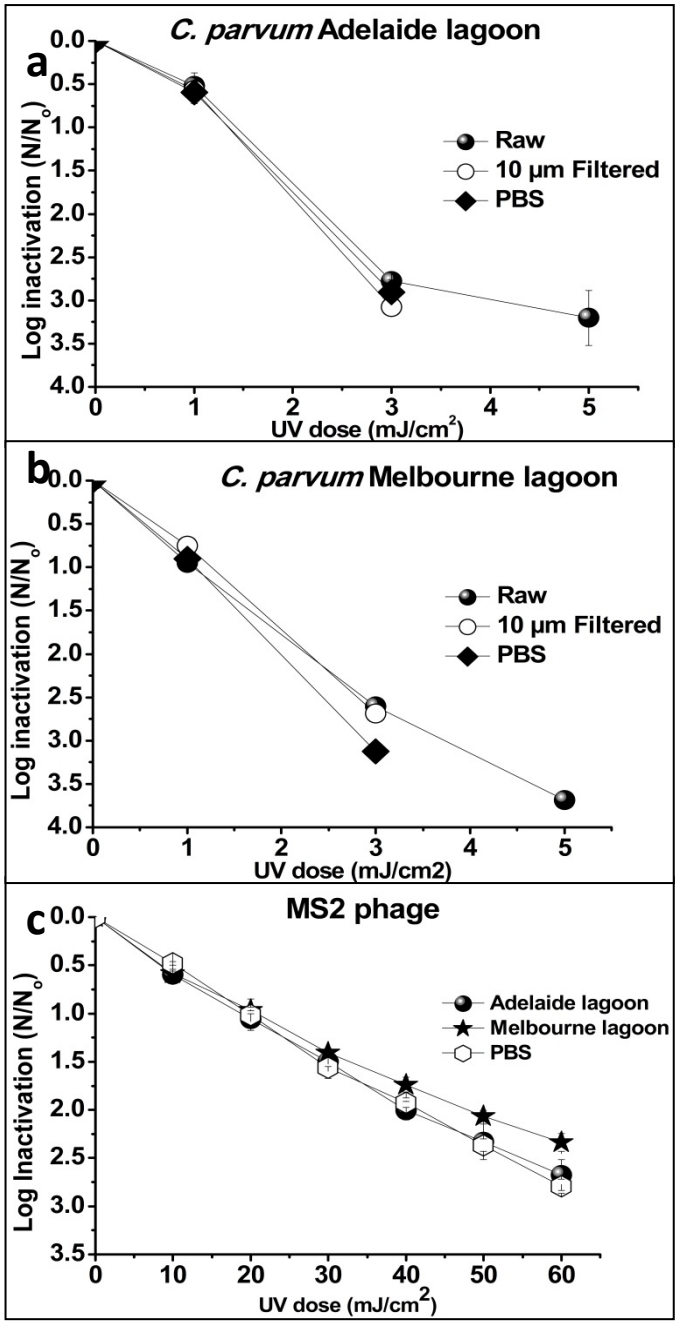

Figure 6.3 U.V Disinfection of *C. parvum* oocysts and MS-2 phage

Figure 6.3 U.V Disinfection of *C. parvum* oocysts and MS-2 phage after ultraviolet disinfection of wastewater effluents. Oocysts in raw (parent) lagoon effluents from Adelaide and Melbourne, effluent samples treated by filtration (through 10 µm) and particle-free PBS (phosphate buffered saline) controls were disinfected at predetermined UV doses. MS-2 phage in raw parent lagoon effluents from Adelaide and Melbourne (not filtered) was disinfected at predetermined UV doses. The $\log_{10}$ inactivation was calculated as N/N_o, where N was the mean concentration of microbes after each exposure and N_o was the mean concentration of microbes without any exposure. Two-way analysis of variance was performed to analyse the differences between the log inactivation at different doses using Tukey's multiple comparison test.

6.3.3 Chlorination:

E. coli: There was a 2 $\log_{10}$ inactivation of *E. coli* in Melbourne lagoon effluent after 0.5 minutes at a dose of 2 mg/L (Fig 6.4) which was faster than than the Adelaide lagoon effluent in which there was only a 1-$\log_{10}$ inactivation in the same time but at a higher dose of 3 mg/L. The Adelaide sample was dosed with a higher concentration of chlorine to account for the increased chlorine demand that would be caused by the higher turbidity and TOC/DOC in this sample. Both the raw lagoon effluents reached a maximum of 3.5 $\log_{10}$ inactivation after 3 minutes and further exposure did not increase the inactivation (Fig 6.4). Homogenisation resulted in a maximum $\log_{10}$ inactivation of >4 $\log_{10}$ in both the samples and there were no *E. coli* detected after 3 minutes of chlorine exposure. Filtration caused complete inactivation in Adelaide lagoon effluent within 1 minute as compared to over 2 minutes for complete inactivation in the Melbourne lagoon effluent. There was a much higher, faster inactivation in the particle free control (demand-free water) than in the wastewater samples, with 5 $\log_{10}$ inactivation after a 1 minute exposure to 1 mg/L chlorine. The calculated CT values for *E. coli* in all the samples and treatments are shown in Table 6.3. The CT values for 1-$\log_{10}$ inactivation were similar between the locations for raw and filtered water, as were the CT values for 2-log inactivation for the raw waters and Adelaide homogenised water. The CT values for 1-log inactivation for the homogenised samples for both locations were generally lower compared to the othe treatments. Melbourne samples had lower CT values for raw water and homogenized samples but similar CT values for filtered water ascompaed with the Adelaide samples . The CT of Melbourne filtered and homogenized samples did not increase proportionally between 1 and 2 logs, whereas it did for the rest of the samples and sample types analyzed

