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Microbial Pathogen Detection and Removal in Water Reuse Practices

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Environmental Engineering

by

Xiao Huang

Dissertation Committee:
Professor Sunny Jiang, Chair
Associate Professor Diego Rosso
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2015

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DEDICATION

I dedicate this work to my parents, who offered unconditional love and support to me and always encouraged me to follow my heart.

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ACKNOWLEDGMENTS

Five-year graduate study is a long journey of adventure. I am forever grateful for those who have helped me, encouraged me, and accompanied me to the end.

First, to my mom and dad, thank you for all your love and support through the years. You will never know how much it is appreciated. To my longtime advisor and mentor Professor Sunny Jiang, her guidance, inspiration, and help had a tremendous impact on making the completion of this thesis possible.

I would also like to thank the rest of my committee members, Professor Diego Rosso and Professor Amir AghaKouchak, for their guidance and support.

Thanks to all those who worked with me in Applied & Environmental Microbiology Lab. Janet, Wenzhou, Simon, Leda, Keahying, Linda, Ming, Derek, Edgar, Tylor, Kenny, Dana, Sara, Yiwen, Xiaoshu, Shanshan and Haiping. I cherish the time shared with you guys.

The research would not have been possible without funding support from WaterReuse Foundation, National Science Foundation – Partnerships for International Research and Education, and Bill & Melinda Gates Foundation.

To my loving fiancée, Lijie Li, thank you and I love you.

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[1] **Huang, X.**, Qu, Y., Cid, C., Finke, C., Hoffmann, R., Lim, K.-Y and Jiang, S.C., Electrochemical Disinfection of Toilet Wastewater using Solar-Powered Wastewater Electrolysis Cell (Water Research, under review).

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CONFERENCE PRESENTATIONS DELIVERED

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ABSTRACT OF THE DISSERTATION

Microbial Pathogen Detection and Removal in Water Reuse Practices

By

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Doctor of Philosophy in Environmental Engineering

University of California, Irvine, 2015

Professor Sunny Jiang, Chair

Population growth, rapid urbanization, and climate change have been straining our traditional water resources and leading to a global scale water crisis. It is projected that global water demand could outstrip supply by up to 40% by 2030, if no effective water management strategies are quickly adopted. Wastewater reclamation and reuse offer an effective means of conserving our limited freshwater resources and improving water productivity by closing the hydrological loop locally. The advances of water treatment technologies have provided options for treating wastewater to the quality required for any intended uses. The efficiency and sustainability of water reuse practices, however, rely on matching the water treatment process with specific end uses, by which insufficient or over treatment could be avoided.

The primary concern associated with water reuse practices is the public health risk caused by the potential exposure to microbial contaminants, such as pathogenic protozoa, bacteria and viruses. The detection and removal of microbial pathogens is therefore of great

importance to ensure the reclaimed water is “safe” for the intended end uses. This research studied the microbial pathogen removal in three different water reuse schemes. In the first study, a rapid direct virus detection method based on Accuri C6 flow cytometry (FCM) was developed to quantify the virus removal rate in a water reclamation plant using microfiltration-reverse osmosis (MF-RO) process for indirect potable reuse water production. This new method, in combination with online total organic carbon and nanoparticle analysis, has shown to be a viable way for online monitoring of high-pressure RO membrane integrity and the potential breakthrough of viral particles. In the second study, the effectiveness of microbial pathogen removal by constructed stormwater wetlands (CSW) in the U.S. and Australia was investigated using digital droplet PCR (ddPCR) and 454-pyrosequcing techniques. The results showed that the two US CSW and one of the three Australian CSW had good performance in terms of indicator bacteria removal during dry weather flow. The treated stormwater can meet the recreational water quality criteria/guidelines. No *Cryptosporidium* was detected in any of the CSW, while Adenovirus were present at all sites. Human specific HF183 *Bacteroides* were only found in Australian sites indicating the potential contamination from sewage ingression. The microbial community analysis showed a clear increase of *Cyanobacteria* in the outflow of CSW with better performance. The water residence time was determined as a critical factor affecting the efficacy of microbial pathogen removal or inactivation. In the last study, pathogen removal efficiency was investigated in a solar-powered mobile toilet system for decentralized wastewater treatment. The wastewater electrolysis cell (WEC) uses solar energy to generate oxidants via electrochemical (EC) reaction for disinfection of microbial

pathogens. The results showed 5 log_{10} reductions of bacteria (*E. coli* and *Enterococcus*) and viruses (coliphage MS2 and adenovirus) were achieved within 1 h reaction at applied cell voltage of +4V. The dominating role of free reactive chlorine generated *in situ* during EC disinfection process was verified using laboratory model waters. The formation of organic disinfection byproducts trihalomethanes (THMs) and haloacetic acids (HAA5) during EC treatment were found to increase with the rise of applied cell voltage. The EC treated toilet wastewater is suitable for many non-potable reuse applications (e.g., toilet flushing and irrigation) with significantly reduced microbial infection risk. As there is no need for supporting chemicals, the WEC system can be developed into commercial viable, self-sufficient, solar-powered mobile toilets for decentralized wastewater treatment.

CHAPTER 1 INTRODUCTION: WATER REUSE AND WATERBORNE DISEASE

1.1 Background

Water is life. At the time when scientists are dedicating to search light-years away in the universe for the proof of life - the present of the simple molecular [H_2O], at our home planet - the earth, we are facing a tremendous challenge threatening the survival of the whole human community - a global water crisis (Hanjra and Qureshi 2010).

Water is seemingly abundant on earth as 70% of the planet is covered by oceans. However, the real situation is that very limited amount of water is accessible for human consumption. 97.5% of all water on Earth is salty water in the ocean with salinity from 35 to 37 gram per liter leaving only 2.5% as fresh water; 70% of the fresh water is frozen in the icecaps of Antarctica and Greenland; most of the remainder is present as water vapor in the air, as soil moisture in the ground, or lies in deep underground aquifers. It is estimated that only about 1% of the fresh water is actively supporting the life on earth.

The water crisis in essence is the mismatch between fresh water availability and the increasing human demands on water (Bogardi et al., 2012). Many factors have contributed to this mismatch. First of all, the geographic distribution of water resources does not often match the human population distribution. Historically, the four early great civilizations all

grew up in river valleys, where reliable source of water was available. But today, around 30% of world population live in arid and semi-arid areas. Urbanization is intensifying this issue. Large cities become water demand hot spots creating regional water scarcity. Second, in many places, water supplies is heavily depended on precipitation, and thus the water availability varies significantly between dry and wet seasons. Due to the global climate changes, extreme weather events such as extended drought and heat waves has become more frequently. Consequently, seasonal water shortage will be much more geographically widespread (Aghakouchak et al., 2014). Last but not the least, water pollution has added enormous pressure to existing water scarcity problems, especially in developing countries where wastewater treatment facilities are usually too costly to be built and operated (Azizullah et al., 2011). Given the fast increase of world population, the gap between freshwater demand and supply will be about 40% globally by 2030 if no effective water management strategies are adopted soon (Brabeck-Letmathe 2012).

Water crisis is not a single issue. Water, food and energy are tightly interconnected forming a nexus. Worldwide, more than 90% of the freshwater withdrawal is used for agricultural irrigation. In the United States, 39% of water is used for cooling at thermoelectric power plants. Water-related energy use (e.g., wastewater treatment, water transport) in California consumes approximately 20% of the state's electricity (Hanlon et al., 2013). Water shortage will inevitably cause the unbalance of the nexus and consequently, adversely affect the economic development, public health and the environment (Plappally 2012). It has become ever clear that, in the face of urbanization, global population growth and

climate change, ensuring water security is imperative for realizing sustainable socio-economic development.

1.2 Water Reuse

1.2.1 Concept

Water reuse (also known as water recycling, wastewater reclamation) is the use of treated wastewater for beneficial purposes. In many countries like China, Mexico, Peru, Egypt, Lebanon, Morocco, India and Vietnam, raw wastewater has been used for agricultural irrigation over centuries (Drechsel et al., 2009). Sewage farming was also quite popular in United States, France and Germany during the first half of 20th century. In 1920s, more than 70 municipals in California applied their raw sewage to orchard or farms for food production (NRC 2012). Although water reuse is not new, the practice keeps evolving with time. After late 1950s, direct reuse of raw wastewater was banned in most developed countries due to the concern of public health (NRC 2012). "Treatment" and thus, has been emphasized in the concept of wastewater reuse, by which the benefits of wastewater reuse as well as the institutional challenges and risks associated with the practice are addressed. It should be noted that, direct reuse of raw wastewater is still prevalent in many developing countries, where sanitation and waste management are unable to keep pace with urban population growth. In this dissertation, we only focused on planned water reuse, which refers the wastewater is treated to meet certain criteria before it is used for designed applications.

1.2.2 Drivers for water reuse

In developing countries, the main drivers of water reuse are the lack of alternative water sources, the limited wastewater treatment capacity, and the economic incentives for food production (Drechsel et al., 2009). In developed world, drought and regional water scarcity are the leading drivers for water reuse. For example, 80% of the San Diego region's water supply is imported from northern California and the Colorado River via massive piped aqueducts. These import sources are becoming subject to considerable restrictions forcing the city to explore new water supplies such as reclaimed water and desalination. Water reuse is also increasingly seen as a way to enhance resource efficiency. Not all water used for industry, agriculture or municipality purpose needs to be drinking water quality. Instead of discharging the treated wastewater to environment (e.g., rivers, lakes and oceans), using treated wastewater for beneficial applications can improve the water productivity by closing the hydrologic loop locally (Grant et al., 2012). Currently, only 7-8% of the wastewater produced in the United States is reused one way or another. Therefore, there is tremendous potential for expanding the use of reclaimed water in the future. It is expected that more wastewater reuse projects on city scale (centralized systems) as well as community or household scale (decentralized systems) will be implemented in order to augment water supplies and minimize the impact of human activities on the environment (Bixio et al., 2006).

1.2.3 Water pollution

Water is characterized in terms of its physical, chemical and biological compositions. When water is withdrawn and used for certain purpose, various contaminants may enter the used water and change its compositions. The common contaminants in water can be categorized into the following groups: 1) microbial pathogens; 2) oxygen-demanding wastes; 3) suspended solids; 4) nutrients; 5) heavy metals; 6) salts; 7) pesticides; 8)volatile organic chemicals; 9) emerging contaminates; 10) heat (thermal pollution). It should be noted that even the most pristine natural water is not a pure compound. It may already contain certain levels of those contaminants. When the concentrations of contaminants in water are in excess of their normal amount and negatively affect its functionality, water pollution happens. Water usage does not necessarily result in water pollution, yet water quality degradation is the inevitable consequence of water usage as more contaminants are expected in the used water than the raw water. The usefulness of water decreases with the decline of the water quality as the used water becomes "unusable" for certain purpose. In essence, the primary goal of water reuse is closing the hydrologic loop locally to increase the water use cycles before it is discharged into the environment. Contaminants are not always harmful. By properly designing the reuse scheme, it is possible to turn some contaminants into valuable resources. A good example here is nutrient. In metropolitan areas, frequently outbreaks of algal blooms in rivers, lakes and coastal waters have been blamed to the excess nutrient loading by municipal wastewater or stormwater. Consequently, wastewater treatment plants are under growing pressure for nutrient removal which inevitably increases the cost of treatment. If the same water can be used for

irrigation, the problematic nitrogen and phosphorous in the wastewater could become good fertilizers (Mo and Zhang 2013).

1.2.4 Source matters

In cities, municipal wastewater is produced from households, offices, hospitals, and commercial facilities and conveyed through a collection system (sewage pipes) to wastewater treatment plants (NRC 2012). In rural areas, irrigation runoff represents the largest portion of wastewater. The source of wastewater largely decides the characteristics of wastewater. For example, municipal wastewater containing human feces is characterized as high concentration of microbial pathogens; agricultural runoff may carry large amount of pesticides, fertilizers and salts; and industry wastewater usually shows elevated levels of specific chemical or organic pollutants. Pollutants may also enter water from natural sources and from human activities via nonaqueous route. For instance, atmosphere deposition is believed to be the main source of pollutants such as mercury and polybrominated biphenyl ethers (PBDEs) in water. The source of wastewater does not only decide the quality, but also largely determines the quantity of wastewater. In the United States, 121 million m^3/d of municipal wastewater effluent is discharged nationwide. In large cities, the wastewater treated in centralized wastewater treatment plants represents a reliable local water source, and therefore creates a unique opportunity for large scale planned water reuse. In fact, to minimize the energy consumption on water transport, most of the water reclamation plants are built adjacent to traditional wastewater treatment plants. In rural or remote area, where sophisticated sewage collection systems are not

available, decentralized wastewater treatment systems would be more practical to treat relatively small volumes of wastewater and facilitate the reuse of the treated water locally.

Stormwater is another potential source of fresh water in urban areas. Stormwater runoff is generated when precipitation from rain and snowmelt events flows over land or impervious surfaces and does not percolate into the ground (Jiang et al., 2015). In many cities, stormwater is collected by engineered storm drain systems like street gutters and underground concrete channels. In a few old urban areas where storm drains and the sewer collection systems are combined (combined sewage system), the stormwater collected in the system is pumped to the wastewater treatment plants together with municipal sewage for treatment. During dry weather condition, urban runoff (water from landscape and agriculture irrigation, car washes and natural groundwater seepage, etc.) is the main source of stromwater transported by the stormwater drain systems. As stormwater flows over the impervious street surfaces, it carries debris, chemicals, sediments and microbial pathogens to the local receiving water bodies (lakes, rivers, wetlands and coastal waters, etc). In many urban areas, stormwater has become the main nonpoint source pollution resulting the local water quality degradation. In spite of the different contaminants carried in the stormwater, the quality of stormwater is generally much better than raw sewage. Traditionally, the main function of stormwater drain systems is to convey stormwater out of urban areas quickly to avoid flooding problem. Recently, stormwater management has begun to tackle the pollution problem. A variety of best management practices (BMPs) and low impact development (LID) techniques are

employed to reduce the peak flow as well as remove contaminants. In water-scarcity areas (e.g., southeast Australia, southern California), it is therefore logical to move one step forward to harvest and treat stormwater for some beneficial uses to meet the ever increasing water demands.

1.2.5 Reuse applications

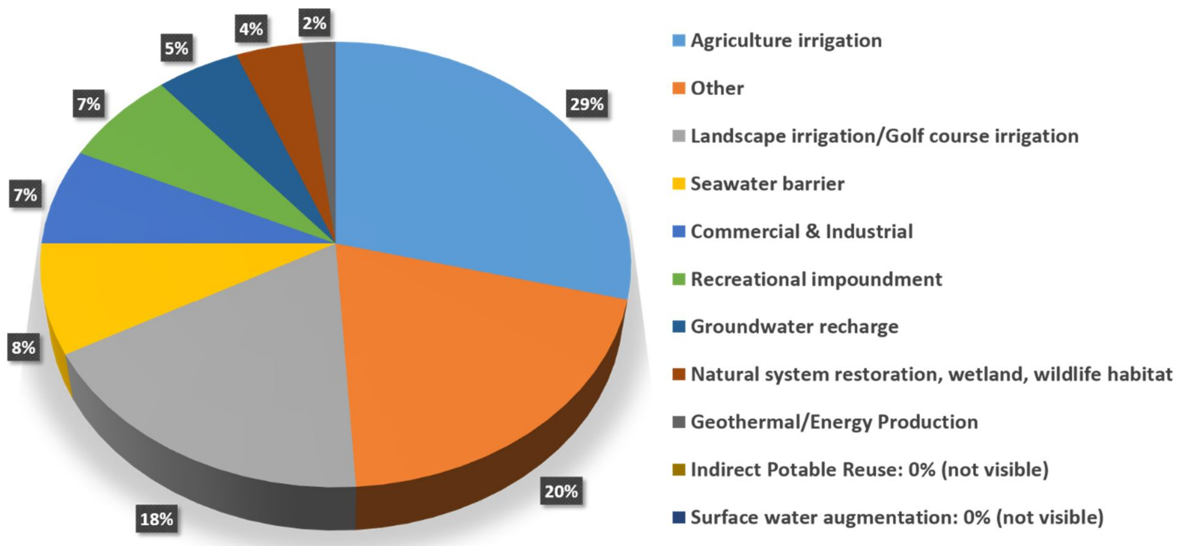

Figure 1-1 Summaries of the United States water reuse applications (adapted from Bryck et al., 2011).

As an alternative to directly releasing treated wastewater into the environment, reclaimed wastewater can be reused for a variety of purposes. The potential reuse applications are largely decided by the reclaimed water quality and availability. Figure 1-1 shows the U.S. nationwide reclaimed water uses in 2010. Clearly, agricultural and landscape irrigation

represent the largest portion of reclaimed water uses. From the demand side, in the U.S., almost 128,000 million gallon freshwater is used for agricultural irrigation every day, which accounts for approximately 37% of all freshwater withdrawals (Maupin et al., 2014). In many parts of the country, irrigation water demand alone is nearing or exceeds the total supply of fresh water. Therefore, using reclaimed water for irrigation is imperative choice to save the limited freshwater resources for other purposes (e.g. potable use). Municipal wastewater typically increases with population growth. After secondary treatment, it normally meets the agricultural irrigation water quality requirements. The practice has also been widely accepted by public and supported by regulatory and institutional policies. For example, in 2009, California adopted "Recycled Water Policy" and "Water Recycling Criteria" to encourage the reuse of reclaimed water in agricultural and landscape irrigation. Yet the success of reclaimed water irrigation is depended on many other factors, such as the reclaimed water quality, the plants' sensitivity, soil characteristics and the irrigation management. Salinity is the key factor determining the suitability of reclaimed water for irrigation. As the salinity of reclaimed water is usually higher than freshwater from traditional sources (e.g., surface water). Without proper management, salt buildup can reduce the crop production and eventually result in the degradation of soil quality. Chlorine residual is another factor need to be considered as chlorine is widely employed for secondary effluent disinfection. Chlorine concentration higher than 5 mg/L can cause severe damages to most plants. For food crops irrigation, especially those eaten raw, higher quality reclaimed water is expected to minimize the potential microbial infection risk.

The second category of water reuse is for environmental or recreational purposes including seawater intrusion barrier, ground water recharge, recreational impoundments and natural system restoration. The quality of reclaimed water used for these applications would be no lower than that of traditional secondary effluent discharged into environment. Advanced treatment processes can be added depending on the potential public and environmental health risks associated with the reuse practices. Based on 2012 U.S. EPA Water Reuse Guidelines, reclaimed water used for applications where no human contact is expected should be disinfected to achieve an average fecal coliform concentration not exceeding 200 CFU (colony-forming unit) per 100 mL, while for uses where direct or indirect contact is expected, no detectable fecal coliform per 100 mL is suggested. Increased attentions have also been given to the impact on environmental health. Among the many pollutants present in treated wastewater, trace organic contaminants (e.g., pharmaceuticals and personal care products) raise the most concerns. Trace organic contaminants are ubiquitous in secondary effluent as the traditional wastewater treatment processes are not designed to remove these contaminants. The environmental toxicity for many of these chemicals has not been fully understood. Therefore, for reuse projects involving sensitive ecological systems, more rigorous assessment is needed to evaluate the site-specific conditions.

Although negligible amount of reclaimed water is currently used for potable purpose (Figure 1-1, less than 1% of the total volume of reclaimed water), the technological and public health interests are the greatest. Indirect potable reuse (IPR) is the main way

practiced in the United States. In IPR, highly treated wastewater is required to store in an environmental barrier (e.g., groundwater basin) for certain period of time (usually longer than 6 months) before it can be used as drinking water sources. In many arid area (e.g., southern California, Florida, Texas), IPR has become an important alternative drinking water source to maintain local water sustainability. For example, the world largest IPR project - Groundwater Replenishment System (GWRS) at Orange County California produces 70 MGD (million gallons per day) drinking water level reclaimed water, which equals 20% of the total water supply to the district's 19 municipal water agencies. Direct potable reuse (DPR) refers that the treated water is distributed immediately to upstream of a drinking water treatment plant or directly into the potable water distribution system. Currently, only two DPR facilities are operating in the United States (Colorado River Municipal Water District in Big Spring, Texas and Cypress Water Treatment Plant in Wichita Falls, Texas). Worldwide, the only example of a DPR system in operation is in Windhoek, Namibia, where highly-treated recycled water is put into a drinking water system that serves 250,000 people. The advantages of potable reuse is obvious. The technologies have been proven and there is no evidence showing that the practice has resulted in the increase of public health risk. However, the implementation of potable reuse projects are facing more public and political barriers, because the public support for water reuse initiatives generally wanes as the likelihood of individual contact with reused water increases. In a recent survey for public perception of DPR in four U.S. cities, about half of the respondents were not in support of using purified water as potable water (Ishii et al.,

2015). This study highlighted the challenge of fostering public acceptance for potable reuse projects in a community.

1.3 Waterborne Disease

Waterborne diseases are illness caused by pathogenic microorganisms that are commonly transmitted by water. Most of microorganisms associated with waterborne diseases are enteric pathogens and therefore the infections are commonly spread by fecal-oral route (human to human or animal to human) due to the consumption of or contact with fecal contaminated water. According to the World Health Organization (WHO), waterborne diarrheal disease alone is responsible for the deaths of 1.8 million people every year making it the leading cause of disease and death around the world (WHO 2014). The majority of victims are children from developing countries, where no safe water supplies are available due to the lack of wastewater treatment facilities, inadequate sanitation and insufficient hygiene. Even in the developed world, people can suffer from waterborne diseases occasionally. In 2007-2008, 134 recreational water illness outbreaks were reported in the U.S., which resulted in at least 13,966 cases of illness (Hlavsa and Brunkard 2011); 4,128 cases of illness and three deaths associated with drinking water contamination were reported by 23 states and Puerto Rico (Brunkard et al., 2011).

The most common concern associated with water reuse is the potential transmission and outbreak of waterborne disease. Wastewater contains human excreta is the main source of waterborne pathogenic microorganisms. As water reuse is increasing in scale and volume,

the probability of human exposure to pathogens is simultaneously elevated. WHO determined that a waterborne disease burden of 10^{-6} DALYs (disability adjusted life years) per person per year is a tolerable risk (Priiss and Havelaar 2001). To control the public health risk within that range, integrated water management strategies, such as wastewater treatment, application techniques, regulations and policies should be considered and implemented with water reuse projects. Understanding the occurrence and concentration of pathogenic microorganisms in reclaimed water, the efficiency of wastewater treatment processes, the potential exposure routes, and the dose-response relationship is of great importance to ensure the safety of reclaimed water and to build public confidence and trust in water reuse.

1.3.1 Waterborne microbial pathogens

10^{8}~10^{12} microorganisms per milliliter are expected in raw sewage. In spite of the fact that untreated wastewater contains a large number of microorganisms, only a sub-portion of these microorganisms are human pathogens. Table 1-1 lists the common waterborne disease causing agents, which include members from viruses, bacteria and protozoa. Among those microorganisms, some are "obligate pathogens", which indicates they can only propagate inside their hosts. Examples of obligate pathogens include *Campylobacter*, *Salmonella*, *Cryptosporidium*, *Giardia* and all of the enteric viruses. These pathogens may survive in environmental waters for an extended period of time, especially for those in the form of spores, cysts or oocysts. But water is more like a way for dissemination rather than their preferred habitat. Other pathogens, such as *Legionella spp.*, *E. coli* and some

Mycobacteria species are capable of surviving and proliferating in environmental waters. They can spend most of their life outside their hosts and therefore are called "environmental pathogens". In some cases of massive waterborne disease outbreaks that cannot be traced to recent external fecal contaminations, environmental pathogens are more likely to be the causing agents. Although environmental pathogens are well adapted to the natural water environment, the reproducing rate outside their hosts are normally much slower due to various environmental stressors (e.g., nutrients, predators and temperature).