MS-2 phage:

In the Melbourne lagoon effluent, an initial chlorine dose of 5 mg/L resulted in 3.5 log_{10} inactivation after 3 minutes and no phage were detected with further exposure (Fig 6.4). In contrast, it was not possible to achieve effective inactivation of MS-2 phage in the Adelaide lagoon effluent even at a higher dosing concentration of 10 mg/L, with a maximum of 0.9 log_{10} inactivation observed throughout the time of the experiment (Fig 6.4). Even though higher chlorine doses were used in these samples (similar to the *E. coli* experiments), the chlorine decay was still relatively rapid and and free chlorine was near detection limit after 3 minutes of contact time (Fig 7.5). FRNA bacteriophage in the particle-free control were rapidly inactivated (Table6. 4), similar to phage in the Melbourne lagoon effluent (4.2-log_{10} after 3 minutes). The CT for 1-log10 inactivation of FRNA bacteriophage in Adelaide lagoon effluent was much higher than in Melbourne lagoon effluent and demand free water (Table 6.3).

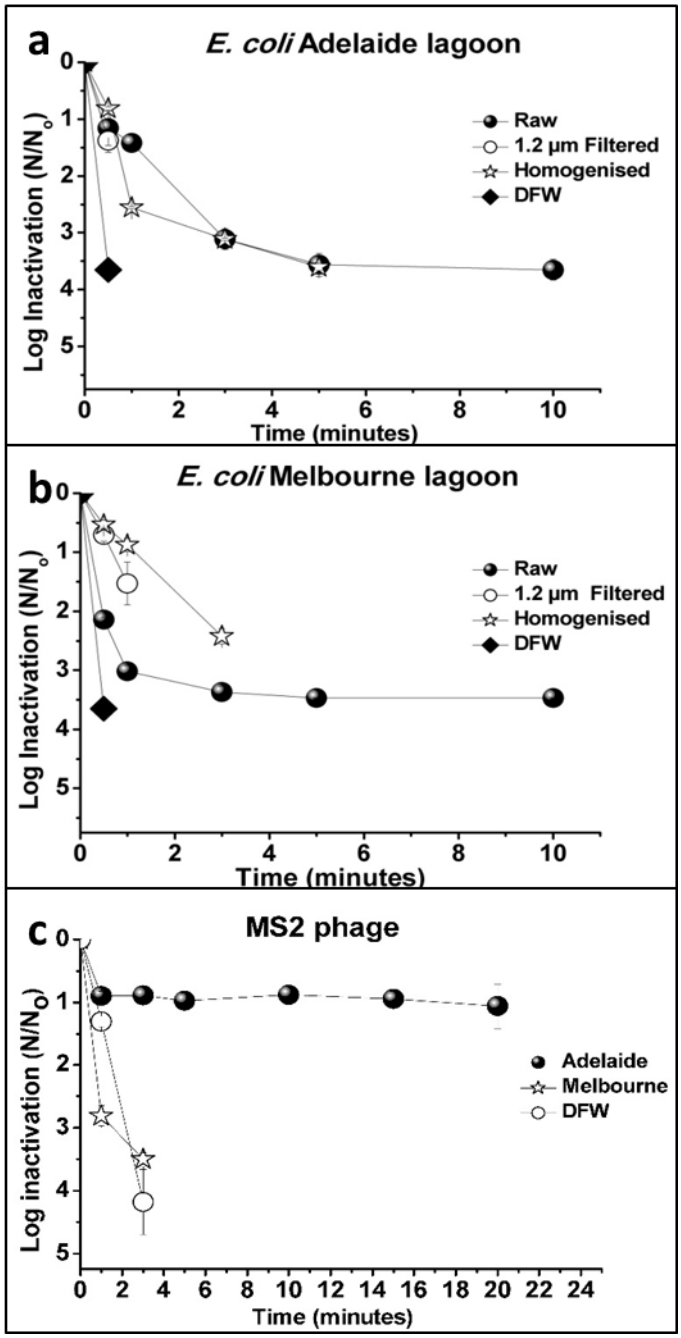

Figure 6. 4 Chlorine Inactivation kinetics of E. coli and MS-2 phage

Figure 6. 4 Chlorine Inactivation kinetics of *E. coli* and MS-2 phage *E. coli* in raw (parent) lagoon effluents from Adelaide and Melbourne and demand-free water was disinfected at 24° C with chlorine at predetermined doses of: Adelaide 3 mg/L, Melbourne 2 mg/L and DFW 1mg/L. MS-2 phage were spiked into raw (parent) lagoon effluents from Adelaide and Melbourne or particle-free demand-free water controls (DFW) before disinfection at 24°C with chlorine at predetermined doses: Adelaide 10 mg/L, Melbourne 5 mg/L, DFW 1 mg/L. The $\log_{10}$ inactivation was calculated as N/N_o, where N was the mean concentration of phages after each exposure and N_o was the mean concentration without any exposure. Two-way analysis of variance was performed to analyse the differences between the log inactivation at different doses using Tukey's multiple comparison test.

Table 6.3 CT values for chlorine inactivation of *E. coli*

Table 6.3 CT values for chlorine inactivation of *E. coli* using 3 mg/L chorine in Adelaide lagoon effluent, 2 mg/L in Melbourne lagoon effluents and 1 mg/L in demand free water at 25 ± 0.5°C. Chlorine doses were selected to account for different initial turbidity values. CTs were calculated using the area under the curve from a plot of residual chlorine concentration against time.

		ADELAIDE LAGOON		MELBOURNE LAGOON		DEMAND FREE WATER	
Log_{10} reduction		1-log_{10}	2-log_{10}	1-log_{10}	2-log_{10}	1-log_{10}	2-log_{10}
Raw	pH	7.5	7.5	7	7	7.4	7.4
	Ct	0.681±0.37	1.080±0.45	0.467±0.06	0.935±0.11	0.094±0.06	0.187±0.12
Filtered	pH	8	8	7.5	7.5	NA	
	Ct	0.681±0.04	NA	0.614±0.07	0.639±0.10		
Homogenised	pH	8.3	8.3	8	8	NA	
	Ct	0.456±0.01	0.912±0.02	0.457±0.02	0.508±0.01		

NA- Not applicable- Demand free water was not treated (filtered or homogenised)

Table 6.4 CT values for chlorine inactivation of MS-2 phage

Table 6.4 CT values for chlorine inactivation of MS-2 phage using 10 mg/L chlorine in Adelaide lagoon effluent, 5 mg/L in Melbourne lagoon effluent and 1 mg/L in demand free water at 24°C. CTs were calculated using the area under the curve from a plot of residual chlorine concentration against time.

LOG_{10} REDUCTION	0.5-LOG_{10}	1-LOG_{10}	2-LOG_{10}	3-LOG_{10}
Adelaide lagoon pH 7.5	3.757±0.08	7.514±0.017	NA	NA
Melbourne lagoon pH 7	0.559±0.09	1.202±0.19	2.488±0.38	3.773±0.58
Demand free water pH 7.4	0.179±0.09	0.359±0.14	0.719±0.24	1.079±0.39

NA- Not applicable (too high for comparison)

Free available chlorine was plotted as a function of exposure time (Fig 6.5) to allow calculation of contact times. Chlorine in the raw and treated lagoon samples had an initial rapid linear decay then tailed near the detection limit after 1 minute of exposure time. The homogenised Adelaide lagoon effluent had slightly more free available chlorine than the filtered and raw effluents. Demand-free water had free available chlorine just below 1mg/L throughout the experiment, showing that the *E. coli* culture spiked into the DFW contributed very little chlorine demand. Chloramine formation occurred in all the wastewater samples, but not in the chlorinated demand-free water (Fig 6.5). The formation of chloramine coincided with the reduction in free chlorine, and accounted for 25-50% of the loss of chlorine residual. Irrespective of location, the filtered and raw samples formed similar levels of chloramine, whereas more chloramine was formed after homogenisation. Treatment appeared to alter the sample pH, especially homogenisation, in which there was an increase of approximately 1 pH unit compared with the pH of the raw sample.