Table 1-1 Characteristics and associated illness of common waterborne microbial pathogens

Pathogens		**Characteristics**	**Associated illness**
Virus	Adenovirus	dsDNA, ~26-46 Kbp, 80-110 nm	Respiratory disease, eye infections
	Astrovirus	ssRNA, ~6.8-7.2 Kbp, 27-43 nm	Gastroenteritis
	Coxsackievirus	ssRNA, ~7.5 Kbp, 30 nm	Herpangina, aseptci meingitus,respiratory illness
	Echovirus	ssRNA, ~7.4 Kbp, 24-30 nm	Fever, rash, Respiratory illness, gastroenteritis
	Hepatitis A	ssRNA, ~7.5 Kbp, 24-30 nm	Infectious hepatitis
	Hepatitis E	ssRNA, ~7.2 Kbp, 30 nm	Fever, malaise, vomiting, anorexia, nausea, abdominal discomfort
	Norovirus	ssRNA, ~7.6 Kbp, 25-35 nm	Gastroenteritis
	Poliovirus	ssRNA,~7.5 Kbp, 30 nm	Paralysis, headache, nausea, vomiting, general discomfort
	Rotavirus	dsRNA, ~18.5 Kbp, 75 nm	Gastroenteritis
Bacteria	*Campylobacteria*	Gram-, microaerophilic, curved, helical-shaped, 0.2–0.8 μm	Diarrhea, fever, abdominal pain, malaise
	E. coli O157:H7	Gram-, facultative anaerobic, rod-shaped, 0.5–2 μm	Acute hemorrhagic diarrhea, abdominal cramps
	Legionella	Gram-, aerobic, pleomorphic, 0.5-2 μm	Cough, fever, muscle aches
	Salmonella	Gram-, facultative anaerobic, rod-shaped 0.7-3 μm	Diarrhea, fever, stomach cramps
	Shigella	Gram-, facultative anaerobic, rod-shaped	Diarrhea, fever, stomach cramps
	Virbrio cholerae	Gram-, facultative anaerobic, comma-shaped , 0.5-0.8 μm	Profuse watery diarrhea, vomiting, muscle cramps
Protozoa	*Cryptosporidium*	Tough outer shell, 4-6 μm	Watery diarrhea, fever, vomiting, nausea
	Giardia lamblia	Flagellated, 10-20 μm	Diarrhea, Stomach or abdominal cramps

The symptoms of waterborne microbial infections may be varied, but diarrhea and vomiting are commonly reported (Table 1-1). The infections mainly occur in the

gastrointestinal tract of the hosts and the matured pathogens are usually shed with the feces of the hosts. Some pathogens may also cause illness unrelated to the gut epithelium. For example, Hepatitis A and B mainly cause disease related to the liver. The infective dose of different pathogens can vary dramatically. Even though the dose-response curves for many microbial pathogens are unknown or not very accurate, the infection dose of viral or protozoan pathogens is generally lower than that of bacterial pathogens. Studies have shown that for certain virus or protozoa, infection dose can be as low as one to ten infectious units (Percival et al., 2013).

Microorganisms in wastewater can be in the form of planktonic cells or attached/imbedded in biofilms. Only bacteria and algae can actively secret extracellular polymeric substance (EPS) to form biofilms, but many viral or protozoan pathogens are able to attach to existing biofilms, that leads to the formation of a complex microbial community. In environmental waters, the forming of biofilms offers many critical advantages for the survival of pathogens associated with biofilms compared to their planktonic counterparts. Studies have presented evidence that the EPS layers can effectively protect embedded pathogens by limiting the diffusion of chemicals, by neutralizing oxidants, and by protecting against dehydration. For example, 9-$\log_{10}$ reduction of planktonic growing *E. coli* O157:H7 in modified tryptic soy agar was achieved within 5 min by treating with 50 mg/mL chlorine. When the same bacteria formed biofilms in minimal salt broth, less than 1-$\log_{10}$ reduction was observed after 5 min treatment at the chlorine concentration of 200 mg/mL (Ryu and Beuchat 2005). The forming of biofilms also facilitates the information and genetic

materials exchanges among the members due to the higher cell density. Quorum sensing, a cell-to-cell communication mechanism regulating the cell density in biofilms, is found to be related to the virulence of many bacterial pathogens. For example, the human pathogens *Pseudomonas aeruginosa* employ autoinducer N-acyl-L-homoserine lactones (AHLs) to control the expression of multiple virulence genes in concert with cell population density (De Kievit and Iglewski 2000). The forming of biofilms provides a unique way in terms of pathogen delivery, which poses a higher infection risk, because the biofilm clumps enter the bulk water can potentially deliver a larger number of pathogens to the host compared to the evenly distributed planktonic pathogens. However, on the positive side, chances are that pathogens associated with detached biofilms or inorganic particles are more likely to be removed by sedimentation or filtration process.

1.3.2 Microbial pathogen detection

1.3.2.1 Microbial pathogen concentration methods

Although the concentration of total microorganisms in raw sewage could be in the magnitude of 10^{12} per milliliter, for specific pathogenic microorganisms, their concentrations are dependent on the epidemic conditions in the watershed, which is normally close or much lower than the detection limit of many available pathogen detection methods. This issue is more evident when dealing with treated wastewater, stormwater and high quality reclaimed water (e.g., reverse osmosis (RO) effluent). In fact, concentrating the microorganisms of interest from a large volume of environmental water

sample into a suitable volume for the downstream detection is sometimes more challenge than the detection itself. Commonly used microbial pathogens concentrating methods are summarized in Table 1-2. The first group of methods are based on membrane filtration. Different group of microorganisms can be separated and concentrated by membranes of different pore sizes. Single layer disc membranes are widely used to concentrate bacteria and protozoa with the working volume from 1 mL to 1000 mL depending on the turbidity of the sample. Disc membranes with extremely small pore sizes (<0.02 m) are developed for concentrating viral size particles. Due to the small pore sizes, these membrane are very easy to be clogged by other large particles, and thus the starting sample volume is usually limited to 10 mL. To overcome this disadvantage, membranes with positive or negative charges but larger pore sizes (usually 0.2 m) are developed to concentrate viruses in larger volume of samples (up to 100 mL). In natural water (pH=5~8), most of the viral particles have a negative surface charge and therefore, they are attracted to the electropositive membranes. Negative charged membranes also rely on the charge interactions, except that the water samples have to be pre-conditioned (adding multivalent cations and adjusting pH) to alter the surface charges of the viral particles. Cartridge style membrane filters can process environmental water samples from 1 L to 1000 L depending on the water quality. The 1 MDS positively charged cartridge filter is recommend by U.S. EPA for recovering human enteric viruses in drinking water. However, these filters are not cost-effective for routine viral monitoring. A much cheaper alternative positively charged cartridge filter (NanoCeram ®, Sanford, Florida) made of nanoalumina (aluminum oxide hydroxide) and glass fibers is developed and has been tested in several studies. The

nanoCeram filters have showed similar efficiency in concentrating virus compared to 1 MDS filters (Francy et al., 2013, Karim et al., 2009). Some researchers also investigated the feasibility of using dialysis ultrafilters (e.g., Hemoflow F80A) to co-concentration of viruses, bacteria and protozoa (Hill et al., 2005). The hollow-fiber ultrafilter is typically run in a tangential flow mode where the retentate is recirculated until the desired concentration factor is achieved. The scouring effect of the cross-flow recirculation decreases the tendency for microbes or other particles to adhere to filter surfaces, and thus reduces the possibility of filter clogging. However, the final volume of the retenate is still larger than 100 mL. A secondary concentration step is needed to further reduce the sample volume for downstream analysis.

Table 1-2 Microbial pathogen concentration methods

		Characteristics	Starting volume*	Ending volume**	Pros	Cons
Filtration	Single layer disc filter	Different pore and membrane sizes available	10~1000 mL	<0.5 mL	Cheap	Small processing volume
	Cartridge Filter	Co-concentrate, electro-adsorption	1~1000 L	~100 mL	Large volume	Expensive, secondary concentration needed
	Hemoflow	Tangential flow, co-concentration	1~1000 L	~100 mL	Large volume	Time consuming, complicated
Centrifugation	High speed centrifuge	Convenient for bacteria and protozoa	50~1000 mL	<0.5 mL	Cheap, user friendly	Cannot concentrate viruses
	Ultra centrifuge	Usually only for lab scale research	1~10 mL	<0.5 mL	Good for viruses	Expensive, time consuming, small volume
	PEG precipitation	Co-concentrate	1~100 mL	< 1 mL	Cheap, user friendly	Small processing volume, time consuming
Combined	Amicon	Co-concentrate, different pore sizes available	0.5~15 mL	<0.3 mL	Fast, convenient	Small processing volume
	Centricon	Co-concentrate, different pore sizes available	15~75 mL	<0.3 mL	Fast, convenient	Small processing volume

*Depending on the turbidity of the water samples. ** Volume of attenuate or wash buffer.

Centrifugation is another way to concentrate microorganisms. The rate of sedimentation depends on the particle diameter, particle density, solution density, volume, angle and speed of rotation. Bacteria and protozoa can be settled with simple high speed centrifugation (< 60,000 g, relative centrifugal force (RCF)), however, direct precipitation of viruses requires ultracentrifugation (> 250,000 g, RCF) and the concentration step may take several hours. Bench scale high speed centrifuge can process as much as 6 L samples per run, while most ultracentrifuge machine can only process less than 60 mL samples per run. The efficiency of centrifugation can be improved by destabilizing the microbial particles by coagulation (e.g., pH change or cations addition) or by increasing their sizes through flocculation (e.g., adding high molecular weight polymers). These two processes are usually employed simultaneously. For examples, polyethylene glycol (PEG) precipitation method are widely used to concentrate viruses in water samples without the need of ultracentrifuge (Lewis and Metcalf 1988, Sánchez et al., 2012).

The filtration and centrifugation schemes can be used in combination. The Centricon Plus-70 Centrifugal Filter Units (Billerica, MA, USA) and Amicon Ultra Centrifugal Filters (Billerica, MA, USA) are devices based on both mechanisms. These commercialized filters are user friendly as they are compatible with regular centrifuge machine and the centrifuge process only takes less than 30 min. 1 ~ 75 mL of water samples can be concentrated to less than 300 L. The small final volume is very helpful for downstream detection step, especially those polymerase chain reaction (PCR) based methods.

1.3.2.2 Culture-based detection methods

Detection and enumeration microorganisms of sanitary importance in environmental waters is an essential prerequisite for evaluating microbial water quality and assessing the effectiveness of treatment processes. The culture-based detection methods, in essence, are based upon providing a combination of nutritional and physicochemical conditions that will support the growth of the microorganisms of interest (Kator and Rhodes 2003). Yet providing a similar environment like the pathogens' warm-blooded hosts could be difficult or in some cases, not possible. For example, no suitable tissue culture assays or animal models are available for studying human noroviruses - one of the most common causes of gastroenteritis worldwide (Papafragkou et al., 2013). Another consideration is the culture assay should be designed to recover all target microorganisms, while it is also supposed to limit the growth of other interfering (background) microorganisms. The selectivity of the growth media can be improved by adding inhibitors, (e.g., bile salts, sodium deoxycholate, and sodium tetrathionate) or control other physical environmental parameters such as temperature, oxygen concentration and pH. Because of the complexity and diversity of microorganisms in environmental samples, an idea culture media only supporting the growth of target microorganisms does not exist. Moreover, some microorganisms may enter a state called viable-but-non-culturable (VBNC) in environmental waters due to various stressors (temperature, osmotic pressure, UV, and disinfectant). A practical consequence of VBNC is that, the results from culture-based methods tend to underestimate the number of the target microorganisms. However, given all these limitations, cultured-based methods are still used as standard methods in many regulations

and guidelines. The most important reason is that other alternatives are not able to provide information on viability and infectivity of the target pathogens, which is critical to evaluate the microbial infection risk. Many commercialized testing kits (assays) are developed for common indicator microorganisms or pathogens. For example, the IDEXX systems (Westbrook, ME, USA) based on the Most Probable Number (MPN) model can provide counts of coliforms, *E. coli*, *Enterococcus* and *Pseudomonas aeruginosa* in 18 -24 h. Membrane filtration coupled with colony counts on special designed solid media (e.g., EPA method 1604 for *E. coli* and method 1600 for *Enterococcus*) may offer better precision when compared to MPN systems. The Biolog system (Hayward, CA, USA) can differentiate over a thousand different bacteria in environmental samples based on their carbon substrate utilization patterns.

1.3.2.3 Non-culture based detection methods

Table 1-3 Non-culture based methods for microbial pathogen detection

		Starting volume	Detection limit*	Time	Pros	Cons
Direct counts	Optical microscope	0.5 mL	10^3~10^4/mL	15 min	Fast, cheap	Cannot detect viruses
	Epifluorescent microscope	0.5 mL	10^3~10^4/mL	45 min	Fast, cheap	Unspecific
	Flow cytometry	0.5~2 mL	10^2~10^4/mL	20 min	Fast, semi-automatic	Unspecific
Genetic based	qPCR	200 μL	1 copy/μL	3~5 h	Specific, fast, sensitive	Rely on standard curve
	ddPCR	200 μL	1 copy/μL	3~5 h	Specific, fast, sensitive	Expensive
	Next generation sequencing	200 μL	Qualitative	1~10 days	Detect unknown species	Complicated
	FISH	< 1mL	Semi-quantification	10~30 min	*In-situ*, fast, specific	Semi-quantification
	Microarray	< 1mL	unknown	5~30 min	Fast, specific, high throughput	Low sensitivity

* Theoretical detection limit

Over the last two decades, the whole areas of environmental microbiology has been going through a fundamentally change due to the emergency of a vast variety of non-culture based techniques. Some of the techniques used for detecting microorganisms in environmental waters are summarized in Table 1-3 with the special focus on techniques employed in our later studies. Direct counts differ from culturable counts in that the cell/viral particle counts are the real numbers of the target microorganisms rather than that estimated from the growth results based on dilution-to-distinction principals. Protozoan and bacterial cells can be easily viewed with optical microscopes and the concentrations are determined by a counting chamber (e.g., hemocytometer, Petroff-Hausser). Viral particles can be viewed with electron microscopes, however, the sample preparation procedures are often complicated and time consuming. The invention of new high-efficiency nucleic acid fluorescent dyes (e.g., SYBR-Green, SYBR-Gold) has facilitate the detection of viruses in environmental waters. Water sample is filtered through a membrane, by which all the viral particles are attenuated on the membrane. The viral particles are then stained with fluorescent dyes and counted with epifluorescence microscope manually or automatically using image processing software (Chen et al., 2001). Flow cytometry is another method for directly detecting and enumerating microorganisms in environmental waters (Wang et al., 2010). It's not a new technology, but its application in environmental studies has been booming in recent years thanks to the improvement on detection limit and the reduced costs. Flow cytometry exhibits some unique technical advantages compared to traditional microscope-based counting methods. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to

thousands of particles per second. Some flow cytometers with sorting capacity can separate particles of interest from the sample for further analysis (Vesey et al., 1994). Combined with commercially available LIVE/DEAD cell staining kits, flow cytometry become very useful tool for quick assessing the bacterial viability in environmental water samples, although the interpretation of results needs to be cautious as the as LIVE/DEAD staining does not necessarily give a clear-cut between live and dead cells based on the FCM files (Berney et al., 2007).

The second group of methods detect the target microorganisms by analyzing their genetic information, most of which are based on amplifying the target microorganism's DNA or RNA by the PCR technique. The advent of PCR based methods have resulted in tremendous paradigm shifts in microbial water quality monitoring. The pathogen detection time has been shorten to several hours instead of days to weeks as traditional cultured-based methods. These tools also provide unparalleled sensitivity and accuracy. PCR assays have been developed and optimized for many of the known waterborne disease causing agents. Commercialized testing kits have simplified the assays in terms of primers design and DNA/RNA amplifying conditions. The evolution of PCR from conventional PCR to quantitative PCR (qPCR), and to the very recent digital PCR, has simplified the assay on pathogen quantification. It is very likely that these methods will be employed as standard methods in water quality guidelines and regulations in the near future. Unlike PCR methods, where the targets are limited to selected known pathogens. The next generation sequencing techniques (e.g., 454 pyrosequencing, Miseq, Iontorrent) with high-throughput

sequencing ability can detect novel pathogens and study microorganisms in community level. These methods have been employed for screening pathogens in environmental water samples and investigating their fates during different water treatment processes (Kumaraswamy et al., 2014). Although getting the genetic information from the samples becomes fasters and cheaper, analyzing the massive data from the sequencing results is challenge, especially for viral pathogens. In a study on viral community in reclaimed water, Rosario et al., (2009) found most of the viruses detected in reclaimed water are novel viruses without matching sequences in the virus database. Bacteriophages dominated the DNA viral community, while none of the established human pathogens (e.g. enteroviruses, hepatitis viruses and caliciviruses) were detected. In application such as direct potable reuse, where rapid identification of multiple pathogens are of great importance to ensure the water safety, microarrays, which based on the hybridization of DNA/RNA probes with the pathogenic targets, have showed great potential in simultaneously detecting multiple pathogens. However, currently, microarrays are still at research stage. The improvement on the detection limit, probably through the combination with water sample concentration and purification techniques, would accelerate the commercialization of this technique.

1.4 Fit for purpose

Although the advances of water treatment technologies have provided options for treating wastewater to the quality required for any intended uses, selecting the appropriate treatment technology for the right level of treatment can be a complex decision, where both insufficient treatment and over treatment should be avoided. Due to the financial

constraints, the reuse of raw or partially treated wastewater is still prevalent in the developing world. In these countries, cost-effective, low energy demanding decentralized treatment systems are more practical to minimize the public health risk associated with water reuse. In contrast, in many highly developed countries, the selection of a higher or more costly level of treatment is often preferred by decision makers, planners and external support agencies. Therefore, the concept of "Fit for Purpose" (Figure 1-2) is highlighted in 2012 U.S. Guidelines for Water Reuse to emphasize the efficiencies realized by designing reuse for specific end applications (EPA 2012). Schimmoller et al., (2015) proposed the use of triple bottom line (TBL) framework (financial, environmental, and social components) to guide the selection of treatment process. By taking factors such as regulations, water quality of the wastewater effluent, water quality goals, end uses of the treated water, and public influence, the designed treatment process can be matched with the intended use without expending unnecessary funds or energy or emitting excess greenhouse gas (GHG) and other air emissions, meanwhile, minimizing other environmental and social costs.

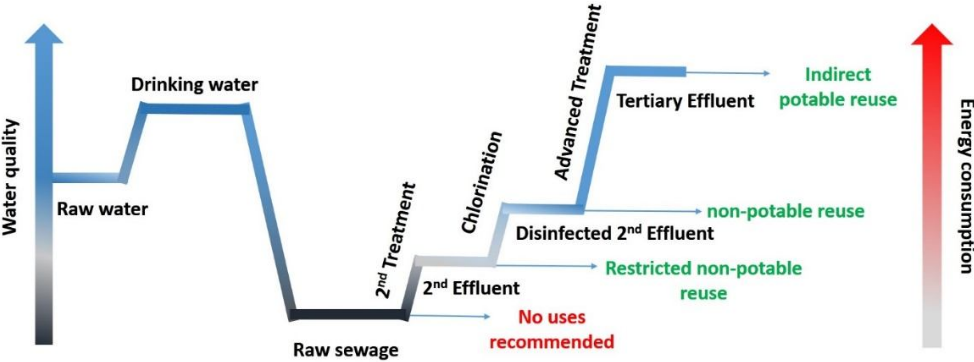

Figure 1-2 Fit for Purpose - Treat the wastewater for potential uses (adapted from 2012 U.S. EPA Guidelines for Water Reuse).

1.4.1 Conventional wastewater treatment

Table 1-4 Pathogen removal rate in conventional wastewater treatment plant

		Inflow	2nd efflunet	Log reduction	Testing method	References
Viruses	Enteroviruses	4.44±0.76	3.13 ± 0.57	0~2.94	MPN-RT-PCR	Ottoson et al., 2006
(log_{10}#/L)		5~6	0~4	1~5	qPCR	Kitajima et al., 2014b
		0.7~3.52	0.7~2.15	0~1	Plaque assay	Hewitt et al., 2011
		2.84~6.67	1.54 ~ 5.28		qPCR	
	Norovirus	3.29±0.26	N.A.*	0.39~1.3	MPN-RT-PCR	Ottoson et al., 2006
		3~6	0~3	1~5	qPCR	Kitajima et al., 2014b
		5~6	4.5~5.5	0.7~1.8	qPCR	Nordgren et al., 2009
	Adenovirus	5~6	4~5	1~2	qPCR	Kitajima et al., 2014b
		1~4.08	0.7~3.26	0~1	Plaque assay	Hewitt et al., 2011
		3.25~8.62	2.97~6.95	0~1	qPCR	
	Hepatitis A	0~6.5	0	4~6	qPCR	Prado et al., 2012
	F-specific phages	N.A.	N.A.	0.74~2.63	ISO method 10705-2	Ottoson et al., 2006
	Coliphages	N.A.	N.A.	0.61~1.86	ISO method 10705-1	Ottoson et al., 2006
		7~8	1~2	6~7	Double layer	Tanji et al., 2002
	Sapovirus	3~7	0~5	1~5	qPCR	Kitajima et al., 2014b
Bacteria	*E. coli*	7.18~7.28	3.2~3.76	3~4	qPCR	Shannon et al., 2007
(log_{10}#/100mL)		N.A.	N.A.	1.2~3.5	IDEXX	Ottoson et al., 2006
	E. faecalis	4.66~4.85	0~1.42	3~4	qPCR	Shannon et al., 2007
		N.A.	N.A.	0.9~2.8	IDEXX	Ottoson et al., 2006
	C. perfringens	5.85~6.09	2.7	3~4	qPCR	Shannon et al., 2007
		N.A.	N.A.	0.62~2.33	Pour plate	Ottoson et al., 2006
	K. pneumoniae	6.31~6.56	2.08~2.23	3~4	qPCR	Shannon et al., 2007
Protozoa	*Cryptosporidium*	0.7~2.5	0.3~2.6	0~1	EPA Method 1623	Castro-Hermida et al., 2008
(log_{10}#/L)		1.3±0.32	N.A.	0.32~0.85	EPA Method 1623	Ottoson et al., 2006
		1.7~2.5	0~1.2	1~2	EPA Method 1623	Montemayor et al., 2005
		1.6~2.9	0~1.6	0.5 ~1	Immunofluorescent	Kitajima et al., 2014a
	Giardia	1.9~3.8	1.9~3.4	0~1	EPA Method 1623	Castro-Hermida et al., 2008
		3.3±0.46		1.6~3.5	EPA Method 1623	Ottoson et al., 2006
		3.3~4.6	2.0~3.0	0.9~1	EPA Method 1623	Cacciò et al., 2003
		3.6~5.6	3.0~3.3	1~2	EPA Method 1623	Neto et al., 2006
		3.4~4.2	0.6~2.17	0.9~2.4	Immunofluorescent	Kitajima et al., 2014a

*N.A., not available

In urban areas, conventional centralized wastewater treatment plants are designed and operated to handle large volumes of wastewater. Although the main goal of primary and secondary treatment is to remove suspended solids and dissolved organic matter (measured as biological oxygen demand, BOD), microbial pathogens can be simultaneously reduced during these processes. Table 1-4 shows the virus, bacteria and protozoa removal rates in traditional wastewater treatment plants before the disinfection process. A large variance in terms of the log removal values of pathogens or indicators are found across the plants, which is likely due to the different treatment technologies used, plant capacity, and environmental conditions. However, the influence by detection methods should not be underestimated, especially for viruses counted by plaque assays. These culture-based methods are generally not comparable as different cell lines (virus hosts) can lead to a 50x difference in numbers of virus detected. Understanding these uncertainties is important to choose the right treatment process to match the potential uses. As a large amount of microbial pathogens may still be present in the non-disinfected secondary effluent (Table 1-4), reuse of secondary effluent should be restricted in order to minimize direct human contact. For example, in California, according to Title 22 regulation, non-disinfected secondary effluent can only be used for irrigation of non-edible plants. For other non-potable reuse applications (e.g., landscape impoundment, golf courses irrigation) disinfection process has to be added to reduce the fecal coliform bacteria levels. Depending on the reuse practices, either 23 MPN/100 mL or 2.2 MPN/100 mL (seven-day median) standard will be enforced. However, the rationale of the standard is still in debate as

farmers can legally irrigate with surface water from free-flowing rivers and lakes, which often have fecal coliform bacteria levels of over 1,000/100 ml (Mara and Horan 2003).