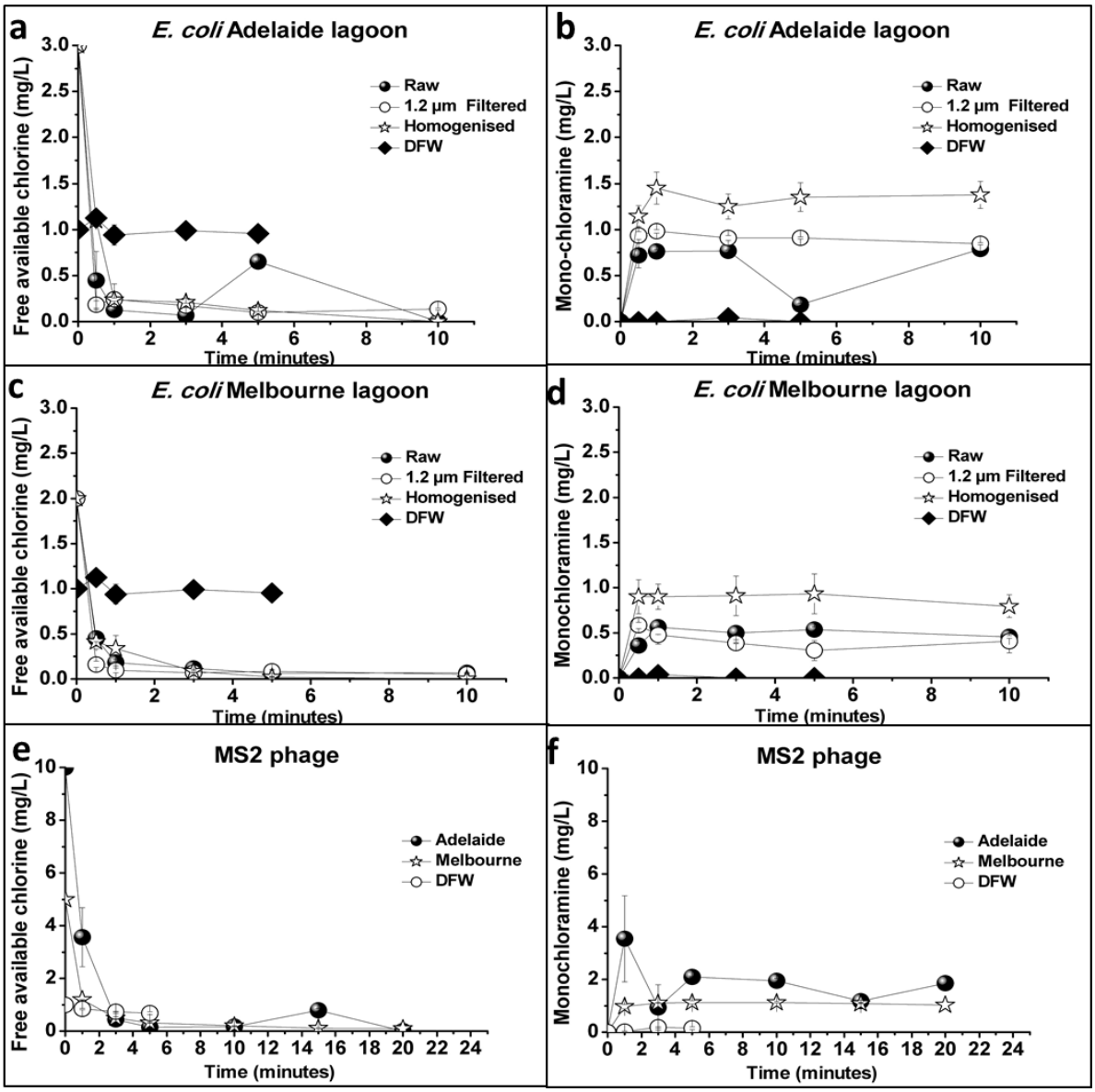

Figure 6.5 Free available chlorine and monochloramine

Figure 6.5 Free available chlorine and monochloramine in lagoon effluents. *E. coli* in raw (parent) lagoon effluents from Adelaide and Melbourne, effluent samples treated by filtration (through 1.2 µm membrane) or homogenisation (raw samples blended at 13,500 rpm for 1.5 minutes) and particle-free PBS (phosphate buffered saline) controls were chlorinated at 24°C as follows: Adelaide 3 mg/L for and Melbourne 2 mg/L for *E. coli*. MS-2 phage in raw (parent) Adelaide and Melbourne lagoon effluent and particle free PBS control were chlorinated as follows: Adelaide 10 mg/L and Melbourne 5mg/L. Free available chlorine was measured using the N,N Diethyl-P-Phenylenediamine-ferrous ammonium sulphate (DPD FAS) colorimetric method and plotted against exposure time. Monochloramine concentration was measured at each time point by adding 5g/L potassium iodide solution and titrating using the N,N Diethyl-P-Phenylenediamine-ferrous ammonium sulphate (DPD FAS) colorimetric method.

6.4 Discussion:

The Victorian guidelines for recycled water use a variety of sources to set disinfection targets for different pathogen groups (USEPA, 1992, Keegan et al., 2012a). However, these studies only consider recycled water with lower turbidity's (5-10 NTU) and may not consider turbidity's (>20 NTU), similar to the ones observed in Adelaide lagoon effluent in this study. The disinfection requirements can change considerably depending on the turbidity and particle characteristics of the water; therefore, our study adds valuable information to the assessment of disinfection of wastewaters. The findings of this study can additionally be applied to similar wastewater treatment plants which lack filtration before disinfection.

E. coli was highly sensitive to UV radiation in the PBS control and doses higher than 5mJ/cm^2 caused complete inactivation. The US EPA Ultraviolet Disinfection Manual (Table 5.2) states that UV doses greater than 5 mJ/cm^2 can result in >2 $\log_{10}$ reduction of *E. coli* (USEPA, 2006) but this was not the case in raw Adelaide lagoon effluent in which only 1.1 $\log^{10}$ inactivation occurred at this dose. Similarly, inactivation of *E. coli* in raw Adelaide and Melbourne lagoon effluents was lower than in control and filtered effluents which lacked particles, suggesting that the free-swimming *E. coli* were readily inactivated with a UV dose of 5 mJ/cm^2. Tailing was observed in both raw lagoon samples when particles were present, suggesting that either particle shielding, or particle association were responsible for this tailing. However, future experiments are needed to gather more data and validate the study. The differences in tailing and efficiency of inactivation between the Melbourne (2.2 $\log_{10}$ inactivation) and Adelaide (1.1 $\log^{10}$ inactivation) raw effluents at the same 5 mJ/cm^2 dose can be attributed to differences in the particle loading of the two waters. The Melbourne lagoon effluent, which had less tailing and better inactivation, had lower turbidity and suspended solids, and lower numbers of particles in all the size classes, than the Adelaide lagoon effluent. Higher particle counts and solids (TSS and SS) have previously been reported to decrease UV inactivation due to higher

scattering of UV light and enhanced shielding effects (Madge and Jensen, 2006a). Participle association can also play a role in decreasing UV effectiveness and particle-associated bacteria are less sensitive to UV disinfection than the free-swimming bacteria (Emerick et al., 2000, Brahmi et al., 2010, Kollu and Örmeci, 2012). An estimation of count of free-swimming *E. coli* in the samples (approximately 1.5 $\log_{10}$) further suggests effective disinfection of the free-living bacteria as the tailing roughly begins around 1.5 $\log_{10}$ reduction. Our previous fractionation study (Chapter 5) of the Adelaide and Melbourne lagoon waters found that *E. coli* were associated with particles in the 1.2 -10 µm size range. The inactivation results for filtered samples in our study agree with previous studies, which have reported increased activation of bacteria in filtered wastewater samples compared to unfiltered samples (Qualls et al., 1985, Örmeci and Linden, 2002b, Madge and Jensen, 2006a). This suggests that removal of particles larger than 1.2 µm, along with any particle-associated bacteria, will greatly improve the performance of UV disinfection, such that the remaining free-swimming bacteria will be inactivated with similar efficiency to that observed for the particle free control water. It is not possible to discriminate between the relative contribution of shielding and particle association to increased bacterial survival from our experiments because the numbers of culturable *E. coli* attached to particles could not be quantified. Fluorescent *In situ* hybridisation (FISH) could be applied to directly enumerate and co-relate the particle associated bacteria before disinfection and surviving bacteria after disinfection (Loge et al., 2002). This could be used in future experiments to obtain detailed information and enumerate pathogens associated with particles. Homogenisation of the samples increased the inactivation of *E. coli* in the Adelaide lagoon sample, allowing complete inactivation with no apparent tailing. Previous studies utilising similar homogenisation techniques have reported that blending can breakdown larger particles and disperses particle-associated bacteria (Örmeci and Linden, 2002b, Caron et al., 2007a). This suggests that blending broke larger particles and increased free swimming bacteria which

resulted in increased inactivation. It was also observed that there was less inactivation in filtered samples than parent samples for *E. coli*,. The reason for this is unclear but may be due to differences in the starting numbers of *E. coli* in the parent and filtered samples, which can influence the accuracy of the counts and the maximum inactivation that can be measured. While there were differences in the inactivation at the low UV dose, there was clear evidence of tailing in the parent samples and complete inactivation in the filtered samples. Interestingly, the PBS control had lower inactivation compared to the Melbourne sample. This may be due to differences in the *E. coli*, because the PBS sample had laboratory cultured *E. coli* (a single strain), whereas the Melbourne sample had faecally-derived *E. coli* (which would also be a mixed population of different strains). While there were some differences in the level of inactivation between parent and treated samples, both the filtration and homogenisation experiments confirm that particle association can hamper UV disinfection.

MS-2 phage were found to be more resistant to UV disinfection than *E. coli* in both the raw lagoon effluents and PBS control. MS-2 phage inactivation for the Melbourne lagoon effluent deviated slightly (but not significantly) from the Adelaide sample at higher doses >30 mJ/cm^2. Further experiments are needed at higher doses (>60 mJ/cm^2) to identify any differences and validate the findings. However, MS-2 phage inactivation followed first order kinetics in all the samples, with no evidence of tailing. These results are consistent with other studies of MS-2 phage UV disinfection in wastewater and buffered water (Havelaar et al., 1991, Tree et al., 1997, Park et al., 2011, Fang et al., 2014). Our previous particle characterisation study of these lagoon samples found no evidence that FRNA bacteriophage were associated with particles and agreed with Batch et.al., who reported that MS-2 phage disinfection was not affected by varying turbidity, particle count or size (Batch et al., 2004). These authors estimated that a UV dose of 57.4 ± 7.9 mJ/cm2 was required for 3-log10 inactivation (Batch et al., 2004). The

findings of this study are consistent with this, with all the samples showing 2.5 $\log_{10}$ inactivation at 60 mJ/cm^2.

C. parvum oocysts were more sensitive to UV radiation than *E. coli* or MS-2 phage. Low and medium pressure UV disinfection is highly effective at inactivating *C. parvum* oocysts (Craik et al., 2001, Linden et al., 2001). A high inactivation of the oocysts was observed in both the raw lagoon samples (~3-$\log_{10}$ at a dose of 3 mJ/cm^2), but for the Adelaide lagoon sample the inactivation rate was lower, suggesting tailing, consistent with tailing effects reported previously for oocysts (Craik et al., 2001, Amoah et al., 2005). Oocysts spiked into filtered lagoon water from either location were completely inactivated, as were oocysts spiked into particle-free buffer. This is supported by our *C. parvum* association study (Chapter 5) that there was slight evidence of association of oocysts with larger particles (>10 μm). There appears to be very little published information describing the particle association of *C. parvum* oocysts in wastewater, with a single study showing that oocysts spiked into and mixed with wastewater can readily attach to wastewater particles (Medema et al., 1998b). There did not appear to be much association of oocysts with particles in our study, considering that only slight tailing was observed.

The inactivation of *E. coli* following chlorination of both the lagoon effluents suggests rapid initial inactivation, followed by apparent tailing. The interpretation of this result is complicated by the rapid formation of chloramine, which coincided with the plateau in inactivation. Chloramine is less reactive and less effective as a disinfectant than chlorine, hence requires much larger CTs for the same inactivation (Gagnon et al., 2004). The loss of free chlorine and the larger CT required by chloramine could account for the tailing. However, the results for the filtered lagoon waters showed that the free-swimming *E. coli* were completely inactivated (>2-2.5 $\log_{10}$) and suggest that the tailing was due to the association of *E. coli* with particles. Tailing has previously been reported after chlorine inactivation of particle-associated bacteria in the

wastewater samples (Dietrich et al., 2003, Dietrich et al., 2007, Winward et al., 2008). While tailing occurred for both UV and chlorine disinfection of *E. coli*, chlorine inactivation achieved an order of magnitude higher inactivation than UV, suggesting that it is a more effective strategy for the inactivation of particle-associated *E. coli*. Others also concluded that chlorine is more effective in inactivating particle-associated bacteria than UV radiation (Örmeci and Linden, 2002b). The cause for this difference has not been elucidated, but chemical disinfectants may be able to diffuse through particles to inactivate associated bacteria, whereas UV can be scattered or fail to penetrate aggregates of cells/particles. Furthermore, the mode of action of both the disinfectants is completely different. UV radiation leaves no residual disinfectant after the dose has been delivered and *E. coli* possess repair mechanisms that can allow them to recover from UV disinfection and regrow (MeiTing et al., 2011).