1.4.2 Advanced wastewater treatment

Advanced wastewater treatment can be defined as any process designed to produce an effluent of higher quality than normally achieved by secondary treatment process or containing unit operations not normally found in Secondary Treatment. These processes are commonly found in treatment plants where higher effluent quality is required. The goals of advanced treatment include: 1) additional organic and suspended solids removal; 2) nutrient removal; 3) microbial pathogens and micropollutants removal. The mechanisms of microbial pathogens removal can be classified as physical removal and disinfection. Physical removal refers that the pathogens are taken out the water through filtration or sedimentation process, while disinfection means the pathogens are killed or lose their infectivity by certain treatment process. For indirect and direct potable reuse, where high quality reclaimed water is desired, the water may go through both disinfection (e.g., chlorination, UV, ozonation) and filtration (e.g., granular activated carbon (GAC), microfiltration, ultrafiltration, RO) processes to ensure the water safety. These engineered systems have been well proven in removing all spectrum of microbial pathogens. The current challenge, however, lies in how to monitor these systems in a real-time manner, and thus quick remediation procedure can be adopted when unexpected system malfunction happens (Rodriguez et al., 2009). The high efficiency of pathogen removal capacity of advanced wastewater treatment process also come with high capital and

operational costs. Besides financial costs, environmental costs, public acceptance and regulatory pressure may also need to be considered to evaluate the designed reuse applications. For example, RO based treatment system is the regulatory mandate for potable reuse projects in California, by which extremely low total organic carbon standard (< 0.5 mg/L) can be achieved. However, in some inland locations, where the disposal of RO concentrate via ocean or sewer system is not available, the concentrate management costs can be prohibitively high. Therefore, in places like Georgia and Virginia, GAC based treatment systems are employed for potable reuse projects (Schimmoller et al., 2015). Although no scientific evidence has shown GAC based system would increase the actual public health risk compared to RO based system, a higher perceived risk to the former may exist showing the importance in public communication and education.

1.4.3 Low energy options

Although treating wastewater biologically at a secondary level has become a minimum requirement in developed countries, similar goal is not achievable in the developing world due to the financial and technological barriers. Hence, treatment processes, such as wastewater stabilization pond, constructed wetlands, and biofilters, are more practical alternatives as they require much less energy and maintenance. These low energy options (LEOs) are efficient at removing all kinds of pathogens without the addition of chemicals. For example, constructed wetlands can remove 1–3 $\log_{10}$ of viruses, 2–6 $\log_{10}$ of bacterial pathogens, and a 1–2 $\log_{10}$ of protozoan (oo)cysts (Davies and Bavor 2000, Karim et al., 2004, Thurston et al., 2001). Because LEOs normally rely on natural factors such as

biodegradation, sunlight, temperature, sedimentation, predation, and adsorption to treat wastewater, a large variation in terms of effluent quality is expected. However, the treated water is still much safer than raw sewage for many reused applications (e.g., irrigation). LEOs also gain attention for stormwater treatment in many developed countries (e.g., Australia and the United States), where stringent standards have been set for the stormwater in order to protect the receiving water bodies as well as the needs for stormwater reuse (Table 1-5). According to the International Stormwater BMP Database (ISBD) 2014, the average removal rate of fecal coliform, *E. coli*, and *Enterococcus* in the five wetlands investigated were 91%, 53%, and 61% (Table 1-5). Similar or slightly higher rates of removal are also reported in two Australian studies (Table 1-5). However, based on the current data, none of the wetland effluent can meet the primary human water contact bacteria criteria (30-day geometric mean for *E. coli* < 126 CFU/100mL, *Enterococcus* <35 CFU/100mL), which indicates potential health risk could be associated with the wetland-treated water. Many of current studies are monitoring fecal indicator bacteria (FIB). However, studies have shown that the behavior of microbial pathogens in environmental waters can be significantly different from FIB (Harwood et al., 2005, Wu et al., 2011). So far, there is no reported study on specific pathogen removal in the stormwater wetland, which is likely due to the lack of tools for detecting low concentration of human microbial pathogens in stormwater. Compared to wetlands, biofilters generally provide better microbial removal efficiencies with smaller footprints (Table 1-5). Studies showed that plants play a crucial role in the removal of microbial pathogens. Dry and wet weather conditions also significantly influence the performance of biofilters, and thus a submerged

zone is suggested in the new design of biofilters to alleviate the adverse impact during extended dry weather condition (Li et al., 2012). Stormwater treated by LEOs has been used for many non-potable applications in Australia (e.g., irrigation, toilet flushing and firefighting), while there is currently no national stormwater reuse guidelines in the United States. The situation may change soon as many states have been carrying out related studies on the feasibility of stormwater harvesting and reuse. For example, the District of Columbia is currently developing rules and water quality requirements for stormwater reuse. Projects are currently proved on a case-by-case basis.

Table 1-5 Removal rate of microbial pathogens and indicators by low energy stormwater treatment systems (adapted from Jiang et. al., 2015)

Treatment		**Removal efficiencies**	**Location**	**References**
Wetland	*E.coli*	33%~96%	U.S.	Hathaway et al., 2009
		53%	U.S.	Leisenring et al., 2014
	Enterococcus	1 log	Australia	Davis et al., 2000
		61%	U.S.	Leisenring et al., 2014
	Fecal coliform	56~98%	U.S.	Hathaway et al., 2009
		91%	U.S.	Leisenring et al., 2014
		0~2 Log	Australia	Bavor et al., 2001
Wet pond	*E.coli*	46%	U.S.	Hathaway et al., 2012
		54~99.8%	Lab scale	Rusciano et al., 2007
	Fecal coliform	84%	U.S.	Leisenring et al., 2014
		7%	U.S.	Hathaway, et al., 2011
		0~0.5 Log	Australia	Bavor et al., 2001
Biofilter	*E. coli*	79-93%	Australia	Chandrasena et al.,2012
		3 log	Israel	Zinger et al., 2011
		1~2 log	Lab scale	Li et al., 2012
		97%	Lab scale	Bratieres et al., 2008
	Enterococcus	79-92%	Australia	Chandrasena et al.,2012
	Fecal coliform	2 log	Israel	Zinger et al., 2011
	C. perfringens	>97%	Australia	Chandrasena et al.,2012
	F-RNA coliphage	1~5 log	Lab scale	Li et al., 2012
		82%	Lab scale	Bratieres et al., 2008
		1~3 log	Australia	Chandrasena et al.,2012
	Adenovirus	< 1 log	Australia	Chandrasena et al.,2012

In many developing countries, the need for affordable community scale or household wastewater treatment technologies keeps increasing with the population growth. Treating wastewater with solar energy has been proposed in these areas. The simplest solar water disinfection (SODIS) method only needs to fill the water to a transparent plastic or glass containers, which are then exposed to the sun for 6 to 48 h (McGuigan et al., 2012). The microbial pathogens are disinfected based on the combined effect of thermal heating of sunlight and UV radiation. Solar-powered electrochemical (EC) treatment is also gained attention for decentralized wastewater disinfection. A variety of oxidants (e.g., chlorine, hydroxyl radicals) generated during the water electrolysis are responsible for the disinfection of pathogens. Because of the high pathogen disinfection efficiencies, the EC treated water is almost free of live pathogens, and thus can be reuse for many purposes.

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CHAPTER 2 EVALUATION OF METHODS FOR REVERSE OSMOISIS MEMBERANE INTEGRITY MONITORING FOR WASTEWATER REUSE

Abstract

Wastewater reclamation with advanced membrane technology holds great potential to supplement the diminishing drinking water supply for human consumption. High-pressure reverse osmosis (RO) membrane processes offer a high level of pathogen removal capacity. However, the lack of recognized membrane integrity monitoring methods has restricted the pathogen removal credits allocation to the processes. This research investigated the feasibility of using flow cytometry (FCM), dynamic light scattering (DLS) analyzer and traditional water quality analyzers for RO membrane integrity monitoring. BD Accuri™ C6 flow cytometer demonstrated good sensitivity and reproducibility for quantifying virus reduction rate along the treatment processes, which provide direct evidence for RO membrane integrity monitoring. DLS (Nanotrac Ultra) showed promise to be used as a qualitative membrane integrity monitoring tool by characterizing particle size distributions in water. Traditional water quality analyzers were tested online in a pilot RO system with intentional introduced integrity breaches. Total organic carbon (TOC) measurements showed the best sensitivity to reflect different levels of integrity breaches. Feasibility analysis based on the instrument sensitivity, capital, maintenance and operating costs shows that an integrated system including more than one monitoring tools would be more reliable and economical for high-pressure membrane integrity monitoring.

2.1 Introduction

The intensification of potable water scarcity has become a global issue affecting the economic and social development in many countries. With climate change, population growth and increasing water demand, the problem cannot be solved without the exploration of new water supplies. Municipal wastewater is one of the "new" water resources which has not been fully tapped. In the United States, only 7-8% of municipal wastewater effluent is currently being reclaimed and mainly for non-potable applications (Matthews and Prieto 2011). Large scale indirect potable reuse projects are still restricted in a few states (e.g., California, Florida and Texas) even though the situation is likely to be changed very soon (NRC 2012). The primary concern associated with wastewater reclamation is the public health risk caused by the potential exposure to microbial contaminants, such as pathogenic protozoa, bacteria and viruses. Currently, reverse osmosis (RO) membrane is the most widely used technology for high quality reclaimed water production. Microfiltration (MF) or ultrafiltration (UF) is usually coupled with RO to mitigate the fouling problem of RO membranes (Drewes et al., 2003, Tam et al., 2007). Low-pressure membrane systems (MF, UF) can remove most of the protozoa and bacteria, but they have limited virus removal capacity. High-pressure RO systems, in theory, completely remove all microorganisms including viruses, as RO systems are designed for nanosized particles and ion removal. The combination of RO with MF or UF offers a high level of pathogen removal capacity, which can produce reclaimed water meeting drinking water standards. However, pathogens may pass through the membrane barriers when the system

integrity is compromised. For example, oxidation damage to the active layer of RO

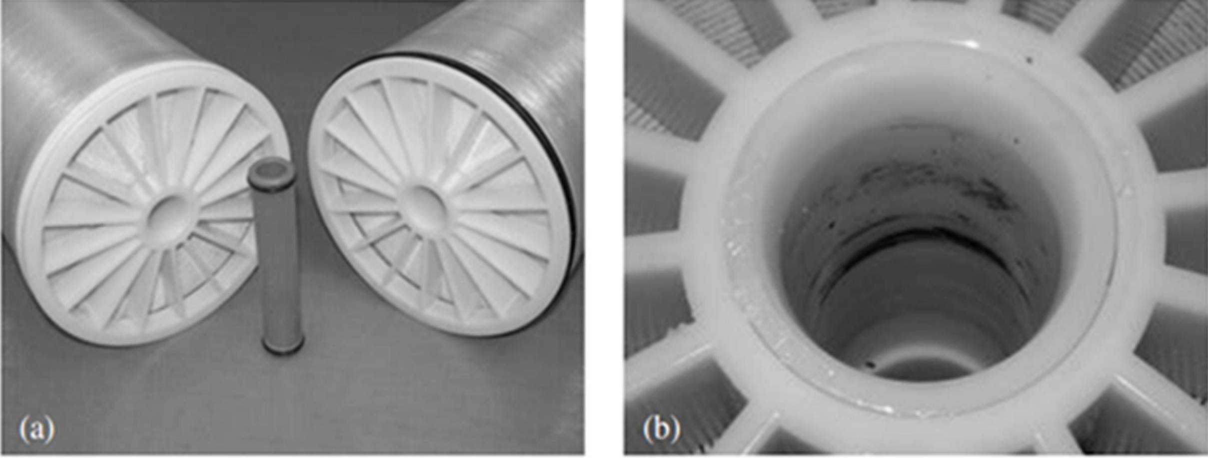

Figure 2-1 Damages which may result in RO integrity loss: (a) standard sliding coupler used to connect the permeate tubes of adjacent elements; (b) residue from O-ring abrasion. Modified from Johnson and Busch (2010).

membranes can happen when the feed water contains chlorine residual or other oxidants (Baker and Dudley 1998). The aging of O-rings used to isolate the feed and permeate streams on the product core tube could also result in the loss of system integrity (Figure 2-1). Due to the lack of means to accurately monitor RO membrane integrity, most states currently do not allocate any specific pathogen removal credits to RO processes (Allgeier et al., 2005). For example, the California Department of Public Health (CDPH) requires 12-$\log_{10}$ removal of viruses from raw wastewater to final effluent for indirect potable reuse. Although 6-$\log_{10}$ virus removal credits were allocated to traditional wastewater treatment processes with disinfection treatments, no removal credit was given to RO processes. The currently approved California potable reuse projects were required to store the highly

purified RO permeate in an environmental buffer system (i.e., groundwater basin) for at least 6 months to acquire additional 6-log_{10} virus decay credits (one month per log) before it can be used as drinking water sources. The rationality of this regulation is currently in debate (NRC 2012). A monitoring system that sensitively detects RO membrane integrity failures will install confidence among regulatory agencies for virus removal credits allocation.

Due to their small sizes (20~220 nm), low infection dose and high resistance to some commonly used disinfection processes, viruses in wastewater are the primary microbiological regulatory targets for high-pressure RO systems (Asano and Cotruvo 2004, Toze 2006). The ultimate goal of RO membrane integrity monitoring is to ensure no or only acceptable level of viruses can pass through the membrane barriers. Membrane integrity monitoring techniques are usually classified as direct or indirect methods. Direct methods refer to tests that are applied to the membrane or the membrane module, such as pressure/vacuum hold, diffusive air flow and bubble point test. These methods are mainly employed by membrane manufacturers before membrane installation, as they can only be conducted off-line. In contrast, indirect methods are based on the feed and permeate water quality. A baseline is first established with intact membranes for certain constituents in permeate. The membrane integrity problems are reflected by the deviations from the established baseline. TOC, turbidity and electrical conductivity (EC) are indirect methods based on constituents naturally present in the feed stream. But their sensitivity has been questioned due to the high purity of the RO permeate (Kumar et al., 2007, Lozier 2003).

Recently, fluorescence excitation-emission matrix (EEM) spectroscopy was proposed for RO membrane integrity monitoring by analyzing the dissolved organic matter (DOM) in RO feed and permeate (Singh et al., 2012). Although EEM provides a wealth of information about DOM, identifying fluorescent signatures for calculating DOM rejection rate can be difficult due to the stochastic nature of DOM in wastewater(Pype et al., 2013). To improve the sensitivity and specificity, seeding studies by adding bacteriophages and artificial fluorescent dyes (e.g., Rhodamine-WT) to the feed water are still used occasionally (Adham et al., 1998). Considering the advantages and disadvantages of currently available methods, an ideal RO membrane integrity monitoring method should include the following features: 1) targeting viruses naturally present in the feed water; 2) sensitive enough to demonstrate the required removal rate of viruses; 3) online capability to provide real-time or near real-time water quality information of the permeate.

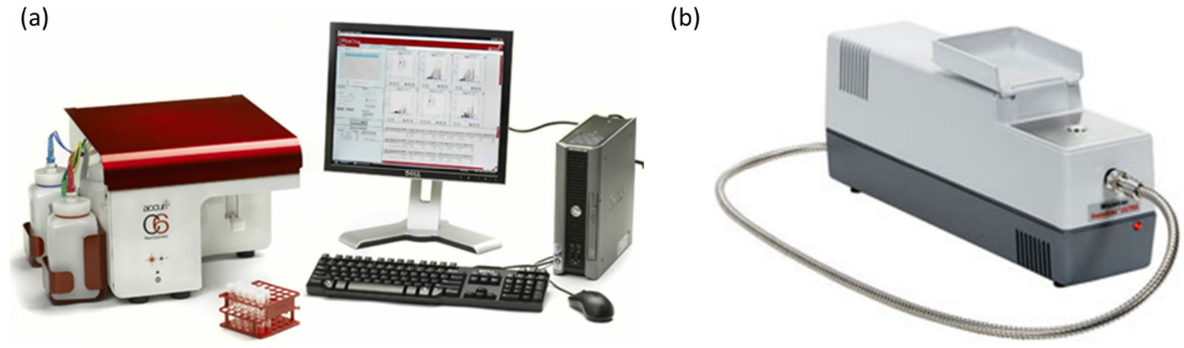

Figure 2-2 New devices proposed to use for RO integrity monitoring: (a) BD Accuri™ C6 bench scale flow cytometer; (b) Nanotrac Ultra dynamic light scattering system with an external probe.

In this study, flow cytometry (FCM) was first time tested as a RO membrane integrity monitoring tool to detect total virus particles including bacteriophages in treated wastewater (Figure 2-2a). FCM is an accurate and fast method for analyzing biological particles in suspension. It has been employed for enumeration of total bacteria in drinking water (Wang et al., 2010). The viability of bacteria can also be assessed by staining the samples with different fluorescent dyes (Ramseier et al., 2011). Using FCM for virus detection is more challenging due to their small sizes. Marie et al., (1999)first reported the enumeration of viruses in seawater by staining samples with SYBR-Green I. Compared to seawater, wastewater contains more fluorescent interference substances, such as organic matter, heavy metals and even some autofluorescent particles. Using FCM for direct virus detection in reclaimed water needs further investigation. A new nanoparticle analyzer, dynamic light scattering (DLS) analyzer, was also used in this study to detect viral sized particles in treated wastewater (Figure 2-2b). Unlike FCM, DLS targets all submicron particles in suspension including inorganic particles. Thus, it can be used as a surrogate method for virus detection. DLS is based on the theory that particles in suspension are under Brownian motion and the speed of the particles is in reverse proportion to their sizes. When moving particles are illuminated with a laser (photons), the scattered light fluctuating rates will be recorded and the particle sizes are determined by using the Stokes-Einstein equation (Brar and Verma 2011). As both FCM and DLS are not able to process continuous water samples at the current stage, grab samples from a wastewater reclamation plant were used during the study. Considering this limitation, new models of water quality analyzers (TDS, turbidity and TOC) with improved sensitivity and online

capability were also tested in a pilot RO system set up in the same plant. A feasibility analysis was carried out at the end of the study based on the instrument sensitivity, capital, maintenance, and operational costs.

2.2 Materials and methods

2.2.1 Description of the water reclamation plant

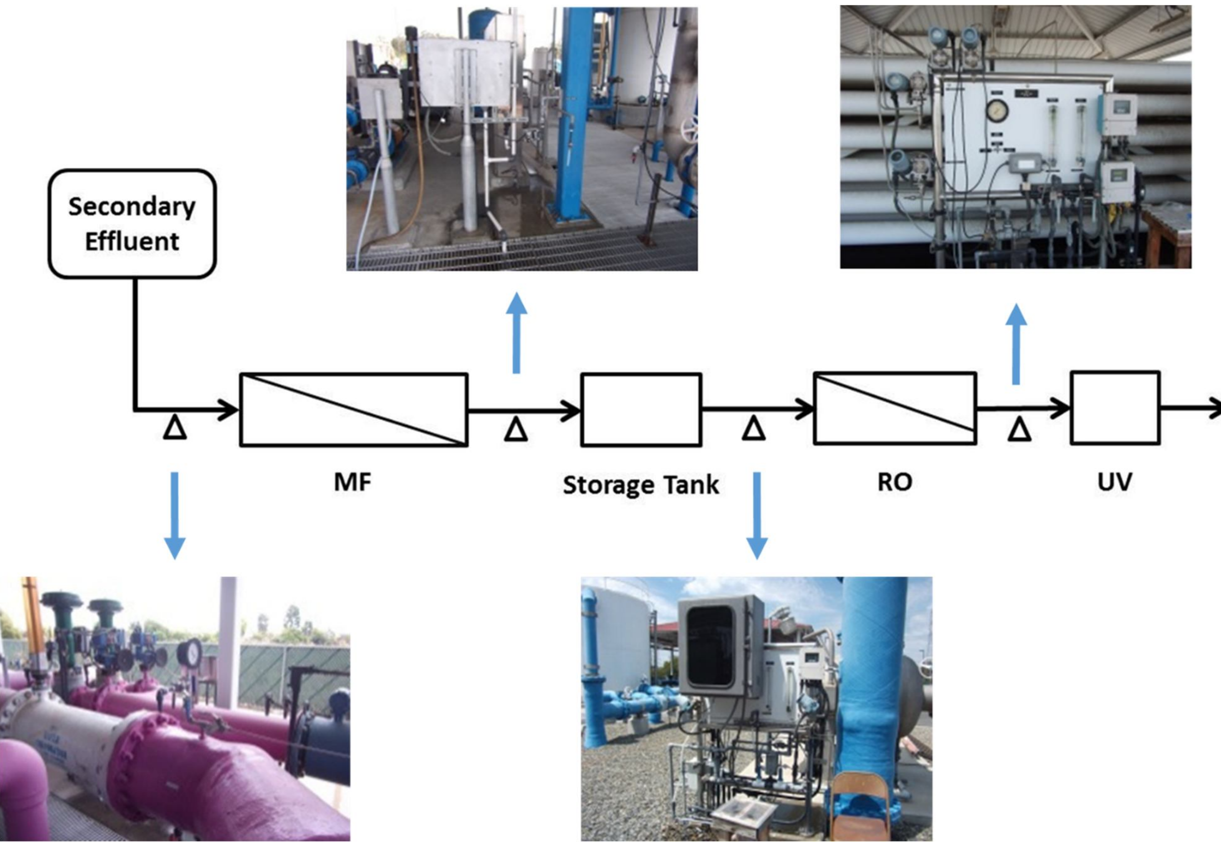

Figure 2-3 Sampling locations at the Leo J. Vander Lans Advanced Water Treatment Facility in Long Beach, California (triangles indicate sampling points).

Water samples were collected from the Leo J. Vander Lans Advanced Water Treatment Facility of Water Replenishment District (WRD) of Southern California. Sampling points (Figure 2-3, indicated by triangles) were selected based on the treatment processes of the plant, which include MF inflow, MF outflow, RO feed and RO permeate. After purging the sampling valve by running the water for 5 min, 1 liter of water was collected in 1.5 liter sterile Whirl-Pak sampling bag and stored in an ice box during transportation to the lab. Fresh samples were tested by FCM and DLS within 4 h after sample collection.

2.2.2 Optimization of direct virus enumeration by FCM

The BD Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) was used in this study for direct virus enumeration(Figure 2-2a). The sample pre-treatment method of Brussaard (2004) was used as "the reference protocol" for trouble shooting issues associated with the testing of reclaimed water. Briefly, 1 mL water samples were fixed with glutaraldehyde (0.5% final concentration); the samples were then flash frozen with liquid nitrogen and stored at -80°C; samples were thawed at 35°C and diluted with TE-buffer (10 mM Tris-HCl,1 mM EDTA; pH 8.0) before testing; the staining was carried out in the dark at 80°C water bath with 0.5X SYBR Green I (final concentration) for 10 min. This initial protocol resulted in high background noise, which could not be clearly separated from the virus signal. To determine the best sample pre-treatment procedures, a MF inflow sample from WRD was tested under different sample fixation, staining and dilution conditions (Table 2-1). The same sample was also filtered with 30 Kilodalton (KDa) molecular mass

cut-off Amicon Ultra-4 Centrifugal Filters (Millipore, Bedford, MA) to remove all the viruses and used as a

Table 2-1 Optimization of sample pre-treatment protocol for enumeration of virus particles using flow cytometry. A microfiltration inflow sample from WRD was tested under different conditions. Data of relative counts were normalized to the highest count obtained (=1).