Adelaide lagoon had higher CT values than Melbourne lagoon effluent, similar to the requirement for higher UV irradiation in the case of *E. coli* inactivation. The pH in the Adelaide lagoon was higher than in the Melbourne lagoon but was comparable to the particle free control. However, the control had much lower CT values than either of the two raw lagoon effluents because of the absence of particles in the control. Filtration and homogenisation increased pH in both the samples but corresponding changes in CT did not occur (the differences were not significant). The changes in pH could be due to removal of certain particles during filtration and break down of particles during homogenisation, which might release different materials. The order of increasing pH was raw< filtered< homogenisation in both the samples but the order of increasing CT was not the same. In Adelaide lagoon the CT increased as filtered<homogenised< raw whereas in Melbourne lagoon it was homogenised<filtered< raw. The differences in particle count, nature and size between the two samples could be the reason for the difference between the behaviour of both the samples as described in Chapter 3. This also suggests that increasing turbidity and pH greatly affected chlorine inactivation of *E. coli*.

Although most of the chlorine was quickly converted to monochloramine in homogenised samples, they still showed higher inactivation and lower CT. This further supports the premise that particle pathogen interactions impact disinfection and removing these interactions using various treatments increases disinfection efficiency.

MS-2 phage chlorine inactivation was completely different in both the samples. The spiked MS-2 phage appeared to be highly resistant to chlorine disinfection in the Adelaide lagoon water, but not resistant in the Melbourne lagoon water, because there was similar inactivation in demand-free water. Our previous fractionation study (Chapter 5) revealed no association of FRNA bacteriophage with wastewater particles. The absence of MS-2 phage particle association is further confirmed by the first order kinetics of UV inactivation and suggests that the tailing of MS-2 phage inactivation observed in Adelaide lagoon water was not due to particle association. MS-2 phage has been reported to be highly susceptible to free chlorine but resistant to chloramines (Havelaar and Nieuwstad, 1985, Tree et al., 2003a). We detected rapid conversion of free chlorine into monochloramine in both the Adelaide and Melbourne lagoon waters, but the monochloramine concentration was not high, so this is unlikely to be the reason for the difference in inactivation of MS-2 phage observed between the Adelaide and Melbourne lagoon waters. Furthermore, we used 10mg/L of free chlorine as the initial dose in the Adelaide lagoon water (twice that used for the Melbourne lagoon water) and 3 mg/L free chlorine was measured after 1 minute's exposure, so additional inactivation was expected. Therefore, it is unclear why there were differences observed between Adelaide and Melbourne lagoon effluents. Future work is needed to address this issue with improved methodology and specific controls. MS-2 phage displayed entirely different inactivation kinetics as compared to *E.coli*. Apart from the fact that the starting concentration of both the organisms were different, sensitivity of individual microbes to the disinfectant could be another reason for the observed differences. This finding is consistent with previous studies that *E.coli* are more readily

inactivated at lower doses of chlorine than viruses in wastewater (Tree et al., 1997, Tree et al., 2003b, Owoseni et al., 2017).

6.5 Conclusions:

1) Both UV radiation and chlorine disinfection were affected by particle-pathogen interactions in different wastewater environments and disinfection efficiency decreased as the particle count increased.
2) Chlorine disinfection is more effective in disinfection of *E. coli* whereas additional barriers such as filtration may be needed for MS-2 phage before any disinfection. The formation of other chlorinated species, such as monochloramines, can highly impact MS-2 phage chlorination.
3) Slight changes in pH did not affect CT values in both turbid wastewater samples.
4) Filtration increased UV and chlorine disinfection efficiency for *E.coli* and *C. parvum* oocysts and homogenisation increased UV and chlorine disinfection efficiency for *E. coli*.

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Chapter 7: General Discussion

7.1. Discussion

The main goals of this project were to a) characterise particles in treated wastewater effluents, b) analyse the association behaviour of pathogens or pathogen surrogates with these particles and c) to determine the impact of particles and particle association on disinfection processes. Wastewater effluents were successfully analysed and particulate differences were identified (Chapter 3), which lead to the investigation of the association behaviour of various surrogates in the same effluents (chapters 4 and 5). Additionally, the impact of these associations on disinfection of surrogates was also determined in these effluents (chapter 6). The individual research objectives of this project were satisfied as follows.

A review of the literature led to the conclusion that a further study was needed to characterize particles and particle-pathogen interactions in wastewater and to better understand the effect of different treatment processes on the nature of particles and their associations with different pathogens. Although tailing effect caused by particles in disinfection processes was well documented in freshwater and stormwater but there was a need to examine the effect of these associations on disinfection of wastewater. The literature review provided important information on the methods used for characterising particles and analysing their association with pathogens. It was identified that most of the particle-pathogen associated studies were conducted in freshwater or stormwater and it would be interesting to extrapolate those methods to wastewater to identify the associated fraction of microbes and the nature (size and composition) of particles that they associate with. There is a need to identify the changes in particle nature as it progresses from one treatment stage to another because this will impact the ways in pathogens would associate to them. It is also important to compare association behaviour of pathogens from environmentally and geographically isolated treatment plants because factors such as temperature and population dynamics would impact pathogen and particle composition of the treatment plant. Combining different partitioning techniques would

provide insights into removal mechanisms of particle-associated fraction. Understanding the disinfection kinetics of particle-associated fraction and particle free fraction of microbes using different disinfectants would help make informed decisions on targeted removal strategies and selection of appropriate disinfectants.

Particles were identified and characterised in secondary effluent and lagoon effluent from the Bolivar treatment plant, and in lagoon effluent from the Western treatment plant. Adelaide lagoon effluent displayed seasonal changes in nutrient concentrations, however, Melbourne lagoon effluent had little seasonal variation. Melbourne lagoon effluent had significantly higher levels of NH_3 and total P than both the other two samples and the levels of these two nutrients were not affected by any seasonal changes in Melbourne lagoon effluent. Both the lagoon effluents had different algal species which had negative zeta potential. Negative charge carried by particles would offer repulsive forces to the negative charged pathogens. The organic particle content was only 20% in winter months and this suggests the effect of colder temperatures on growth conditions favouring less growth in these months. Sun shielding using plastic shading balls or similar floating structures could be used to reduce penetration of sunlight, which would reduce particle counts due to reduced growth. Nutrient harvesting using chemicals or natural ion exchange resins could be another strategy to reduce biological load in wastewater which can be coupled with removal of other inorganics techniques such as filtration to reduce overall particle count and improve disinfection.

E. coli was associated with 1.2 -10 µm particles in the lagoon effluents, FRNA bacteriophage did not show any association with particles in either of the samples. This was supported by the finding that there were higher number of 1.19-3.78 µm particles in the Adelaide lagoon effluent (Chapter 3), suggesting an impact of particle availability on association behaviour. There were higher concentrations of *E. coli* in Melbourne lagoon (bacteria observed in SEM images as well) effluent, higher available nutrient content (NH_3 and total P) and higher association in

Melbourne lagoon which suggests that availability of nutrients and hence microbial concentration can play an important role in particle association. Therefore, materials such as ion exchange agents can be an used to simultaneously remove NH3 and total P (Ma et al., 2011), which would control growth conditions and impact particle association due to particle count reduction. There were differences observed in removal of *E. coli* by centrifugation and filtration in Adelaide samples. Scanning electron microscopy indicated the presence buoyant particles such as Microcystis in Adelaide samples (Chapter 4). Microcystis buoyancy can vary a lot seasonally (You et al., 2017) and therefore associated pathogens might not settle well throughout the year. This suggests that settling alone could not an effective strategy in removal of pathogens. The differences in the association behaviour of *E. coli* and FRNA bacteriophage in the samples from the Bolivar treatment plant suggests that the treatment processes greatly impact the association behaviour. The presence of FRNA bacteriophage in Adelaide secondary effluent and no detection in the lagoon effluent suggests that Adelaide lagoons were effective in removing FRNA bacteriophage. *E. coli* was detected in all the samples and greater particle association was observed in both the lagoon effluents. This means that there was production of particles (1.19-3.78 μm) within the lagoon systems, which increased *E. coli* association. Association of *E. coli* was observed in both the lagoon effluents in spite of the differences in nutrient concentrations and nature of particles (organic vs inorganic). This suggests that particle size and availability can highly impact particle association and additional removal techniques, such as micro-filtration, would benefit pathogen removal. Additionally, it was identified that bacteriophage concentration in the tested effluents was low to below detection limit. Further research is required to identify other potential viral surrogates which are present in vast numbers for easy detection. PCR techniques can be used to increase the detection limit of viral surrogates and also detect any non-culturable viruses. The research successfully identified the size of associated particles, and SEM protocol quantified organic content of the particles, but

the nature of the particles will change throughout the year. It is therefore necessary to identify accurately physical structure of the particles to which the bacteria are associating. Particles can be enclosed in various compartmentalised structures within certain particles as described in Chapter 1. Techniques such as FISH combined with microscopy can be used to confirm this which would explore other removal techniques such as dissociation of particle- associated pathogens.

. Blending or breaking larger particles have shown to release associated pathogens (de Lima Isaac et al., 2014, Torres-Palma et al., 2017). The results of this study suggest that more than one spore was associated with a single particle in all samples. It further suggests that spores can be easily dissociated from particles. Particle structure therefore an important factor that might favour pathogen association by harbouring more than one pathogen on its surface (Chapter 1). There were higher number of 1.19-14 μm in both the lagoon effluents than Adelaide secondary effluent (Chapter 3). Additionally, there were different numbers of settleable particles (chapter 3 and 4) indicating differences in particle densities in all the three samples. Significant association of spores in all the three samples suggest, surface properties of spores such as external appendages (Hijnen, 2010) could have favoured higher spore association overtaking other factors such as particle count and nature. This suggests spores might have higher natural tendency of association due to its surface properties. In comparison, *C. parvum* oocysts did not show any significant particle association. This suggests that anaerobic spores are not a good surrogate for *C. parvum* oocysts in particle association studies, or as a surrogate for treatment processes. Further work is needed to identify other potential surrogates such as aerobic spores which might provide a better correlation than anaerobic spores.

Furthermore, it was observed throughout the studies that there were higher numbers of smaller particles and lower numbers of larger particles in all the samples. *E. coli* was associated with

smaller particles and *C. parvum* was shielded by larger particles (>10 µm). Homogenisation was helpful in releasing associated particles but it also increased the numbers of smaller particles. It is easier to remove larger particles by coarse filtration screens whereas smaller particles become harder to remove and require finer filtration screens. Therefore, homogenisation might not be the most suitable method and further work is needed to explore other dissociation methods, which would dissociate pathogens without physical breakdown of particles. Alternatively, homogenisation can be coupled with conventional coagulation and filtration with addition of a chemical precipitant which can clump finer particles which can be easily removed by coarse filtration.