	Fixation					Dye						Liquid N2 frozen		Incubation		Diluent		Relative counts
	Glutaraldehyde Concentration					SYBR-Green I			SYBR-Gold									
	0	0.5%	1%	2%	5%	0.5x	1x	2x	0.5x	1x	2x	-	+	RT [a] (15min)	80° C (10min)	TE	MQ [b]	
Best				*					*			*		*		*		1
1	*								*			*		*		*		88.9%
2		*							*			*		*		*		83.1%
3			*						*			*		*		*		87.8%
4					*				*			*		*		*		102.7%
5	*					*						*		*		*		71.6%
6		*				*						*		*		*		71.7%
7			*			*						*		*		*		79.7%
8				*		*						*		*		*		85.9%
9					*	*						*		*		*		83.6%
10	*								*				*	*		*		44.8%
11		*							*				*	*		*		66.4%
12			*						*				*	*		*		80.2%
13				*					*				*	*		*		85.3%
14					*				*				*	*		*		92.9%
15	*					*							*	*		*		19.4%
16		*				*							*	*		*		30.5%
17			*			*							*	*		*		44.3%
18				*		*							*	*		*		42.2%
19					*	*							*	*		*		54.8%
20				*						*		*		*		*		94.9%
21				*							*	*		*		*		82.5%
22				*		*						*		*		*		94.4%
23				*			*					*		*		*		79.5%
24				*				*				*		*		*		69.7%
26				*					*			*			*	*		88.9%
26				*					*				*		*	*		80.6%
27				*					*			*		*			*	21.2%

[a] RT, room temperature. [b] MQ, Milli-q water.

blank to assess the reagent noise as well as determine the detection limit of the method. The BD Accuri C6 flow cytometer threshold was set at 450 nm in fluorescence channel 1 (FL1) to exclude the instrument electronic noise. Green and red fluorescence were

collected in the FL1 channel (533±30 nm) and the FL3 channel (> 670 nm) on a logarithmic scale, respectively. Data analysis was carried out using the BD CFlow® software. The total virus counts were obtained by subtracting the counts in blank from the counts in the sample. To validate the accuracy of virus counts by FCM, pure cultures of bacteriophage T4 (ATCC 11303-B4) and MS2 (ATCC 15597-B1) were seeded in autoclaved 1x phosphate-buffered saline (PBS buffer) as positive control samples. The results from FCM were compared with those obtained by the double agar layer titration method (Clokie 2009) and epifluorescence microscopy (EFM) direct counting method (Noble and Fuhrman 1998). The optimized protocol was then used to monitor the virus removal efficiencies of membrane processes at WRD for a month long period.

2.2.3 Detection of nanosized particles by DLS

Grab samples from WRD were tested with the Nanotrac Ultra (Microtrac, Montogomeryville, PA) DLS system (Figure 2-2b). The instrument theoretically can detect particle size from 0.8 to 6,500 nm and is optimized to detect particles in low concentration suspensions. The manufacturer's testing procedure was followed for the reclaimed water samples. First, the sampling cell was thoroughly flushed with distilled water to remove any residual particles before testing. Analysis time of 90 seconds was used for all the samples and the cleaner samples (with low particle concentration) were always tested first in multiple sample runs. The particle size distributions were displayed based on % volume of total particles. To determine the concentration threshold of detection, RO feed samples from WRD were diluted in distilled water to the final concentrations of 2%, 4% 12% and

20% (volume). The threshold was determined when a signal above the background noise was detected.

2.2.4 RO pilot Study

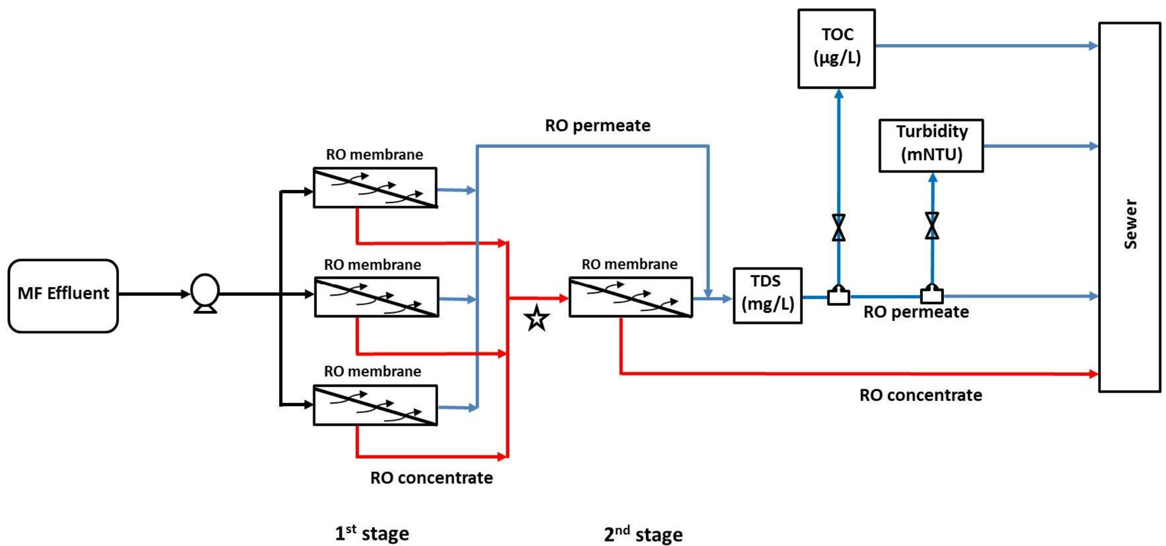

Figure 2-4 RO pilot layout. Star on the graph indicates the location of the compromised O-ring.

A pilot scale RO system was set up at WRD to evaluate the feasibility of using new models of water quality analyzers for online membrane integrity monitoring. The MF outflow of the plant was used as feed water for the system. The pilot system is a two-stage RO system using a 3:1 array (Spiral wound, Polyamide membrane, CSM, Anaheim, CA), where the concentrate from the first stage membranes is treated again by the second stage membrane. The pilot system includes built-in permeate and concentrate flow meters, feed

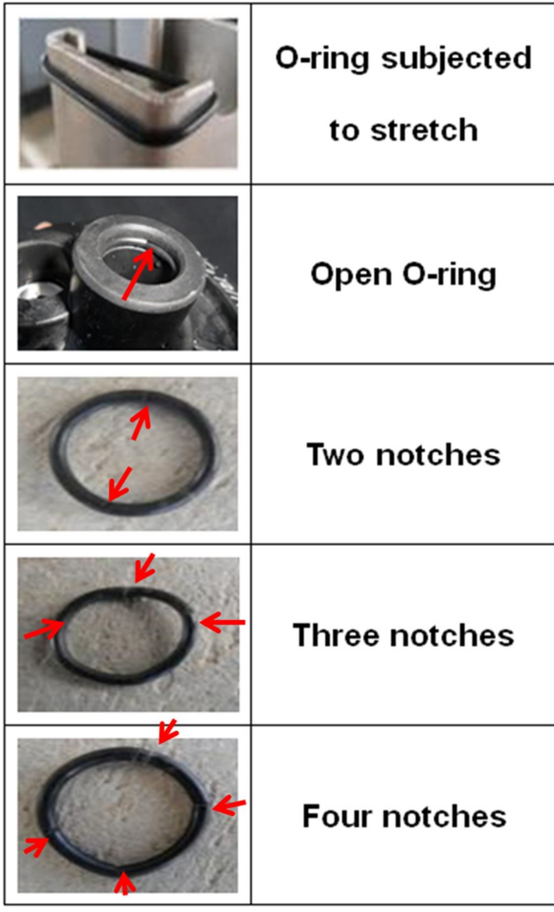

Figure 2-5 Five levels artificial damages to the O-ring used on the high pressure end (feed inlet) of the second pass pressure vessel (stretched, cut off, two notches, three notches and four notches).

and permeate pressure gauges and a conductivity meter, which monitored TDS results continuously with data recorded hourly. Figure 2-4 shows the schematics of the RO pilot layout. Valves were installed at the end of the permeate lines through T-connectors to throttle the flow to the moitoring instruments. A GE Sievers 900 Online TOC Analyzer (GE Analytical Instruments, Boulder, CO) was installed to record the TOC changes of RO

permeate with an interval of every 4 minutes. The operating range was set from 1µg/L to 1000 µg/L. A HACH FilterTrak 660sc (Hach Company, Loveland, CO) was employed to measure the turbidity of the RO permeate flow at an interval of every 5 minutes. The operational range of the analyzer is 0-5000 mNTU with a resolution as low as 0.3 mNTU. After system start up, the RO pilot system was continuously operated and the baseline conditions were established for different monitoring parameters in the first 24 h. The system operating conditions are summarized in Table 2-2. Later, the O-ring on the high pressure end (feed inlet) of the second pass pressure vessel was artificially damaged (Figure 2-4, the star indicates the location of damaged O-ring) to simulate a typical failure of RO systems (Kumar et al., 2007). Five levels of damage were introduced to the O-ring (stretched, cut off, two notches, three notches and four notches). The pilot was run under each testing condition for 24 h. The changes of TDS, turbidity and TOC were recorded continuously and the sensitivity of different monitoring parameters was compared with the

Table 2-2 Summary of pilot RO system operational conditions

Parameters	Values
Pilot Feed Pressure	179 kPa to 207 kPa
Temperature	22 to 29 ^{o}C
Recovery	80%
Permeate Flux	38.7 L/m^2/hr
Permeate Flow Rate	20.8 L/min
Concentrate Flow Rate	5.7 L/min
Inflow TOC	6 ~ 8 mg/L
Inflow Conductivity	800 ~1000 µS/cm
Inflow Turbidity	60 ~ 100 mNTU

baseline. The turbidity data for the last two days could not be retrieved from the turbidity meter due to a malfunction of the HACH controller. To remedy the lost data, turbidity values measured from grab samples were entered. FCM and DLS were not tested during the pilot study due to the unavailability of the equipment at the time.

2.3 Results and discussion

2.3.1 Optimize FCM assay for reclaimed water

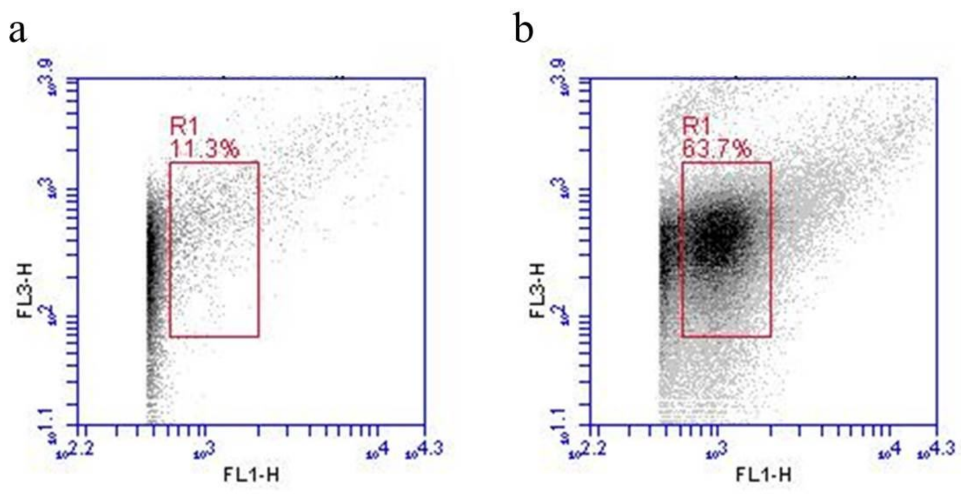

Figure 2-6 Flow cytometric analysis of virus samples using FL1 vs. FL3 density plots. The samples were stained with 0.5X SYBR-gold (final concentration). FL1 is fluorescence channel 1 that captures green fluorescence and FL3 is fluorescence channel 3 that captures red fluorescence. Gate R1 was used to separate viruses from other particles and background noise. (a) 30 KDa membrane filtered microfiltration inflow sample from WRD was used as control to show regent background within the gated region; (b) a

microfiltration inflow sample from WRD demonstrates the separation of virus signal from background noise. The total virus count was obtained by subtracting the counts in the blank (a) from the counts in the sample (b).

Although the sensitivity of FCM has been significantly improved by the utilization of high efficient fluorescent nucleic acid dyes such as SYBR-Green and SYBR-Gold, the detection of viruses is still approaching the detection limit of the instrument (Wang et al., 2010). A total of 28 testing trials were carried out in an attempt to optimize the sample pre-treatment protocol for reclaimed water (Table 2-1). The results showed that samples stained with SYBR-Gold generally yielded higher particle counts than those stained with SYBR-Green I (Table 2-1). This observation is in agreement with the report by Chen et al., (2001), who employed EFM for virus enumeration. Besides staining, the sample fixation procedure also significantly affected the virus counts (Table 2-1). Glutaraldehyde is widely used as a preservative to prevent the degradation of viruses and to improve the incorporation of dye into the viral DNA/RNA (Wen et al., 2004). Based on current results, 2% glutaraldehyde fixation was necessary to separate the virus signal from the background noise. Although 5% glutaraldehyde fixation had slightly higher counts than 2% fixation (Table 2-1), it also resulted in the increase of background noise in certain samples (data not shown). Thus, 2% glutaraldehyde fixation was chosen and used for all samples in the later tests. Freezing samples with liquid nitrogen and heating samples at 80°C for 10 min were expected to improve the combination of dye and viral DNA/RNA(Brussaard 2004). However, both procedures resulted in lower virus counts, and thus are not suggested for reclaimed water samples (Table 2-1). A nearly 80% decrease of virus counts was found in samples diluted

with Milli-q water compared to those diluted in TE buffer, which indicates the importance of maintaining pH between 7.5-8.0 (preferably 8.0) as both SYBR-Green I and SYBR-Gold are pH sensitive (Table 2-1). Among three different dye concentrations tested, 0.5X dye concentration gave the highest counts. Beyond this value, increasing dye concentrations resulted in lower virus counts due to the simultaneous increase in background noise. Side scatter (SSC) *vs.* green fluorescence (FL1) plots were used to separate viruses from background noise in the reference protocol (Brussaard 2004). However, in this study, we found that using green fluorescence (FL1, 533±30 nm) *vs.* red fluorescence (FL3, > 630nm) plots provided better discrimination when samples were stained with SYBR-Gold (Figure 2-6). The emission spectrum of SYBR-Gold is 500-700 nm in comparison with 500-625 nm for SYBR-Green I. This shift towards red fluorescence by SYBR-Gold may explain the better discrimination when using FL1 *vs.* FL3 plot (Hammes et al., 2008). Figure 2-6 shows one of the MF inflow samples tested under the optimized sample pre-treatment protocol. The Virus signal was clearly separated from background noise in the gated R1 region (Figure 2-6b), while the virus-free blank had minimal interference within the same gated area (Figure 2-6a). Based on the background counts (background noise) in R1 region of the virus-free blank (Figure 2-6a), the detection limit of FCM for viruses in reclaim water was determined as ~$6x10^4$ VLPs/mL. A previous study by Tomaru and Nagasaki (Tomaru and Nagasaki 2007) showed that FCM and EFM counts of large DNA algal viruses (~200nm) were similar to each other. However, FCM underestimated smaller DNA and RNA viruses (~40 nm) because the FCM counts were lower than the results from the culture-based titration method (most-probable-number). In the current study, viruses counted by EFM

and FCM were generally comparable and they were both higher than plaque assay counts for double stranded DNA coliphage T4 and single stranded RNA coliphage MS2 (Table 2-3). The standard deviations of FCM counts were always less than 5%. Current results indicate that the BD Auccri C6 can effectively capture DNA (T4), RNA (MS2) viruses as well as viruses naturally presented in reclaimed water.

Table 2-3 Comparison of viral counts by different methods

	Plaque assay (PFU/mL)	Epifluorescence microscopy (VLPs/mL) [a]	Flow cytometry (VLPs/mL)
Phage MS2	$(8.3 \pm 1.3) \times 10^{9}$	$(1.3 \pm 0.3) \times 10^{10}$	$(9.4 \pm 0.06) \times 10^{10}$
Phage T4	$(7.7 \pm 2.6) \times 10^{8}$	$(2.6 \pm 0.1) \times 10^{9}$	$(3.0 \pm 0.02) \times 10^{9}$
MF influent	N.A. [b]	$(7.3 \pm 1.2) \times 10^{7}$	$(1.4 \pm 0.02) \times 10^{8}$
MF effluent	N.A.	$(7.7 \pm 2.5) \times 10^{6}$	$(7.3 \pm 0.06) \times 10^{6}$
RO feed	N.A.	$(1.3 \pm 0.3) \times 10^{6}$	$(6.2 \pm 0.09) \times 10^{6}$
RO permeate	N.A.	B.D. [c]	B.D.
Detection limit	1	1×10^{5}	6×10^{4}

[a] VLPs, virus like particles; [b] N.A., data not available; [c] B.D., below detection limit.

2.3.2 Monitoring virus removal efficiency by membrane processes

A month long monitoring study was carried out to examine the virus removal efficiency by membrane processes using grab samples (40 total) from WRD. Total virus particles in MF inflow ranged between 7 $\times 10^{7}$ to 2$\times 10^{8}$ /ml over the one-month sampling period. The virus counts for MF outflow and the RO feed were nearly identical (Figure 2-7). The MF removed one to two logs of viruses, which agrees with previous studies using other counting methods (Huang et al., 2012). No viruses were detected by FCM in the ten RO permeate samples collected during the testing period (Figure 2-7). The result was also confirmed by

EFM (data not shown). Overall, current results indicate that FCM can reliably quantify virus concentration changes in water reclamation processes. Considering the virus concentration in MF inflow and the detection limit of FCM, at least 3 logs removal of total virus particles for the MF/RO systems can be demonstrated by FCM, although the actual virus removal rate could be higher. To further characterize the removal efficiency of RO membranes, virus concentration methods using nano-fiber filters (Li et al., 2010) or a microfluidic system (Lien et al., 2007) could be employed to increase the detection limit of FCM. Recently, a lab-scale online FCM system has been developed for bacterial detection in drinking water (Hammes et al., 2012). With the flow injection and s Development and laboratory-scale testing of taining units, the sampling and staining steps are fully automated. Similar units may also be connected with the Accuri C6 system to realize online detection of viruses.

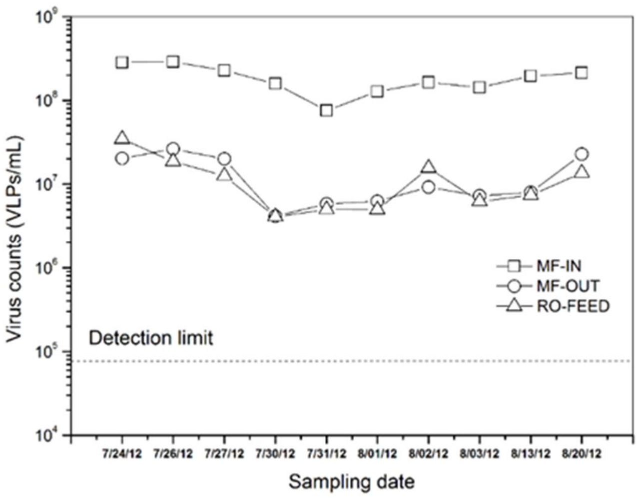

Figure 2-7 Variations in concentration of virus-like particles (VLPs/ml) at different sampling points of WRD. Grab water samples were tested by FCM using optimized protocol.

The standard deviations of FCM counts were always less than 5% and are not plotted on the graph.

2.3.3 Particle size distributions by DLS

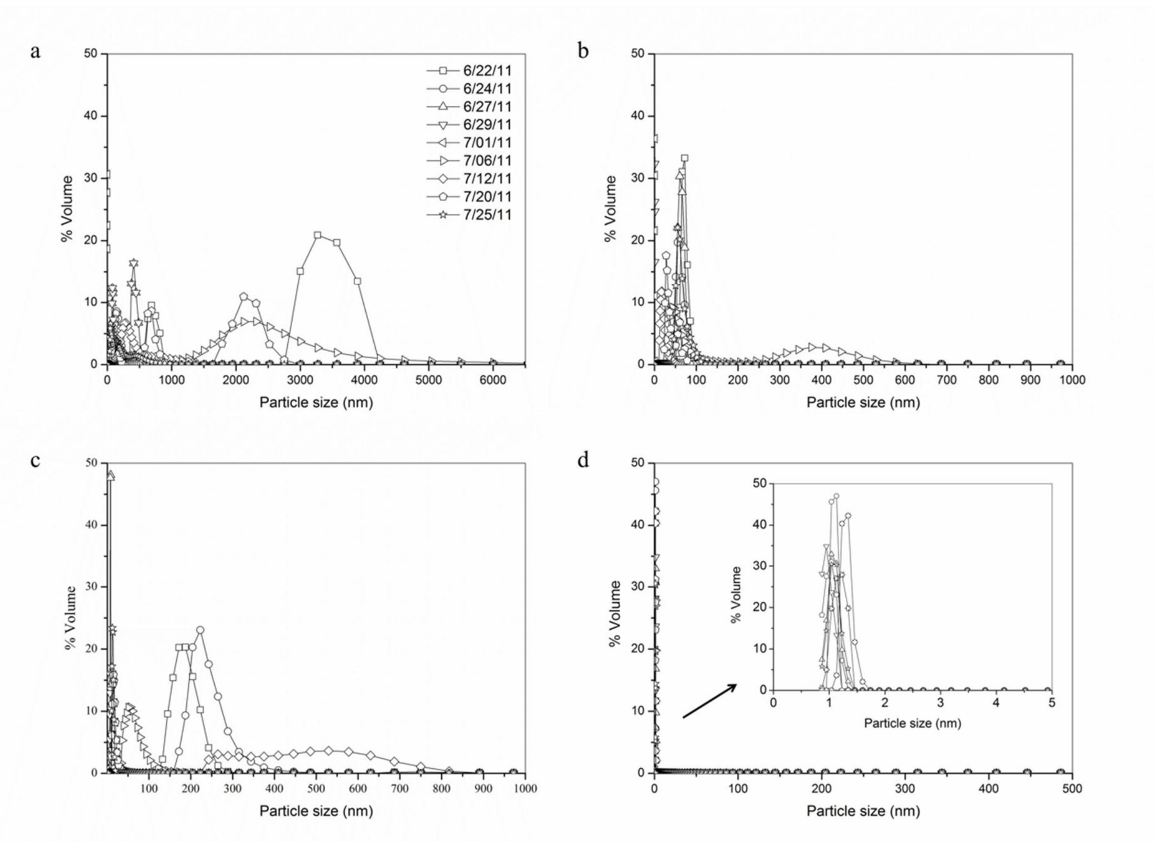

Figure 2-8 Particle size distributions obtained from Nanotrac Ultra for samples from each sampling location: (a) MF inflow; (b) MF outflow; (c) RO feed; (d) RO permeate.

The particle size and particle size distributions (% volume) analyzed by Nanotrac Ultra for samples from WRD are shown in Figure 2-8. MF inflow, which is the secondary effluent from the adjacent wastewater treatment plant, displayed a wide range of particle peaks (Figure 2-8a). The results demonstrate the heterogeneous nature of particles in the

secondary effluent. In a few samples, particles as large as 6,500 nm (the upper detection limit) were detected, which could be unsettled activated sludge flocs. Commercial MF membranes generally have nominal pore size range from 0.1~0.5 μm. Theoretically, particles larger than the pore size (e.g., most bacteria) are removed by MF through sieving. The buildup of a cake layer on the membrane surface during the course of filtration may further improve the removal of particles (USEPA 2001). A well-defined separation for particles larger than 100 nm was observed for MF outflow (Figure 2-8b), which agrees with the size exclusion feature of MF. However, some particles larger than 100 nm were detected in RO feed samples (Figure 2-8c). Considering there is a storage tank between the sampling point of MF outflow and RO feed, those large particles detected in the RO feed may indicate the regrowth of bacteria in the tank. No distinct peaks larger than 2 nm were observed in the RO permeate samples (Figure 2-8d). Similar profiles were observed when testing with the distilled water. As those observed peaks (< 2 nm) were close to the theoretical detection limit (0.8 nm) of the instrument, they could be caused by vibrations or other background interferences. Since the majority of viruses are greater than 20 nm in diameter, setting the size threshold at 10 nm should allow the distinction of viral size particles from the instrument noise. If particles larger than 10 nm are detected in RO permeate, it is likely that the system integrity is compromised.

Besides particle size, the detection limit of DLS is also related to particle concentrations. According to DLS manufacturer's research, the concentration threshold of 30 nm diameter biological particles is about 0.1 ppm (volume) (Plantz 2006). However, the result was

based on single size/property particles. It cannot be simply extrapolated to environmental water samples, which normally contain particles of varied sizes and different properties (organic/inorganic). As a particle's ability to scatter light is proportional to the sixth power of its diameter, the presence of a few large particles in a sample can significantly lower the concentration threshold, thus detection limit of DLS is sample-specific. In current study, when the RO feed sample from the WRD was diluted in distilled water, 12% (volume) of the RO feed sample was required to obtain signal above the background noise. Future development on lowering the concentration threshold of DLS would benefit the application of DLS as a RO integrity monitoring tool.

2.3.4 Online monitoring of membrane integrity

TDS, turbidity and TOC measurements collected from the RO pilot system are presented in Figure 2-9. In the first 24 hours of operation, the turbidity in the permeate line was between 11.93 to 13.09 mNTU, while the TDS was stable at 5 mg/L. The TOC baseline was between 36.3 to 55.4 µg/L, indicating some low molecular weight organics are able to pass through the RO membranes. An intentional system breach by using a stretched O-ring was insufficient to compromise the system integrity (Figure 2-9). However, a more aggressive damage, by cutting the O-ring completely (open O-ring), showed that all three online water quality parameters significantly deviated from the baseline (Figure 2-9). Further experiments with notches cut on the O-ring also resulted in detectable system integrity breaches. Current results showed that the new generations of water quality analyzers are sensitive enough to characterize the ultra-pure RO permeate, but TOC is more sensitive in

showing the subtle water quality changes in the permeate line. The highest TOC level was observed at noon, while the lowest point was at mid-night. The "U" shape curve of each

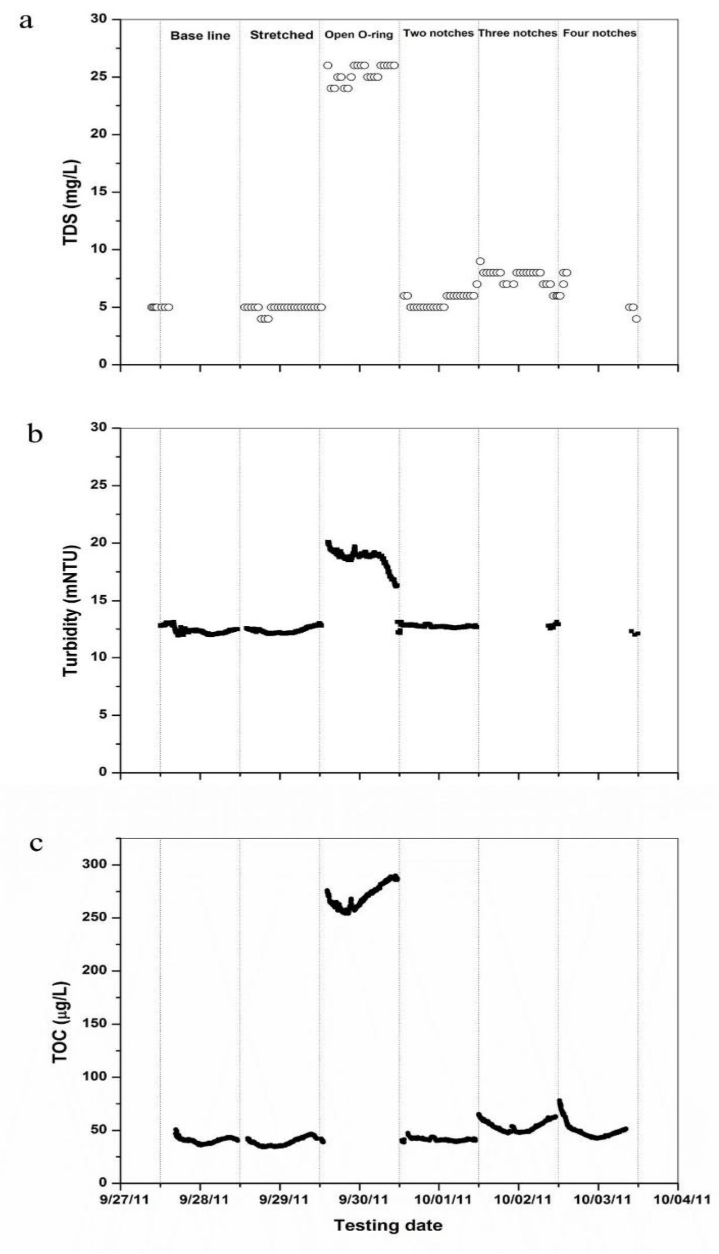

Figure 2-9 Online monitoring of pilot RO membrane integrity using TDS (a), Turbidity (b) and TOC (c).

testing day is believed to follow the daily TOC fluctuations of the feed water (Asano 2007). The temperature variation could be another factor as TOC rejection is higher at lower temperature at night.

In addition to the lower limit of detection, the removal rate of the targeted water quality parameter by intact RO membranes is another important factor that needs to be considered, because the integrity breach is indicated by the deviation from the baseline. In this context, turbidity was not a viable option for integrity monitoring due to the limited cross membrane changes (e.g., less than 30% at WRD). The turbidity increase caused by integrity problems could be masked by the normal water quality fluctuations. The removal rate of TOC and TDS (based on EC) are normally much higher (e.g., 95% and 80% at WRD, respectively), and therefore they are more sensitive to reflect small integrity breaches. It should be mentioned that, in this study, the integrity breaches were only introduced by damaged O-rings, further study may be necessary to determine the instruments' sensitivity to detect other types of integrity loss (e.g., damage to membranes).

2.4 Feasibility Analyses

All instruments tested in the current study were commercially available. Based on the instrument sensitivity and costs, a feasibility analysis for each instrument in terms of full-scale installation is presented in Table 2-4. FCM is the only method in this study which can directly quantify virus particles. The log removal rate of virus particles is the most convincing evidence to demonstrate the membrane integrity and potential virus

breakthrough. However, FCM is also the most expensive method in maintenance and operating costs. DLS has lower maintenance and operating costs but, currently, it can only be used as a qualitative membrane integrity monitoring tool with limited sensitivity. Both FCM and DLS hold the potential to be modified for online monitoring. The new Nanotrac Ultra is already equipped with a sampling probe for automatic sample collection. Together with the minimal needs for sample pretreatment, an on-line DSL system can be easily developed for membrane integrity monitoring. The new generations of traditional water quality analyzers also yield useful information on membrane integrity. Among them, TOC analyzer provides the best sensitivity among the tested online analyzers with a moderate cost. It should be noted, however, TOC is a bulk measurement of organics, the relationship between TOC changes and potential virus breakthrough needs to be further investigated.

Table 2-4 Overall performance and feasibility analysis of instruments for monitoring of membrane integrity

Instrument	Target	Sensitivity	Approx. Capital Cost	Maintenance	Operational Cost	On-line Option
DLS	Nano particles	Moderate	High (~$40K)	Low	Low	Possible
Flow cytometer	Viral particles	High	High (~$40k)	Moderate	Moderate	Possible
TOC analyzer	Organics	High	Mod. (~$22k)	Moderate	Moderate	Yes
Turbidimeter	Water clarity	Low	Low (~$2.5k)	Low	Low	Yes
Conductivity meter (TDS)	Ions	Moderate	Low (<$1k)	Low	Low	Yes

2.5 Conclusions

The results and analyses of this study showed that using more than one monitoring technique is more practical and reliable to ensure the sensitive detection of integrity failure of the RO membrane system. A monitoring system that includes TOC analyzer, DLS, and FCM can provide a feasible way to realize the online and real-time monitoring of RO membrane integrity. For example, when TOC deviates from the baseline for a certain range, DLS and FCM can be employed to determine if there are virus particles leaking into the RO permeate. However, the integration of these methods as a monitoring system would require further optimization.

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CHAPTER 3 INVESTIGATION OF MICROBIAL PATHOGEN REMOVAL AND MICROBIAL COMMUNITY IN CONSTRUCTED STORMWATER WETLANDS

Abstract

Constructed stormwater wetlands (CSW) have been used worldwide for controlling stormwater pollution as well as mitigating the flooding problem in urban area. The microbial pathogen removal rates and the microbial community were investigated in two US and three Australian CSW. The two US CSW (Forge and Old Laguna) and one of the three CSW in Australia (Royal Park) showed high efficiency on indicator bacteria removal. The dry weather outflow from these CSW can meet the primary recreational contact criteria (*E. coli* < 126 cfu/100 mL; *Enterococcus* <35 cfu/100 mL). The removal rate of indicator bacteria was significantly lower under wet weather condition due to the short water residence time. No *Cryptosporidium* were detected in all wetlands by droplet digital PCR, while Adenovirus were ubiquitous in all CSW. Human-specific HF183 *Bacteriodes* were found in all Australian CSW suggesting the potential contamination of sewage ingression. The 454-pyrosequencing results showed that inflow water samples were dominated by *Proteobacteria*. For those CSW with higher indicator bacteria removal rates, an increase of *Cyanobacteria* was observed in the outflow samples. Principal Coordinate Analysis (PCoA) showed clear shift of microbial communities in the inflow and outflow samples from CSW with higher indicator bacteria removal rates, while no such changes were found in CSW

with lower indicator bacteria removal suggesting microbial community can be potentially used as an indicator to monitor CSW performance.

3.1 Introduction

Urbanization inevitably leads to an increase of runoff rates and volumes due to the added impervious surfaces (e.g., roofs, roads and parking lots). Urban stormwater carries a significant amount of chemical and microbial pollutants has been identified as the major non-point source (NPS) pollution impairing the receiving waters bodies. Urbanization also creates a large regional water demand. Many large cities are facing severe water scarcity due to the ever-growing population and extended drought. As traditional water supplies are unlikely to satisfy the increasing demand on high quality water, harvesting and treating stormwater for non-potable uses is considered as a reasonable and imperative choice for sustainable urban water management.

Constructed stormwater wetlands (CSW) have been used worldwide for controlling urban NPS pollution as well as mitigating the flooding issue. CSW combines biological, chemical, and physical mechanisms to treat polluted stormwater when it flows through the system. Many studies have investigated the removal of suspended solids, nutrients, and heavy metals in stormwater by CSW (Birch et al., 2004). The removal or inactivation of microbial pathogens by CSW, however, is still not well understood. Hathaway et al., (2009) compared the indicator bacteria removal efficiency of two CSW in Charlotte, North Carolina. The average removal rates of fecal coliform and *E. coli* by one wetland were about 98% and

96%, while the removal rates were significant lower (56% and 33%, respectively)by the other one with higher vegetation coverage. The authors concluded that the wetland with lower vegetation coverage had a better sunlight exposure, and thus leading to a higher bacterial die-off rates. A similar result was found in a pilot scale wetland study, where the combination of water temperature and light were thought as the governing factors determining the inactivation of indicator bacteria (Struck et al., 2008). However, controversial results were reported in some other studies. Davies and Bavor (2000) indicated that sedimentation was the main mechanism responsible for bacterial removal by CSW. Therefore, extensive vegetation can improve the removal rate as it impeded the water flow and thus enhanced the sedimentation of fine particles (< 2 mm), to which bacteria were predominantly adsorbed. The variability observed in these studies is likely caused by the size of the wetland, the residence time, the quality of influent, and other local conditions.

Indicator bacteria (e.g., fecal coliform, *E. coli* and *Enterococcus*) are widely employed to study the fate of human pathogens in CSW, which bears some inherent limitations. Many studies have shown that microbial pathogens, especially viruses and protozoa, are more resistant to environmental stressors than indicator bacteria (Savichtcheva and Okabe 2006). The removal or inactivation of indictor bacteria does not warrant a same level of microbial pathogen reduction. Consequently, the health risk of microbial infection during the stormwater reuse is likely to be underestimated. In addition, traditional culture-based methods are not able to determine the origins of indicator bacteria. Animal sources (e.g.,

birds, dogs, pigs) can contribute to high levels of indicator bacteria in stormwater. Therefore, high concentrations of indicator bacteria observed in CSW treated stormwater may not necessarily contain pathogens that pose a significant health risk to humans (Pitt et al., 2001, Sauer et al., 2011). These drawbacks underscore the importance of including methods directly targeting microbial pathogens in the study of CSW.

In the last few years, quantitative polymerase chain reaction (qPCR) methods have been increasingly employed to quantify the concentrations of various microbial pathogens in stormwsater. The results indicate sewage ingressions into the urban stormwater runoff are more frequent than expected (Chong et al., 2013, Sauer et al., 2011). Compared to culture-based methods, qPCR provides faster sample-to-result time, higher specificity, and greater flexibility in the type and number of targets that may be detected and quantified (Cao et al., 2015). Despite its popularity, the accuracy of qPCR heavily relies on the quality of standard curves, which may result in approximately half a log difference. qPCR is also susceptible to inhibitors commonly found in environmental water samples (e.g., organic matter, heavy metals). The inhibition problem may offset the effort on concentrating large volumes of stormwater intended to lower the detection, because inhibitors are co-concentrated during the process (Rajal et al., 2007). These limitations are addressed in the new digital PCR technology. In digital PCR, one bulk PCR reaction is partitioned into thousands to millions of sub PCR reactions either by a microfluidic chips or water-in-oil droplets (Hindson et al., 2011). The target concentration is calculated based on the positive sub reactions at the end of the reaction by Poisson statistics. As an end-point approach, digital PCR has shown a

higher tolerance to PCR inhibitors (Hindson et al., 2013). The quantification of target microbial pathogens is also more accurate as no standard curves are needed. As such, direct comparison of results from different studies becomes possible.

CSW in essence is a complex bioreactor (Lee et al., 2009). As one of the most important components, the microbial community interacting with other biological (plants, animals) and abiotic (sediments, water) components mediates the pollution removal processes in CSW. Growing evidences have shown that microbial pathogens in many instances do not live an isolated planktonic life in environmental waters, but are entangled in a 'city of microbes' (Watnick and Kolter 2000). Viewing the microbial pathogens in the community level is therefore crucial to get a better understanding of their fate in CSW. Considering the overwhelming genetic diversity of microbial community in environmental samples, the high-throughput next generation sequencing (NGS) technology is promising as it provides enough sequencing depth to cover the complex microbial communities. 454 pyrosequencing is one of the popular NGS systems, which can generate more than 400,000 effective reads with average read length up to 1000 base pairs with high accuracy rate (> 99.5 %) (Quince et al., 2009). This technology has been applied to characterize the soil microbial community of constructed wastewater wetland (Ansola et al., 2014) and the bacterial community shift in municipal wastewater treatment plant (Ye and Zhang 2013). However, the microbial community diversity and abundance in CSW has not been studied extensively.

In this study, stormwater water samples were collected from two US and three Australian CSW. The indicator bacteria concentrations were analyzed by IDEXX system; microbial pathogens including adenovirus, *Cryptosporidium* as well as human specific HF183 *Bacteroides* were quantified using droplet digital PCR (ddPCR). The microbial community structure was characterized using 454 pyrosequencing. To the best of our knowledge, this is the first study employed ddPCR and 454 pyrosequencing to study the microbial pathogens and microbial community in CSW. The goal of the study is to find a feasible microbial indicator for assessing CSW performance.

3.2 Materials and methods

3.2.1 Wetlands sties and sampling schedule

Table 3-1 Summary of sampling sites, data and, weather conditions.

Sampling site (ID)	Sampling data	Season	Weather condition	Sample ID
Old Laguna (OL)	6.23.2014	Summer	Sunny	OL.I, OL.M, OL.O
Forge (FO)	6.25.2014	Summer	Sunny	FO.I1, FO.M1, FO.O1
Royal Park (RP)	7.03.2014	Winter	Sunny	RP.I, RP.M, RP.O
Lynbrook (LB)	7.03.2014	Winter	Sunny	LB.I, LB.M, LB.O
Hampton Park (HP)	7.07.2014	Winter	Sunny	HP.Ia, HP.Ib, HP.M, HP.O
Forge (FO)	2.26.2015	Spring	Sunny	FO.I2, FO.M2, FO.O2
Forge (FO)	3.02.2015	Spring	Rainy (1.47 cm)	FO.I3, FO.M3, FO.O3
Forge (FO)	3.05.2015	Spring	Sunny	FO.I4, FO.M4, FO.O4

Two CSW in the United States and three in Australia were sampled in 2014 under dry weather condition (Table 3-1). One of the wetlands in the U.S. (Forge) was also sampled during February to March, 2015 to catch a rain event (Table 3-1). The sampling sites for

each wetland were chose to represent the inflow, middle and outflow water quality and named as [SiteID.I], [SiteID.M] and [SiteID.O], respectively. Only Hampton Park has two inflow streams, which were labeled as [HP.Ia] and [HP.Ib]. The samples collected from Forge (FO) at different time were differentiated by numbers, such as [FO.I1], [FO.I2]. Figure 3-1 shows the sampling sites and the direction of stormwater flow in the CSW.

Figure 3-1 Aerial view of constructed stormwater wetlands (CSW) and sampling locations (adapted from Google map, lines show the water flow direction).

3.2.2 Sample collection and concentration

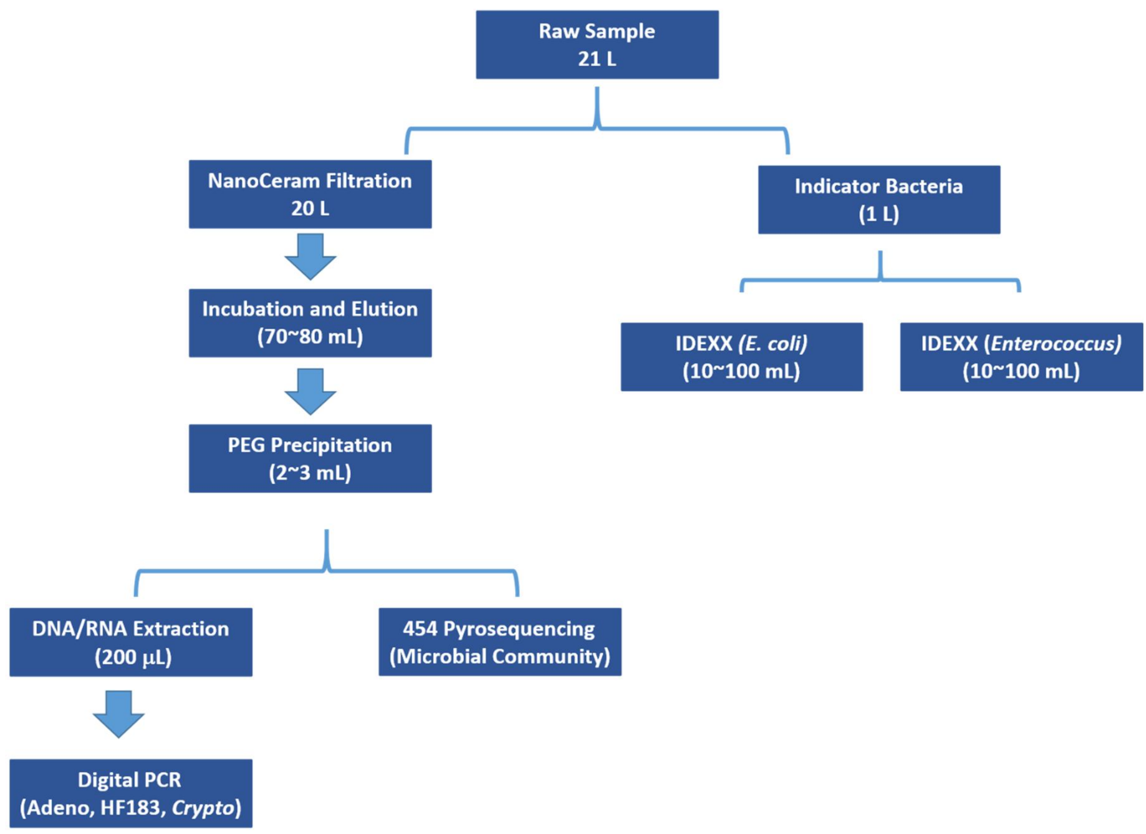

Figure 3-2 Stormwater samples concentration and analysis procedures

Figure 3-2 shows the stormwater samples concentration and analysis procedures. At each sampling location, 1 L water sample was collected in a 1.5-L sterile water bag (Whirl-Pak, Fort Atkinson, WI) for investigating the concentration of *E. coli* and *Enterococcus*. All the samples were kept in 4 ^{o}C cooler and transferred back to the lab within 6 h after sampling. The enumeration of *E. coli* and *Enterococcus* were carried out using the IDEXX Colilert and Enterolert assays (Westbrook, Maine) immediately upon arrival in the lab.

Another 20 L stormwater sample was collected into sterile carboy and filtered with a NanoCeram® Virus Sampler (Argonide, Sanford, FL) in the field. Viruses, bacteria and protozoa were simultaneously attenuated by the positively charged NanoCeram cartridge filter (VS2.5-5) through size exclusion and charge interaction. After filtration, the cartridge filter was transferred into a sterile water bag (Whirl-Pak, Fort Atkinson, WI) with 70 mL elution buffer. The elution buffer was made of phosphate-buffered saline (PBS) (3.8 mM Na_2HPO_4, 6.5 mM KH_2PO_4, pH=9.3) with 1.0% sodium polyphosphate (NaPP) and 0.05 M glycine(Ikner et al., 2011). The filter was soaked in the elution buffer and thoroughly shacked to release the attenuated microorganisms. All filters were transferred to the lab in 4°C cooler within 6 h after sampling. The filters were then incubated in a 4 ^{o}C refrigerator overnight to maximize the elution efficiency. After incubation, the pH of the elution buffer was adjusted to 7.2 with HCl and then transferred to two 50-mL high-speed centrifuge tubes. The eluted microorganisms were further concentrated by PEG precipitation method (10% PEG 8000 and 2% NaCl, 10k ×*g* centrifugation at 4°C for 2h) (Sánchez et al., 2012). The PEG final concentrates (2~3 mL) were used for ddPCR assays and 454 pyrosequencing analysis.