Disinfection kinetics were in agreement with the association studies in that, disinfection of *E. coli* showed tailing in both the wastewater samples. MS-2 phage did not show any associations (chapter 4) and the UV disinfection followed first order kinetics suggesting no effect of particles. MS-2 phage chlorination was, however, different and it was concluded that the presence of chloramines can negatively highly impact MS-2 phage disinfection by reducing the amount of free available chlorine needed. Additionally, MS-2 phage was spiked at a concentration of (10^5/mL) and even at the highest dose of 60mJ/cm^2, it was not completely inactivated. For both *E. coli* and *C. parvum* oocysts, tailing was observed when the residual microbial concentration was minimal. This suggests that if the dose was increased further, there might be some tailing observed. Starting MS-2 phage concentration could be lowered before disinfection to examine this. *C. parvum* oocysts were the most sensitive to UV radiation than *E.coli* and MS-2 phage and most of them ($3\log_{10}$) were inactivated at much lower doses of > 5mJ/cm^2 with slight tailing at 5 mJ/ cm^2 low doses. When the samples were filtered through 10 µm filter membranes oocysts were completely inactivated at doses above 3 mJ/cm^2. Oocysts were therefore physically shielded by larger particles (> 10 µm) due to scattering of UV light. There were lower numbers of larger particles (10-100 µm) than the smaller particles (1.19-

3.78μm) in all the samples. This supports the finding that there was more tailing of *E. coli* than oocysts as *E. coli* were associated with small particles (1.2-10 μm). The overall results successfully showed that the higher particle numbers negatively impacted the disinfection processes and therefore particle removal techniques such as micro-filtration are necessary to enhance disinfection processes.

Additionally, the nutrients concentrations of NH_3 and NO_3^- was different in Melbourne lagoon effluent in the samples used in Chapters 3,4,5 to the sample used in Chapter 6. There was no NH_3 detected in Melbourne lagoon effluent used for disinfection, whilst in other studies the NH_3 levels were higher than the Adelaide samples. Similarly, there was high NO_3^- concentration in Melbourne lagoon effluent used for disinfection, whilst in other studies (chapter 3, 4 and 5), the NO_3^- levels were significantly lower than Adelaide samples. Apart from NH_3 and NO_3^- all the other nutrient contents were comparable in all the studies and the microbial concentrations were also comparable. The differences in nutrient concentration could be due to the differences in sample location and types of treatment at each location. As discussed in chapter 3, warmer temperatures may be more conducive of higher microbial and algal growth and hence higher particle counts. Solutions, such as mechanical aerators to create turbulence in lagoons, could be used to control algal growth and reduce particle counts during summers or shade balls as mentioned earlier could be a beneficial option. Additionally, the turbidity levels were higher for both the lagoon effluents used in chapter 5 than the lagoon effluents used in chapter 4, however the concentration of VS was comparable in both the two chapters. All the studies used the samples collected in winter. Further work is needed to analyse the association trends and determine any correlations with turbidity or particle size distribution for other months and seasons. This would further validate the work presented in this thesis.

There was equal concentration of organic and inorganic particles in Melbourne lagoon effluent but Adelaide lagoon effluent had higher inorganic content. This slightly indicates that *E. coli*

could be associated with organic content in both the lagoon effluents (higher the organic content and higher the association). Additionally, there were algal particles as identified by SEM in Melbourne lagoon and the organic content of Adelaide secondary effluent was different to both the lagoons. Therefore, there is slight evidence that the pathogens could be associated with algal particles. There was slightly higher $\log_{10}$ reduction of *E. coli*, and *C. parvum* oocysts in Melbourne lagoon by UV disinfection (Chapter 6), which suggests pathogens associated with organic particles could be easier to disinfect with UV radiation than pathogens associated with inorganic particles. Chlorination had similar $\log_{10}$ reduction of *E. coli* in both the lagoon effluents. This suggests that chlorine disinfection can have similar effect on both organic and inorganic particulate environments. The differences in the chlorine CT values for E. coli between the lagoon samples and the demand-free water control demonstrate that particulates, and perhaps other aspects of lagoon water chemistry, impact the efficiency of disinfection, requiring larger CT values for the raw lagoon samples. Overall, various strategies (described above; shade balls, mechanical aerators, micro-filtration) could be applied upstream to disinfection to control organic particle load which would reduce particle pathogen associations and increase the disinfection efficiency for both UV radiation and chlorine disinfection. Cost benefit analysis for individual scenarios will then further determine the most suitable solution.

7.2 Conclusion:

It was found that the interaction of *E. coli* with particles had negative impact on both UV irradiation and chlorine disinfection. MS-2 phage had no particle association and the presence of particles had no impact on their disinfection. Anaerobic spores were not a suitable surrogate for *C. parvum* oocysts and oocysts did not show any association. Oocysts were slightly protected by larger particles (>10 µm), which can be easily controlled by removing these particles by filtration. The results of this study suggest that micro-filtration (0.1 µm to 1 µm)

can be an important control strategy to remove the particle associated pathogens. Microfiltration might be enough to remove larger particles, which otherwise offer protection to protozoans, and it will also remove particle associated bacterial populations.

7.3 References

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