3.2.3 Droplet digital PCR

The concentrations of *Bacteroides* HF183, *Cryptosporidium* and adenovirus in stormwater samples were analyzed with ddPCR. The ddPCR assays were followed the protocol by Cao et al., (2015) and briefly described below. For each stormwater sample, 200 L final PEG concentrate was used for DNA extraction with PowerSoil® DNA Isolation Kit (Carlsbad,

CA). 3 L of the extracted sample DNA was used for each 25 mL ddPCR reaction, which contained 1×Droplet PCR Supermix (Bio-Rad) with 900 nmol/L of each primer and 250 nmol/L of the probe. The water-in-oil droplets were generated with Droplet Generator (Bio-Rad, Hercules, CA) and transferred to a standard 96-well PCR plate, which was heat sealed with foil plate seal (Bio-Rad) and placed on a Bio-Rad CFX96 thermocycler for PCR amplification using the following conditions: 10 min at 95 ^{o}C, followed by 40 cycles of 30 s at 94 ^{o}C and 60 s at 60 ^{o}C, followed by a 10 min hold at 98 ^{o}C. Upon completion of PCR, the plate was transferred to a Droplet Reader (Bio-Rad) for automatic measurement of fluorescence in each droplet in each well (approximately 2 min per well), with the RED (rare event detection) setting. ddPCR data was analyzed in QuantaSoft™ software (Bio-Rad) following the manufacturer's recommendation.

Table 3-2 Digital PCR primers and probes used for human-specific HF183 *Bacteroides*, adenovirus and *Cryptosporidium* detection.

Assay	Primer or probe	Oligonucleotide name	Sequence (5′-3′)	Fluorophore
HF183	Primer	HF183	ATCATGAGTTCACATGTCCG	
	Primer	BFDRev	CGTAGGAGTTTGGACCGTGT	
	Probe	BFDFAM	(FAM)-CTGAGAGGAAGGTCCCCCACATTGGA-(BHQ-1)	FAM
Crypto	Primer	COWP P702 F	CAAATTGATACCGTTTGTCCTTCTG	
	Primer	COWP P702 R	GGCATGTCGATTCTAATTCAGCT	
	Probe	COWP P702 P	HEX-TGCCATACATTGTTGTCCTGACAAATTGAAT-(BHQ-1)	HEX
Adeno	Primer	JTVXF	GGACGCCTCGGAGTACCTGAG	
	Primer	JTVXR	ACIGTGGGGTTTCTGAACTTGTT	
	Probe	JTVXP	FAM-CTGGTGCAGTTCGCCCGTGCCA-BHQ	FAM

3.2.4 454-pyrosequencing and bioinformatics analysis

The microbial community structure was investigated by high-throughput 454 pyrosequencing technology (Roche 454 FLX). For each sample, 500 L of final PEG concentrate was shipped on dry ice to Research and Testing Laboratories (RTL) (Lubbock, TX) where the amplicon libraries were prepared and sequenced. Bacterial diversity was evaluated with the 939f-1492r bacterial 16S assay (5’-TTGACGGGGGCCCGCAC-3’ and 5’-TACCTTGTTACGACTT-3’) (Coats et al., 2014), which corresponds to variable regions 6-9 of the 16S rRNA gene.

Data analysis was conducted using QIIME VERSION 1.9.0 (Kuczynski et al., 2012). Sequences that were <200 bp in length were excluded from the analysis, as were sequences containing ambiguous characters, quality scores <25, a non-exact barcode sequence, or any mismatches to the primer sequences. Chimeric sequence detection and OTU selection at 97% sequence similarity were conducted using USEARCH (Edgar 2013). Taxonomy assignment of the 16S rRNA reads was conducted using 2013 Greengenes ribosomal database. The alpha and beta diversity were calculated with alpha_diversity.py script and jackknifed_beta_diversity.py workflow.

3.3 Results and discussion

3.3.1 Removal of Indicator bacteria by CSW

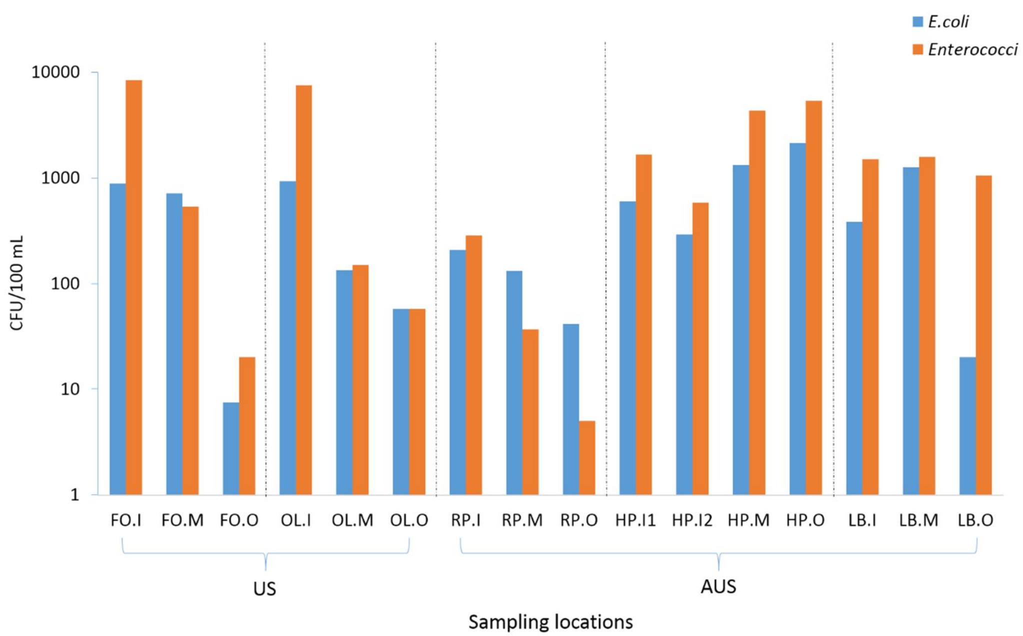

Figure 3-3 The removal rates of *E. coli* and *Enterococcus* in the US and Australian constructed stormwater wetlands (FO, Fordge; OL, Old Laguna; RP, Royal Park; HP, Hampton Park; LB, Lynbrook).

Figure 3-3 shows the removal rates of *E. coli* and *Enterococcus* in the five CSW under dry weather condition. In all inflow samples, the concentrations of *Enterococcus* were higher than that of *E. coli*. The concentrations of both indicator bacteria in the two US CSW were higher than that specified in the 2012 US EPA recreational water quality criteria (RWQC) (Table 3-3). According to Australian recreational water quality guidelines, all inflow water

Table 3-3 The US and Australian recreational water quality criteria recommendations

	E.coli		*Enterococcus*	
US	GM[a] (cfu/100mL)	126	GM (cfu/100mL)	35
	STV[b] (cfu/100mL)	410	STV (cfu/100mL)	130
AUS	Primary (cfu/100mL)	150	Primary (cfu/100mL)	35
	Secondary (cfu/100mL)	1000	Secondary (cfu/100mL)	230

[a] GM: geometric mean; [b] STV: statistical threshold value.

samples from Australian sites had *Enterococcus* concentrations higher than the number recommend for secondary contact, while the *E. coli* concentrations were above the recommended primary contact level (Table 3-3). The two US CSW had 1~2-$\log_{10}$ removal of *E. coli* and 2~3-$\log_{10}$ removal of *Enterococcus* for all dry weather samples. However, for the CSW in Australia, only Royal Park had a good removal of both indicator bacteria (about 1-$\log_{10}$). The indicator bacteria concentrations in the outflow of Hampton Park (HP.O) were even higher than that in the inflow (HP.I). The removal of *E. coli* and *Enterococcus* generally showed similar trend except for *E. coli* in Lynbrook, where 1-$\log_{10}$ removal of *E. coli* was observed, but the removal of *Enterococcus* was very limited (Figure 3-3). In general, the two indicator bacteria showed good correlation (R^2=0.8009) with each other as reflected in Figure 3-4.

The comparison of indicator bacteria removal rates in Forge under dry and wet conditions was presented in Figure 3-5. The results showed that, before the rain event, the inflow

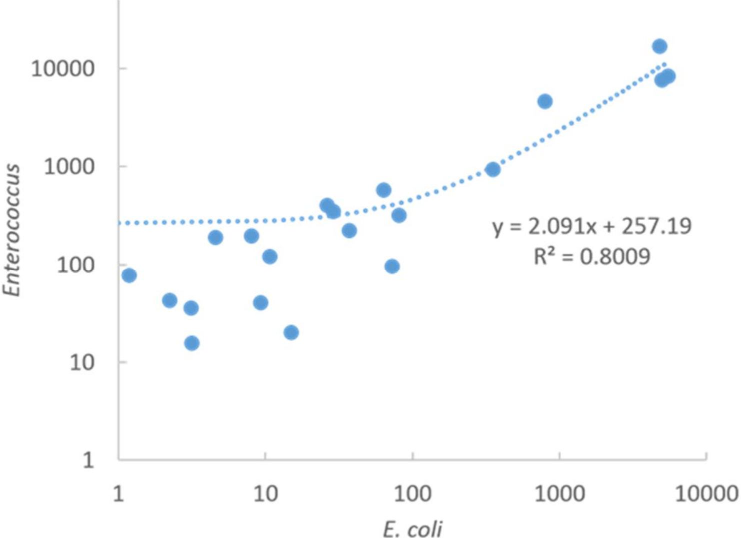

Figure 3-4 Correlation analysis on the concentrations of *E. coli* and *Enterococcus* in all tested samples

(FO.I2) collected in wet season (spring, 2015) had relatively lower concentrations of indicator bacteria than that in the dry season (FO.I1), especially for *Enterococcus* (about 10 times less). The wetland showed very good removal efficiency for both bacteria (1~2-log_{10}) and the outflow (FO.O2) water quality can meet the RWQC (Figure 3-5). During the rainy day, however, a spike in the number of both indicator bacteria were detected in the inflow (FO.I3). This is likely due to the flush effect of the rain, which has been reported in many previous studies (Hathaway and Hunt 2011, McCarthy et al., 2012). The wetland lost its capability for bacterial removal, as the concentration of indicator bacteria in inflow (FO.I3), middle (FO.M3), and outflow (FO.O3) were almost the same (Figure 3-5). However, the bacterial removal efficiency was restored fairly quickly after the rain. About 1-log_{10}

removal of both indicator bacteria were observed 3 days after the rain event, although the *Enterococcus* concentration in the outflow was still higher than the RWQC (Figure 3-5).

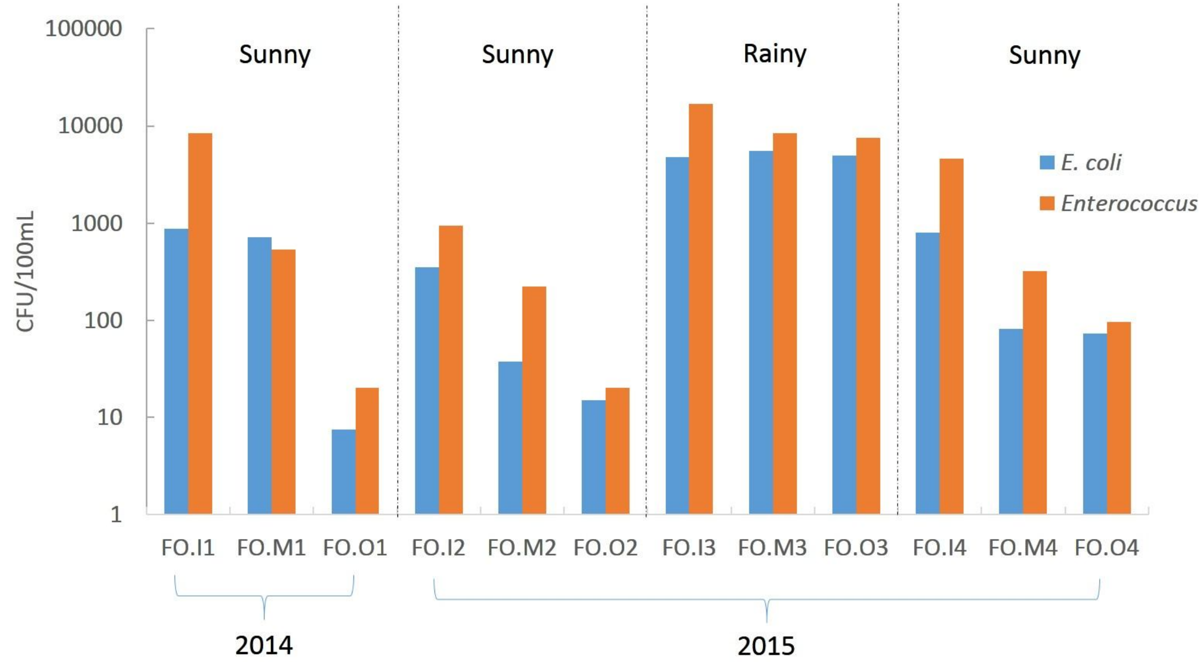

Figure 3-5 *E. coli* and *Enterococcus* removal rates in wetland Forge under dry and wet conditions.

Although limited numbers of samples were collected and tested for indicator bacteria in the present study, their concentrations in these CSW and the removal efficiencies generally followed the trend found by the long term monitoring data collected by the wetland management agencies (data not shown). Sedimentation, sunlight exposure, water temperature, and the adsorption to biofilms are considered as main factors governing the removal of indicator bacteria in CSW (Malaviya and Singh 2012, Sims et al., 2013). Precisely explaining the mechanisms of bacterial removal in CSW is difficult due to the complexity of

the system. However, some of the factors may play a more important role. Sunlight exposure (UV) and temperature are the first factors needs to be considered, as June-July is winter in Australia, while it is summer in southern California. The two US CSW had very good sunlight exposure due to the limited vegetation in the wetlands and the design of shallow water channel (0.3~0.5 m) in the middle of the wetlands (IRWD 2005). In most of the time, the actual water depth in the channels was less than 0.1 m due to the limited inflow water. In contrast, Royal Park and Hampton Park were much deeper with an average depth of 0.5~1 m. The Lynbrook wetland has the highest vegetation coverage, and thus received lowest sunlight exposure. Similar results have been found in other studies suggesting higher indicator bacteria removal efficiencies were positively correlated with better sunlight exposure (Cheng et al., 2013). Another important factor needs to be consider is the water residence time in the CSW. Struck et al., (2008) found the removal of fecal indicator bacteria (FIB) generally followed the first-order decay model as a function of time. The decay rate is significantly faster in the first 50 h over the 100-h study period. This has been clearly reflected by the samples collected during the rain event in Forge (Figure 3-5). Based on the design guidelines of the two US CSW(IRWD 2005), the optimum treatment of low flows requires a detention period of 10 to 14 days, with 10 days considered to be a minimum. During periods of rain, the water residence time is significantly reduced. According to the Municipal Separate Storm Sewer System (MS4) permit, a detention period of about 36-48 h is required for the stormwater flows into CSW. In fact, the actually water resident time in the CSW under moderate and heavy rain conditions may be even shorter. The stormwater passes through the CSW quickly, when the

system is inundated (Figure 3-6). With limited residence time, neither sedimentation nor sunlight exposure will be sufficient to treat the rapid flow of stormwater. Besides these generic impact factors, some site specific conditions should also be considered. For example, the poor performance of Hampton Park is likely due to the maintenance work during sampling period. It usually takes several months for wetland systems to function properly after initial built or retrofitted (LePage 2011). The higher indicator bacteria concentrations in the outflow of Hampton Park and Lynbrook may also be related to the animal origin fecal contaminations (e.g., water fowls, dogs), which was practically hard to be differentiated from fecal pollution of human origin by the culture-based methods. Therefore, further studies on microbial pathogens and microbial community would be necessary to evaluate the potential health risk associated with the reuse of these CSW treated stormwater.

Figure 3-6 Constructed stormwater wetland (Forge) during sunny and rainy days. The wetland was inundated after a moderate rain (1.47 cm).

3.3.2 ddPCR

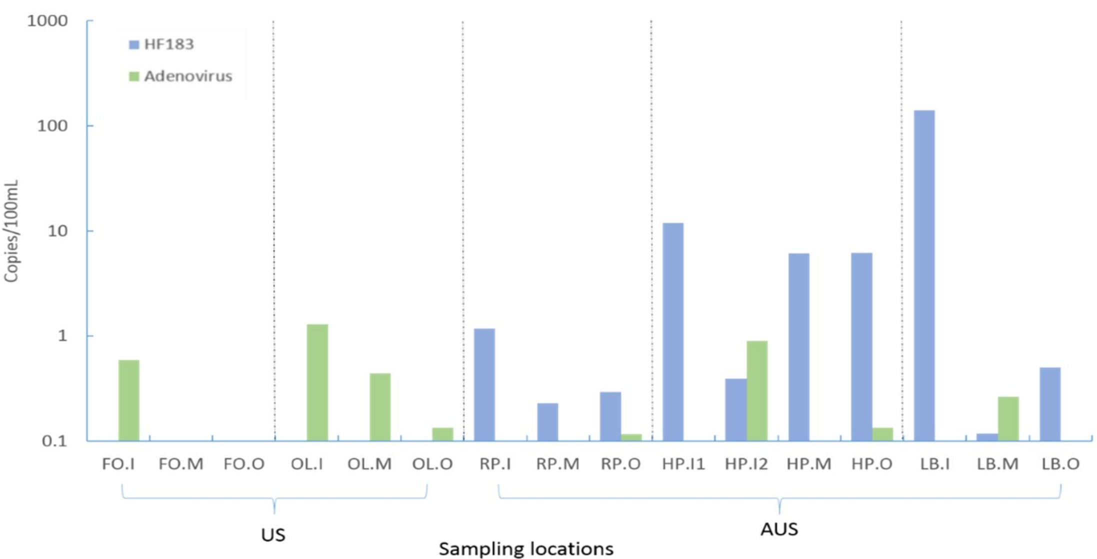

Figure 3-7 Concentrations of HF183 and adenovirus in stormwater samples from the US and Australian wetlands.

Figure 3-7 shows the ddPCR results of human-specific HF183 *Bacteroides* and adenovirus during 2014 sampling period. No *Cryptosporidium* was detected in all of the stormwater samples by ddPCR. On the contrary, adenovirus were found in all of the wetlands, although the concentrations were very low (<1 copy/100 mL). The result agrees with previous study by Rajal et al., (2007), who found only 1 of the 63 stormwater samples collected in California was positive for adenovirus. HF183 were only detected in Australian CSW, where the concentrations from 1 to 100 copies/100 mL were found in the inflow samples. In a recent study by Sidhu et al., (2013), adenovirus and HF183 were found positively in 91% and 96% of the stormwater samples collected from six urban catchments across Australia.

The high concentrations of HF183 as well as the high levels of indicator bacteria found in samples from Hampton Park and Lynbrook wetlands (Figure 3-3) clearly indicated cross contamination of sewage in these stormwater wetlands. The results suggest that other treatment processes should be included to reduce levels of microbiological contaminants, if the stormwater is designed for higher value end-uses.

3.3.4 Bacterial community analysis

As shown in Table 3-4, a total of 149,342 reads were obtained from the 25 stormwater samples by 454 pyrosequencing. After filtering the low quality reads using the RDP Initial Process in Pyrosequencing Pipeline (PP) and trimming the adapters, barcodes and primers, denoising and filtering out chimeras, there were 116,134 effective bacterial sequences and 104,477 of them were assigned as operational taxonomic units (OTUs) determined at distance levels of 3% for each sample. The samples contained OTUs numbers ranging from 1100 (FO.I3) to 8584 (FO. 02). The alpha diversity (within samples) was reflected by Shannon diversity index and Chao 1 richness index (Table 3-4), from which no clear pattern was found among the inflow, middle and outflow samples. Similar result was also showed by the rarefaction curves (Phylogenetic Diversity - Whole Tree analysis) of the 25 samples at distance cutoff levels of 3 % (Figure 3-8). At sequencing depth of 1,000/ sample, the curves becomes flat suggesting at the current sequencing depth, the assay was able to cover the majority of the microbial species within the samples. As expected, the microbial diversity of the stormwater water samples were significantly lower than those reported in

raw wastewater (Shanks et al., 2013) and water samples from wastewater treatments (Ye and Zhang 2013).

Table 3-4 Summary of sequence counts and microbial diversity estimates

Sampling sites	Raw sequence	Effective	OTUs	Shannon diversity	Chao 1 richness estimation
FO.I1	7839	5586	4745	4.090	420.012
FO.M1	4564	4194	4110	0.850	191.596
FO.O1	2631	2441	2302	2.920	331.269
FO.I2	5839	3489	3023	3.428	303.719
FO.M2	3530	2430	2030	4.247	101.221
FO.O2	13575	9118	8584	2.580	307.870
FO.I3	1682	1428	1100	5.014	412.111
FO.M3	2500	2181	1674	5.123	223.612
FO.O3	4355	3749	3107	4.057	149.056
FO.I4	3837	2577	2156	4.762	205.995
FO.M4	4232	2633	2357	3.405	288.789
FO.O4	7654	6512	5829	3.965	358.742
OL.I	6408	5888	4581	4.560	542.734
OL.M	8043	7457	7316	0.667	32.897
OL.O	5621	5260	5187	0.720	56.870
RP.I	5397	5001	4549	3.754	257.922
RP.M	5165	2497	2425	1.862	132.849
RP.O	17949	8294	7974	1.828	134.020
HP.Ia	6316	5836	5322	3.514	318.783
HP.Ib	1972	1842	1651	4.039	152.202
HP.M	4151	3852	3521	3.623	339.108
HP.O	9511	8733	7876	3.219	337.804
LB.I	9096	8271	7355	3.647	306.327
LB.M	2352	2181	1806	4.206	235.989
LB.O	5123	4684	3897	5.361	454.885

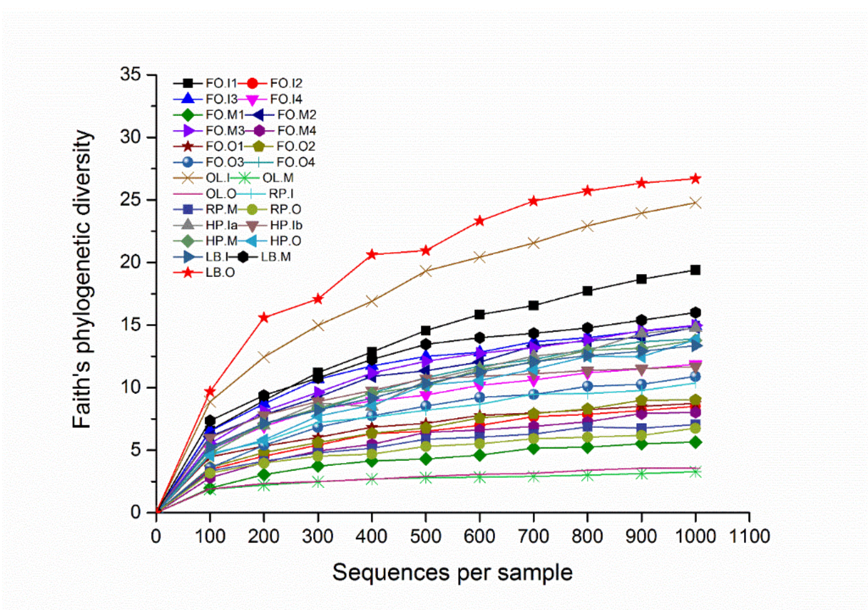

Figure 3-8 Faith's phylogenetic diversity calculated after rarifying samples to equal sequencing depth in QIIME.

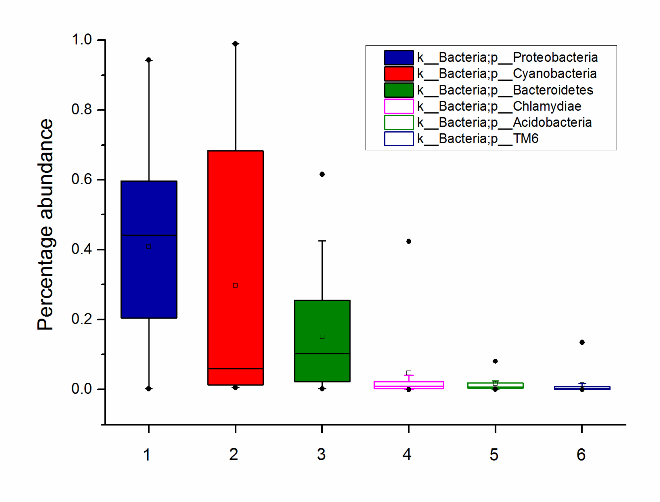

Figure 3-9 Phylum relative abundance box plot for all phyla as determined by Ribosomal Database Project (RDP) classifier in 25 stormwater samples. Only top six phyla were

plotted in the graph. The bottom and top boundary of the box indicates the 25th and 75th percentile, the line within the box represents the median, whiskers above and below the box indicate the 10th and 90th percentiles. The dot symbols denote outlier measurements.

The effective bacterial sequences in each stormwater samples were assigned to different taxa levels (from phylum to genus) using Ribosomal Database Project (RDP) classifier with Qiime. Figure 3-9 depicts the top 6 taxon assignments at the phylum level including *Proteobacteria* (40.8%), *Cyanobacteria* (29.8%), *Bacteroidetes* (15.0%), *Chlamydiae* (4.71%), *Actinobacteria* (1.7%), and TM6 (1.4%). *Proteobacteria* and *Bacteroidetes* were also found to dominate in sewage (McLellan et al., 2010, Shanks et al., 2013) and soil (Roesch et al., 2007), while the high percentage of *cyanobacteria* were not reported in those samples.

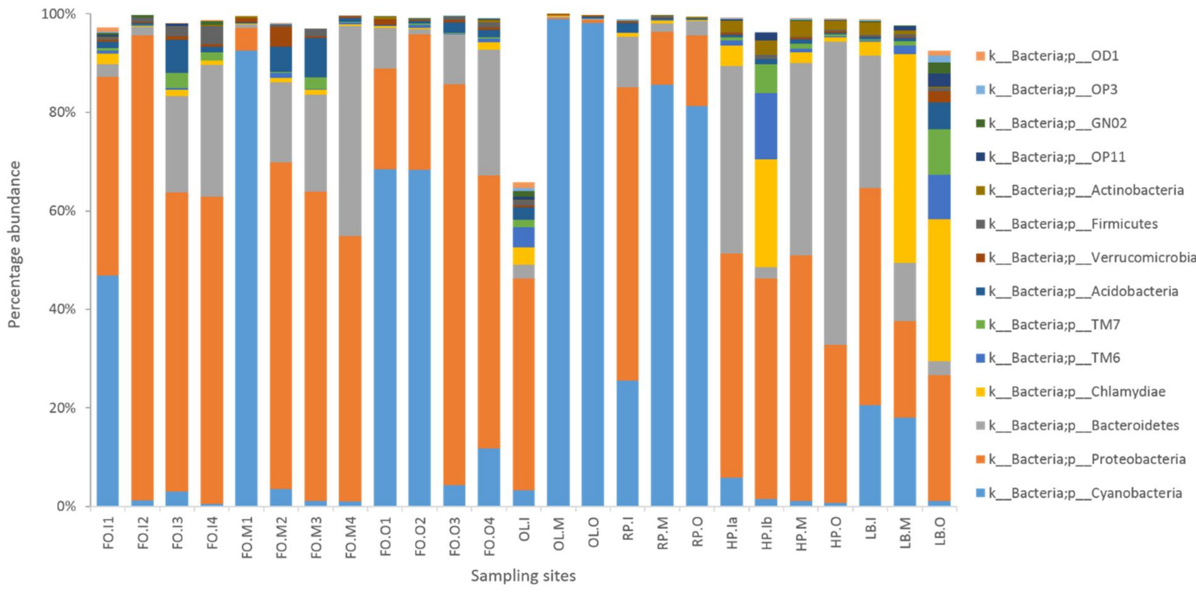

Figure 3-10 Relative abundances of different phyla in samples from different wetlands

The relative abundances of different phyla in the 25 samples were shown in Figure 3-10. From the phylum assignment result, it is interesting to find that for those CSW with better performance (Forge, Old Laguna, Royal Park), the abundance of *cyanobacteria* was significantly increased in the outflows, while the abundance of *Proteobacteria* was decreased (FO.O1, FO.O2, OL.O and RP.O). Similar shift was not observed in Forge during the rainy and after rain samples (FO.O3, FO.O4). In a previous study on the impact of plant density and microbial composition to the performance of a CSW in Orange County, CA, Ibekwe et al., (2007) found that *Cyanobacteria* dominated in the water column, while the plant rhizosphere was dominated by *Proteobacteria*. However, the authors did not find out the microbial community shift in the raw and treated stormwater due to the small size of the clone library (300 sequences). *Cyanobacteria* are key players of photosynthesis and play an important role in nitrogen fixation in systems like oligotrophic wetland ecosystems (Rejmánková and Komárková 2000) and hot spring microbial mats (Steunou et al., 2006). The outgrowth of phototrophic bacteria than heterotrophic bacteria indicate the depleting of organics in the treated water, which agrees with the results from indicator bacteria enumeration.

Principal coordinates analysis (PCoA) was conducted to evaluate the microbial community differences among the 25 samples (beta diversity) using weighted UniFrac, which incorporates the degree of divergence in the phylogenetic tree of OTUs into PCoA (Hamady et al., 2010). We hypothesized that there would be significant differences among the stormwater samples collected from different geographical locations (US and Australia).

However, the results of PCoA did not support this hypothesis. All the inflow water samples were in the positive side of PCoA1, the variation of among them were mainly explained by PCoA2. It is also reasonable to speculate that the bacterial community structure shifts in the inflow, middle and outflow samples should be observed in CSWs with better

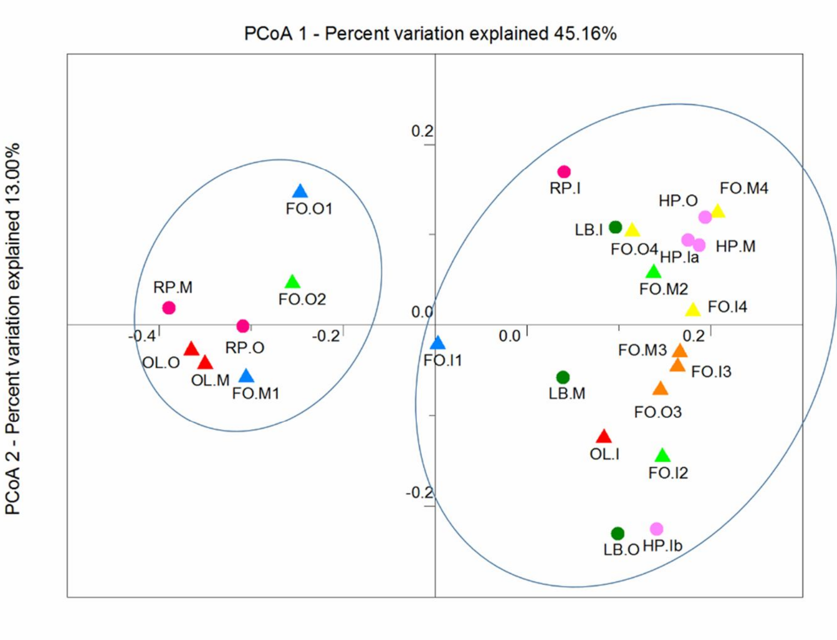

Figure 3-11 Principal coordinate analysis (PCoA) of 25 samples based on the composition of microbial communities. Microbial communities can be grouped into two clusters (circled) corresponding to the performance of wetlands on indicator bacteria removal.

performance. The speculation was confirmed by the current result as shown in Figure 3-11. The outflow of Forge (FO.O1, FO.O2), Old Laguna (OL.O) and Royal Park (RP.O), plus some samples from the middle of these CSW (FO.M1, OL.M, RP.M) clustered in the negative side

of the PCoA1. The bacterial community structure shift was also observed in Lnybrook, but the changes were mainly in the PCaA2. The inflow (HP.Ia), middle (HP.M) and outflow (HP.O) samples collected from Hampton Park clustered together in suggesting they were very similar to each other. Similarly, samples collected from Forge during rain event also clustered together (FO.I3, FO.M3 and FO.O3) (Figure 3-11), which echoed the results from indicator bacteria enumeration showing the wetland lost its bacterial removal capability during the rain.

3.4 Conclusions

- Two CSW in the U.S. and one in the Australia showed high removal rates on indicator bacteria. CSW treated stormwater can meet the primary recreational contact criteria when the system functioning properly.
- Adenovirus were found in all CSW, but human specific HF183 *Bacteroides* were only found in Australian CSW suggesting the potential sewage cross contamination to stormwater in those sites.
- The removal of indicator bacteria by CSW was significantly decreased during rainy day due to the short stormwater residence time.
- The microbial communities in the inflows were dominated by *Proteobacteria*. A clear increase of *Cyanobacteria* was observed in those CSW with higher indicator bacteria removal rates. This shift can be potentially used as an indicator for assessing wetland restoration and management practices.

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CHAPTER 4 ELECTROCHEMICAL DISINFECTION OF TOILET WASTEWATER USING SOLAR-POWERED WASTEWATER ELECTROLYSIS CELL

Abstract

The paucity of proper sanitation facilities has contributed to the spread of waterborne diseases in many developing countries. The primary goal of this study was to demonstrate the feasibility of using a solar-powered wastewater electrolysis cell (WEC) for toilet wastewater disinfection. The treated wastewater was designed for reuse in toilet flushing and agricultural irrigation. Laboratory-scale electrochemical (EC) disinfection experiments were performed to investigate the disinfection efficiency of the WEC with four seeded microorganisms (*E. coli*, *Enterococcus*, recombinant adenovirus serotype 5, and bacteriophage MS2). In addition, the formation of organic disinfection byproducts (DBPs) trihalomethanes (THMs) and haloacetic acids (HAA_5) during the EC disinfection process was also investigated. The results showed that at an applied cell voltage of +4V, the WEC achieved 5-log_{10} reductions of all four seeded microorganisms in real toilet wastewater within 60 min. In contrast, chemical chlorination (CC) disinfection using hypochlorite [NaClO] was only effective for the inactivation of bacteria. Due to the rapid formation of chloramines, less than 0.5-log_{10} reduction of MS2 was observed in toilet wastewater even at the highest [NaClO] dosage (36 mg/L, as Cl_2) over a 1.0 h reaction time. The dominating role of free reactive chlorine generated *in situ* during EC disinfection process was verified using laboratory model waters, while the production of hydroxyl radicals [·OH], and other reactive oxygen species by the active bismuth-doped TiO_2 anode were negligible under the

same electrolytic conditions. The formation of THMs and HAA_5 were found to increase with an increase in applied cell voltage. Based on the energy consumption estimates, the WEC system can be operated using solar energy stored in a DC battery as the sole power source.

4.1 Introduction

In highly developed countries, flush toilets and well-managed sanitation systems have been used for more than 80 years. Wastewater is collected in massive sewer systems and subsequently treated at large-scale centralized wastewater treatment plants before discharged into receiving waters or land. Advanced, tertiary wastewater treatment plants are now capable of producing high quality reclaimed water for indirect or even direct potable reuse (Wetterau et al., 2013). In contrast, approximately 2.5 billion people in the developing world lack to improved sanitation and 1.8 billion people use a source of drinking water which suffers from fecal contamination (Bain et al., 2014, WHO 2014). In many developing countries, the discharge of untreated or partially treated domestic wastewater to the aquatic environment severely threatens public health and socio-economic development. It is abundantly clear that, in many parts of the world the infrastructure required for conventional centralized wastewater systems is prohibitively expensive. The development of cost-effective, decentralized waste and wastewater treatment systems is an important step toward the eradication of waterborne diseases and to ensure water sustainability in the developing world (Massoud et al., 2009).

Conventional flush toilet wastewater (i.e., a mixture of urine, feces and flushing water) is characterized by high levels of microbial contaminants (e.g., pathogenic viruses, bacteria

and protozoa) derived from human excrement. Untreated flush toilet wastewater is considered as health hazard and potential vector of infectious waterborne diseases. On the other hand, toilet wastewater also contains high concentrations of macro- and micro-nutrients essential for plant growth. Urine contributes up to 80% of nitrogen and 60% of phosphorus found in wastewater in spite of contributing only 1% by volume of the total load (Karak and Bhattacharyya 2011). With proper treatment, toilet wastewater can be used as a good liquid fertilizer that could help reduce the use of synthetic chemical fertilizer in the developing world (Drechsel et al., 2009).

A primary challenge of toilet wastewater reuse is how to effectively remove or inactivate microbial contaminants. Many disinfection technologies (e.g., chlorination, UV and ozonation) have been utilized in large-scale wastewater treatment plants. However, the adaptation of conventional disinfection systems to smaller decentralized plants is often challenging due to the financial constraints or technological barriers (Schmalz et al., 2009). In recent years, electrochemical (EC) disinfection has been considered as a viable alternative for use in decentralized systems. EC disinfection has been reported to be capable of disinfecting a wide spectrum of microbial pathogens in various water matrices (Cano et al., 2012, Li et al., 2002). The on-site generation of disinfectants can be environmentally-sound and user friendly in terms of energy consumption and ease of operation. In addition, EC disinfection systems can be powered either totally or partially by the output of photovoltaic (PV)-panels as self-sufficient sanitation facilities, which is critical in many regions of the world where reliable energy supplies are not available (Cho et al., 2014b).

During EC disinfection, microorganisms are killed by a variety of oxidants that are produced during water electrolysis. When chloride is naturally present (e.g., seawater or toilet wastewater) or artificially added, reactive chorine species (RCS) such as free chlorine ([Cl_2], [HOCl], [ClO^-]) and chlorine radical species ([·Cl], [$·Cl_2^-$]) are generated and recognized as primary disinfectants. Therefore, human urine could serve as an excellent electrolyte as it may contain chloride at relatively high levels (50-150 mM) (Kim et al., 2013, Putnam 1971). In addition, reactive oxygen species (ROS) including hydroxyl radicals ([·OH]), hydrogen peroxide ([H_2O_2]), ozone ([O_3]), and superoxide anion radicals ([$·O_2^-$]) generated during electrochemical water splitting can enhance the overall disinfection efficiency (Bergmann et al., 2008, Jeong et al., 2006). Another major factor governing the generation of oxidants is the composition of the anode (Jeong et al., 2009). Anodes made from antimony-doped tin oxide (ATO), PbO_2 and boron-doped diamond (BDD) electrodes, are known to favor the formation of free [·OH]. In contrast, Pt-, IrO_2-, RuO_2- anodes, known as dimensionally stable anodes, form surface bound [·OH], which mediates the facile formation of RCS (Chen 2004). Operational parameters, such as cell voltage, current density, reaction time, temperature, and pH are also important in optimization EC disinfection systems. In many previous studies, bacteria (usually *E. coli* or total fecal coliform) were exclusively used as model microorganisms to evaluate the efficiency of EC disinfection systems since these microorganisms are specified in water quality standards or guidelines (Jeong et al., 2009, Schmalz et al., 2009). However, it is often unknown whether or not human enteric viruses are simultaneously inactivated along with the indicator bacteria during EC disinfection. Given the low infectious doses and potentially

high resistance to commonly used disinfectants, viruses should be considered routinely in the evaluation of system disinfection efficacy to understand the quality of treated water.

Furthermore, the formation of disinfection by-products (DBPs) in chlorination process is of concern because of their possible association with cancer and adverse reproductive outcomes (Nieuwenhuijsen et al., 2000). Depending on the reaction conditions, inorganic or organic DBPs may also be produced during EC disinfection. For instance, chlorite ([ClO_2^-]) is known to be produced electrochemically either by oxidation of chloride or free chlorine ([HOCl] or [ClO^-]) (Ghernaout et al., 2011). High overpotentials are often employed to electrochemically treat refractory organic pollutants. However, the use of high applied potentials in EC disinfection may lead to the formation of chlorate ([ClO_3^-]), perchlorate ([ClO_4^-]) persulfate ([SO_5^{2-}]), and perphosphate ([PO_5^{3-}]). These particular oxidants are toxic to humans and plants even at very low levels (Bergmann and Rollin 2007, Kraft 2008), and thus may restrict the reuse of the treated water. Even though the formation of chlorinated organics, such as trihalomethanes (THMs) and haloacetic acids (HAA_5) during chlorination process is well known, the formation of organic DBPs during EC disinfection has not been studied extensively. Higher concentrations of chloride were found to produce higher concentrations of THMs during the EC disinfection of secondary wastewater effluent (Cano et al., 2012, Perez et al., 2010). Schmalz et al., (2009) reported levels as high as 1000 g/L of organically-bound halogens were produced during EC disinfection as a direct function of the applied electric charge per volume (Q/V). Compared to domestic wastewater effluents, toilet wastewater has much higher concentration of

dissolved organic carbon (DOC), which serves as the precursors of organic DBPs. Consequently, higher concentrations of organic DBPs may be expected.

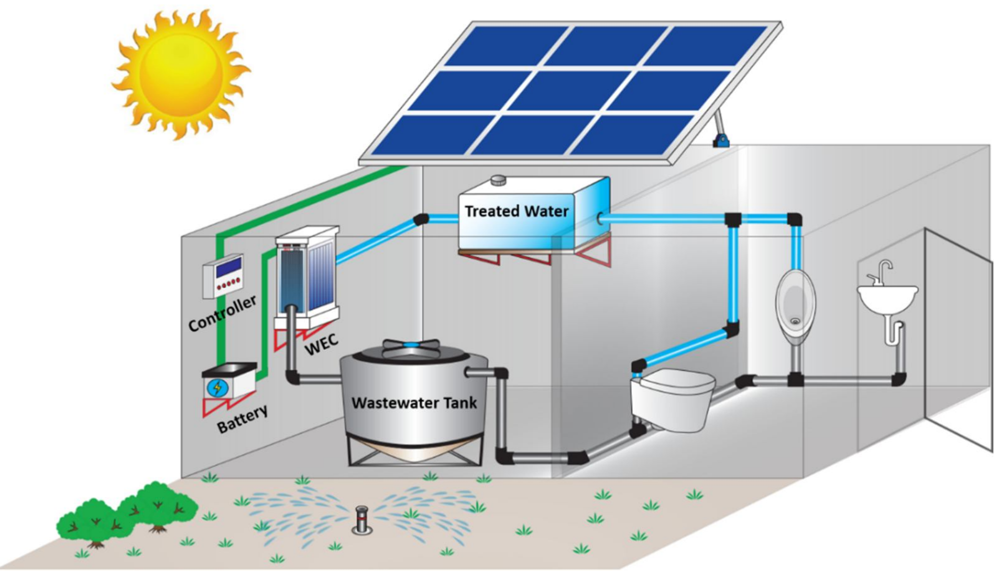

Figure 4-1 Schematic of a solar-powered mobile toilet using wastewater electrolysis cells (WEC) for toilet wastewater treatment.

Utilizing an array of mixed-metal oxide semiconductor anodes with stainless steel cathodes, we developed a variety of wastewater electrolysis cells (WEC), which can be powered by PV-panels for decentralized toilet wastewater treatment (Figure 4-1). The treated wastewater was designed for reuse in toilet flushing and agricultural irrigation. The principle objective of this study is to assess and optimize the disinfection capability of the aforementioned WEC for the treatment toilet wastewater treatment. In addition, the primary disinfection pathways for microbial inactivation were investigated along with the formation of THMs and HAA_5 under typical operational condition.

4.2 Materials and methods

4.2.1 Toilet wastewater and model waters

Table 4-1 Characteristics of raw toilet wastewater.

Parameter	Value
pH	6.7~8.3
Conductivity (mS/cm)	3.2~3.4
Chloride (mM)	12~20
NH_4^+(mM)	4.5~4.7
Total Culturable Bacteria (CFU/mL)	3.2×10^5
Total Bacteria (FCM)(Cells/mL)	2.49×10^7
Total Viruses (FCM) (VLP/mL)	3.74×10^8
E.Coli (mTEC) (CFU/mL)	4.1
Enterococcus (m-EI) (CFU/mL)	6.4×10^2

Forty liters of toilet wastewater were extracted from the wastewater storage tank of a pilot-scale PV-powered toilet located at California Institute of Technology (Figure 4-1). The prototype self-contained toilet and treatment system was in continuous operation when the wastewater sample was collected. The composition of the raw toilet wastewater is given in Table 4-1. All the microbial parameters were tested within 4 h after sample collection. Aliquots of wastewater were stored at 4 ^oC refrigerator before use. In addition to raw toilet wastewater, three laboratory model waters (MW1, 2, 3) were prepared and tested in order to characterize the role of RCS and ROS in EC disinfection. MW1 was phosphate buffered saline (PBS) solution containing only an inert electrolyte monopotassium phosphate ([KH_2PO_4]) in order to minimize the formation of oxidants other than ROS during EC reaction. MW2 was PBS buffer amended with 15 mM sodium chloride ([NaCl]). Free chlorine would equal to total chlorine in MW2 because of the absent

of [NH_4^+]. In MW3, 15 mM of [NH_4Cl] was added to PBS buffer to provide [NH_4^+] and [Cl^-], which ensured that free ammonia was readily available for reaction with [HOCl] during the first 30 min of EC reaction. All model waters were free of organics and other reducing agents. The general characteristics of the toilet wastewater and model waters were summarized in Table 4-2.

Table 4-2 Chemical parameters of toilet wastewater and model waters

	pH	Electrical Conductivity (mS/cm)	Cl^- (mM)	NH_4^+ (mM)
Toilet water	6.7~8.3	3.2~3.4	12~20	4.6~4.7
MW1	7.4~7.5	3.2~3.4	n.d.[a]	n.d.
MW2	7.4~7.5	3.2~3.4	15	n.d.
MW3	7.4~7.5	3.2~3.4	15	15

4.2.2 Model microorganisms

Due to the low concentrations of culturable bacteria and viruses in the raw toilet wastewater (Table 4-1) and the natural decay during refrigerator storage, pure cultured model bacteria or viruses were seeded to the testing waters before each test to maintain a consistent initial level of target microorganism. *Escherichia coli K12* (ATCC 10798) and *Enterococcus faecalis* (ATCC 29212) were employed as model bacteria in the disinfection experiments. Both bacteria were cultured in Luria-Bertani broth at 37 ^{o}C for 18 ± 2 h. The bacterial suspensions were centrifuged at 3,000 × *g* for 15 min and re-suspended in 1× PBS, which were used as bacterial stock. Before each experiment, a freshly prepared 0.1 to 1 mL bacterial stock suspension was seeded to water samples to give an approximate final

concentration of 10^5 to 10^6 CFU/mL. *E. coli* and *Enterococcus* concentrations in the water samples were quantified using membrane filtration via US EPA method 1063 and method 1600, respectively. Parts of the samples were also analyzed using a flow cytometer (FCM, Accuri Cytometers, Ann Arbor, MI, USA) to assess the bacterial nucleic acid injuries caused by EC disinfection. The samples were fixed with 2% glutaraldehyde (final concentration) and stained with 0.5x SYBR-Gold (Invitrogen Corp., Carlsbad, CA). The FCM instrument setting and data acquisition procedure were described in detail in our previous study except the threshold was set as 10,000 in fluorescence channel 1 (FL1) (Huang et al., 2015). Green fluorescence and side scatter (SSC) light were collected in the FL1 channel (533 ± 30 nm) and the SSC channel on a logarithmic scale, respectively. Data analysis was carried out using the BD CFlow® software. FL1 vs. SSC density plots were used to differentiate different bacterial populations as well as the background noise.

Coliphage MS2 (ATCC 15597-B1) was propagated using *E. coli* - 3000 (ATCC 15597) as host. In this case, 0.1 mL (10^7 pfu/mL) MS2 was inoculated into 20 mL actively growing *E. coli* host suspension. The infected bacteria were continuously aerated at 37 ^oC for 36h. The host-associated MS2 suspension was then centrifuged at 3,000 × *g* for 20 min to remove the bacterial cells and debris. The supernatant containing the MS2 phages was further purified by 0.2 µm syringe filter (GE Whatman, Pittsburgh, PA). The filtrate was diluted 1,000x with 1× PBS and stored in -80 ^oC freezer, which was used as MS2 stock. The seeding level of MS2 was 10^5 to 10^6 PFU/mL. The concentration of MS2 in water samples was titrated by the double agar layer method (Clokie 2009).

Recombinant adenovirus serotype 5 (rAd5) with the E1A gene replaced by the green fluorescent protein (GFP) gene was also employed as model virus in the seeding study. A human embryonic kidney cell line (HEK-293A) was used as rAd5 host for the propagation and the quantification of rAd5. Viral infectious units were quantified using a flow cytometer by detecting the GFP positive cells as described in detail by Li et al., (2010). The seeding level of rAd5 was 10^4 PFU/mL due to the relatively low concentration of rAd5 stock.

4.2.3 Experimental procedures

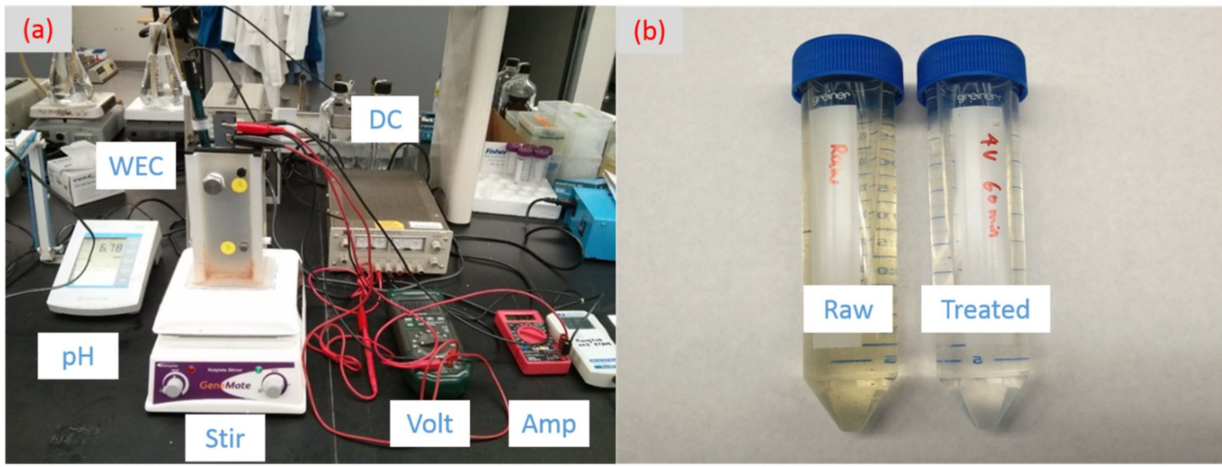

Figure 4-2 Experiment setup and results (a) bench scale water electrolysis cell and (b) raw toilet wastewater and electrochemical treated water (4V, 1 h).

EC disinfection experiments were carried out in a bench scale WEC with a working volume of 250 mL (Figure 4-2). The electrode module used for this study consists of a semiconductor anode (Nanopac, Korea, 13.5 × 6.7 cm) and a stainless steel cathode (Hastelloy C-22, 13.5 × 6.7 cm). The details about the preparation and characteristics of the

$BiOx/TiO_2$ anode can be found in our previous reports (Cho et al., 2014a, Cho et al., 2014b). The electrode pairs were installed in the reactor with a separation between anode and cathode of 0.5 cm. The setup was connected to a direct current power supply (HP-6236B Triple Output Power Supply, Palo Alto, CA) and operated in potentiostatic mode at 3V, 4V and 5.5V providing current density values of 0.39, 1.2 and 2.4 mA/cm^2. All the experiments were carried out at room temperature (21 ± 0.5 ^oC). Before water electrolysis, target concentration of model microorganisms were seeded into 250 mL toilet wastewater or laboratory MWs and stabilized for 30 min. In a sub-set of the experimental trails using MWs, an excessive amount of *tert*-Butyl alcohol (*t*-BuOH) (30 mM), a well-known [·OH] scavenger, was employed to assess the presence and role of [·OH] in EC disinfection (Jeong et al., 2006). During the EC reaction of 30 to 60 min, the electrolyte was well mixed by a magnetic stir bar. Samples were withdrawn at time intervals to measure the concentrations of oxidants or to count the number of viable microorganisms. The change of the volume and wetted electrode surface area due to the sample withdraw from the reactor was negligible. For microorganism enumeration, samples were quenched immediately with excess [$Na_2S_2O_3$] (10 mM) to eliminate the residual disinfectants. The level of inactivation was expressed as a log reduction of the microbe survival ratio (N/N_0) during the disinfection experiments. All experiments were repeated at least twice to ensure reliability. In preliminary disinfection experiment with toilet wastewater, inactivation of microorganisms was not observed under 3V, while the inactivation rate of MS2 was similar under 4V and 5.5V. Thus, in the later EC disinfection experiments, the applied cell voltage was set at 4V unless specified.

Chemical chlorination (CC) was carried out for comparison to EC disinfection in order to investigate the role of different chlorine species (free/combined chlorine) in disinfection. Similar to the EC disinfection experiments, microorganisms were seeded in water samples contained in sterile capped glass bottles. Samples were stabilized for 30 min before adding freshly prepared [NaClO] stock solution (1000 mg/L) to the desired final concentrations. A magnetic stir bar was used to keep the samples well mixed over the course of reactions. Water samples were taken and tested following the same procedures as in EC disinfection experiments to quantify the chlorine residual and viable microorganisms.

4.2.4 Chemicals and analysis

The concentration of total chlorine and free chlorine in water samples were determined as mg/L [Cl_2] by N,N-diethyl-p-phenylenediamine (DPD) colorimetric method using a OMEGA photometer (HHWT-11). It is important to mention that in EC disinfection, besides chlorine, other potential oxidants (e.g., [O_3], [H_2O_2], [ClO_2]) produced by EC reaction can also oxidize DPD to form the semiquinoid cationic Würster dye that accounts for the magenta color in the colorimetric test, and thus the total chlorine results in fact reflect the total oxidizing capacity of EC produced oxidants in the sample (expressed as mg/L Cl_2) (Danial 2002).

The formation of THMs and HAA_5 was measured by an EPA certified laboratory using US EPA method 524.2 and US EPA method SM 6251 B by gas chromatography (Eurofins Eaton Analytical, Inc., Monrovia, CA). Briefly, unseeded toilet wastewater was treated with WEC under applied cell voltage of 4V and 5.5V for 60 min. At the end of the reaction, the treated

water was divided into two portions. The first portion was immediately transferred to sampling bottles with quencher [$Na_2S_2O_3$] (for THMs) or [NH_4Cl] (for HAA_5) to stop the reaction (0 h samples). The other portion was incubated in amber glass bottles capped with Teflon-faced septa at room temperature (21 ± 0.5 ^oC) in the dark for 24 h before transferred to sampling bottles (24 h samples). For comparison, the formation of DBP_s during CC disinfection were also tested with [NaClO] addition at the total chlorine concentration of 5 mg/L (as Cl_2), which was at equivalent to the chlorine concentration at the end of 60 min EC disinfection under 4V. Similar 0 h and 24 h samples were collected and tested in the same way as in EC disinfection.

4.3 Results

4.3.1 Electrolysis production of oxidants in toilet wastewater and model waters

The influence of applied cell voltage on oxidants generation in toilet wastewater is shown in Figure 4-3a. Very limited oxidants (< 0.5 mg/L) were detected under the applied cell voltage of 3V. At cell voltage of 4V, the concentration of oxidants increased to 4 mg/L within 10 min and stabled at 5-6 mg/L during rest of reaction time. When the applied cell voltage was raised to 5.5V, the oxidants generation curve showed resemblance to the direct chlorination process (breakpoint chlorination). At both 4V and 5.5V, free chlorine (> 1 mg/L, data not shown) was detected by the DPD method after 5 min of reaction. Due to the interference of chloramines, DPD method cannot precisely quantify the free chlorine level in the grab samples (the magenta color increased with time resulting in the overestimation

of free chlorine) (Spon 2008). However, the existence of free chlorine before breakpoint (with the present of free ammonia in the system) during the EC reaction was confirmed in our 20 L prototype WEC by an online free chlorine probe (Chemtrac, Norcross, GA). The kinetics of free chlorine evolution and pH changes in the WEC are shown in Figure 4-4.

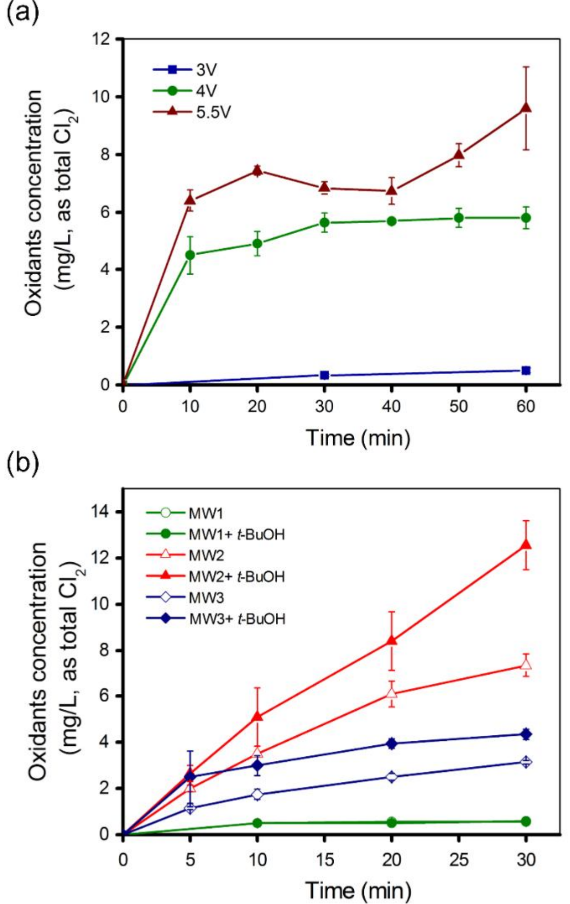

Figure 4-3 Oxidants generation during electrochemical (EC) reaction (expressed as mg/L, Cl_2) in (a) toilet wastewater under different applied cell voltage and (b) laboratory model waters (MWs) at applied cell voltage of 4V (MW1: PBS; MW2: PBS+15mM NaCl; MW3: PBS+15 mM NH_4Cl).

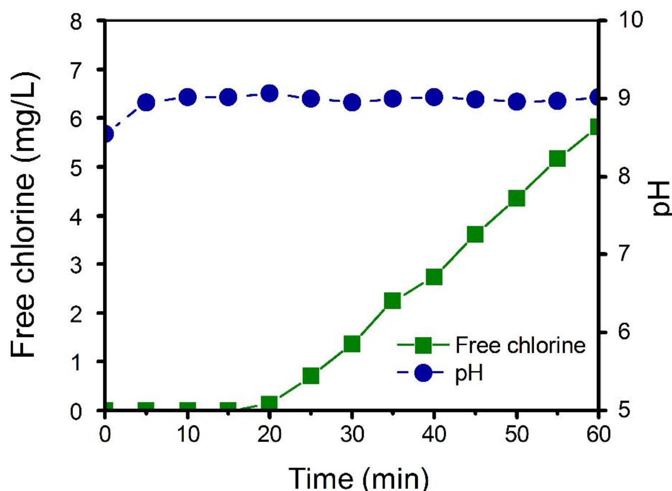

Figure 4-4 Free chlorine generation and pH changes in 20 L pilot scale wastewater electrolysis cell detected by online free chlorine and pH probes during toilet wastewater disinfection experiment (Applied cell voltage 3.8V).

The analysis of individual oxidants during electrolysis of toilet wastewater is extremely difficult due to the complexity of the water as well as the potentially fast reactions of oxidants with ammonia or other reducing substances (e.g., organic matter). The EC experiments using laboratory model waters provided additional evidence for explaining the role of different oxidants in disinfection (Figure 4-3b). In the absence of [Cl^-], less than 0.5 mg/L total oxidants were detected in MW1. In MW2 and MW3, total chlorine concentration steadily increased with the reaction time (Figure 4-3b), although the chlorine evolution rate in MW3 was obviously slower than that in MW2. The addition of excess *t*-BuOH (30 mM) was intended to quench [·OH] in order to inhibit the chlorine production mediated by [·OH]. Unexpectedly, *t*-BuOH in fact promoted the chlorine

evolution in both MW2 and MW3 (Figure 4-3b). However, the similar stimulating effect was not observed in MW1, in which less than 0.5 mg/L total oxidants were detected.

4.3.2 Inactivation of microorganisms in toilet wastewater by EC and CC disinfection

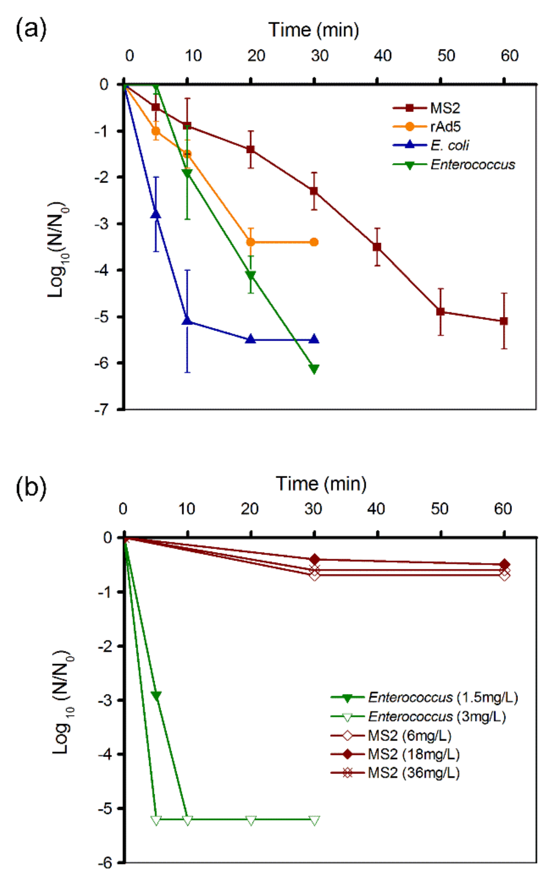

Figure 4-5 Inactivation kinetics of microorganisms in toilet wastewater by (a) electrochemical (EC) disinfection at applied cell voltage of 4V and (b) chemical chlorination (CC) disinfection using different concentrations of hypochlorite (as mg/L Cl_2, indicated in the legend).

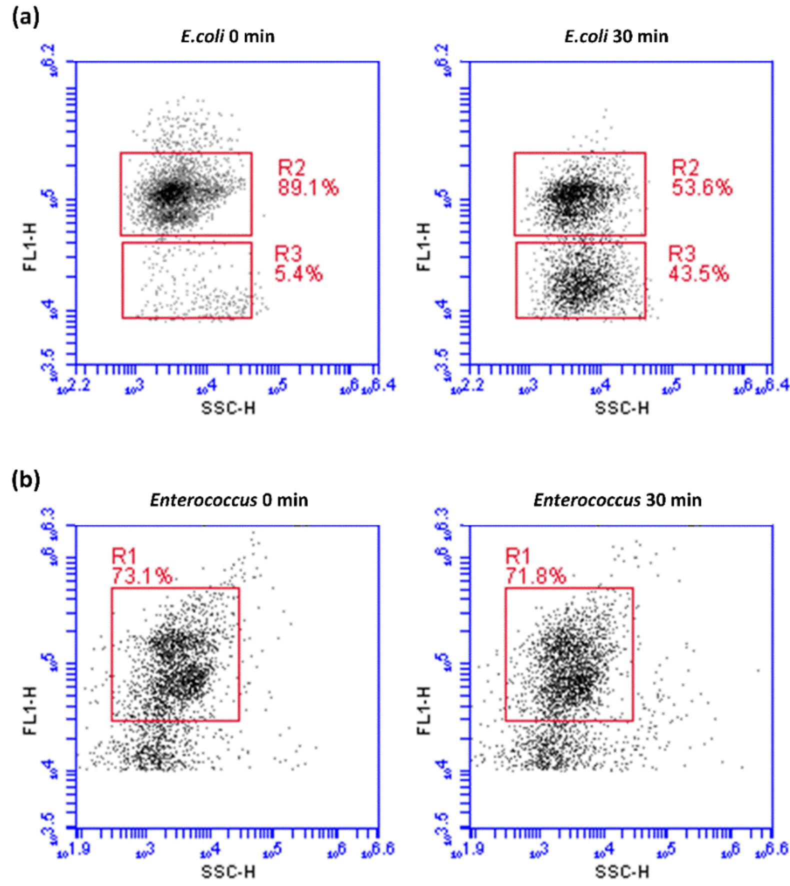

Figure 4-6 Flow cytometry (FCM) density plots of florescence channel 1 *vs.* side scatter (FL1 *vs.* SSC) showing (a) *E. coli* and (b) *Enterococcus* before (left, 0min) and after (right, 30 min) electrochemical (EC) disinfection in toilet wastewater at applied cell voltage of 4V. Samples were stained with 0.5x SYBR-gold. Gate R1-R3 were used to differentiate different bacterial population based on their fluorescence intensity.

The successful inactivation of all four seeded microorganisms was achieved within 60 min at the applied cell voltage of 4V (Figure 4-5a). *E. coli* were highly susceptible to EC disinfection. A 2-log_{10} reduction of *E. coli* was observed within the first 5 min and no viable

E. coli was detected after 20 min of reaction. For *Enterococcus*, a clear lag phase (0 - 5 min) prior to the expected pseudo first-order kinetics was observed indicating their mild resistance to low level of EC produced oxidants. All *Enterococcus* were inactivated to below the detection limit after 30 min. The differences in resistance to EC disinfection between *E. coli* and *Enterococcus* were also reflected by the FCM results (Figure 4-6). After 30 min of EC reaction, no significant change of the particle fluorescence intensity of *Enterococcus* cells was observed (R1, Figure 4-6b), while about 40% of the *E. coli* cells shifted from high fluorescence region (R2, Figure 4-6a) to lower fluorescence region (R3, Figure 4-6a). For both types of bacteria, the fluorescence total cell counts decreased about 10 - 15% indicating they may have become ghost cells (lost DNA integrity) or been totally destroyed after EC disinfection. Due to the lower seeding level, only a 3-log_{10} reduction of rAd5 was shown in Figure 4-5a, yet it demonstrated higher resistance than both types of bacteria. The inactivation of MS2 was the slowest taking about 60 min to reach a 5-log_{10} reduction.

In the comparative CC disinfection experiments, a 5-log_{10} reduction of *Enterococcus* was achieved within 10 min at the total chlorine dosing level of 1.5 mg/L (Figure 4-5b). On the contrary, the inactivation rate of MS2 was significantly lower (< 0.5-log_{10}) even at the highest [HClO] dosage (36 mg/L, as Cl_2). Considering the [NH_4^+] concentration in the toilet wastewater (Table 4-2), monochloramine was the main disinfectant under all [NaClO] dosing levels, although momentarily free chlorine may exist in the system at the beginning of [NaClO] dosing.

4.3.3 Inactivation of MS2 by EC and CC in model waters

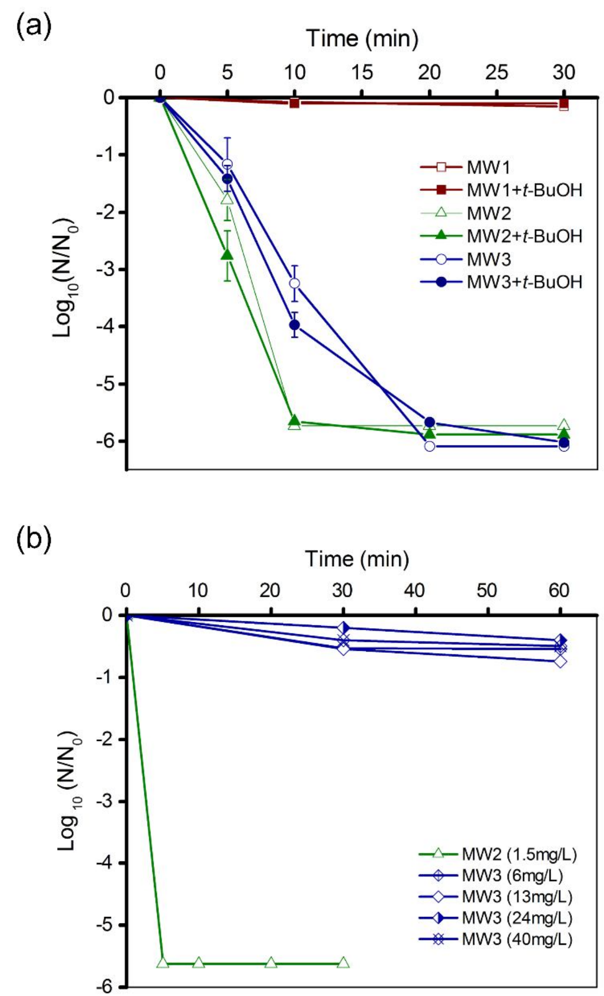

Figure 4-7 Inactivation kinetics of MS2 in model waters by (a) electrochemical (EC) disinfection (applied cell voltage: 4V) and (b) chemical chlorination (CC) disinfection using different concentrations of hypochlorite (as mg/L Cl_2, indicated in the legend).

The inactivation kinetics of MS2 by EC disinfection in MWs are shown in Figure 4-7a. No MS2 reduction was observed in MW1 electrolysis, while a 5-log_{10} reduction of MS2 were achieved within 10 min and 20 min in MW2 and MW3, respectively. The addition of *t*-BuOH (30 mM) slightly enhanced the inactivation rate in MW2 and MW3, but it did not change the

inactivation rate of MS2 in MW1. Figure 4-7b shows the inactivation of MS2 in MWs by CC disinfection. Similar to the toilet wastewater (Figure 4-5b), in MW3, less than 0.5-log_{10} reduction of MS2 was observed at the chlorine concentration as high as 40 mg/L (combined chlorine, as Cl_2). In contrast, in the absence of [NH_4^+], a 5-log_{10} reduction of MS2 was achieved within 5 min in MW2 at the chlorine concentration of 1.5 mg/L (free chlorine, as Cl_2) (Figure 4-7b).

4.3.4 The formation of trihalomethanes (THMs) and haloacetic acids (HAA_5)

Figure 4-8 shows the formation of DBPs in toilet wastewater after EC and CC disinfection. It is found that under both treatment processes, the composition of THMs and HAA_5 appears to be quite similar. For example, trichloromethane [$CHCl_3$] accounted for more than 90% of THMs, while trichloroacetic acid [$C_2HCl_3O_2$] and dichloroacetic acid [$C_2H_2Cl_2O_2$] were the most abundant compounds of HAA_5. Only a small amount of brominated DPBs were detected in the treated water samples (less than 5%). However, the results also demonstrated that the formation of DPBs during EC disinfection was significantly higher than in CC disinfection, although the measured total chlorine concentrations in EC disinfection (4V, 60 min) was equivalent to that in CC disinfection (5 mg/L, as Cl_2). For EC disinfection, when the applied cell voltage was raised from 4V to 5.5V, the concentrations of THMs and HAA_5 were almost doubled (Figure 4-8). The impact of incubation time on DBPs formation was limited. Most of the DBPs had been generated during the reaction (0 h samples), except for the THMs formed during CC disinfection, which increased about 45% after a 24 h-incubation.

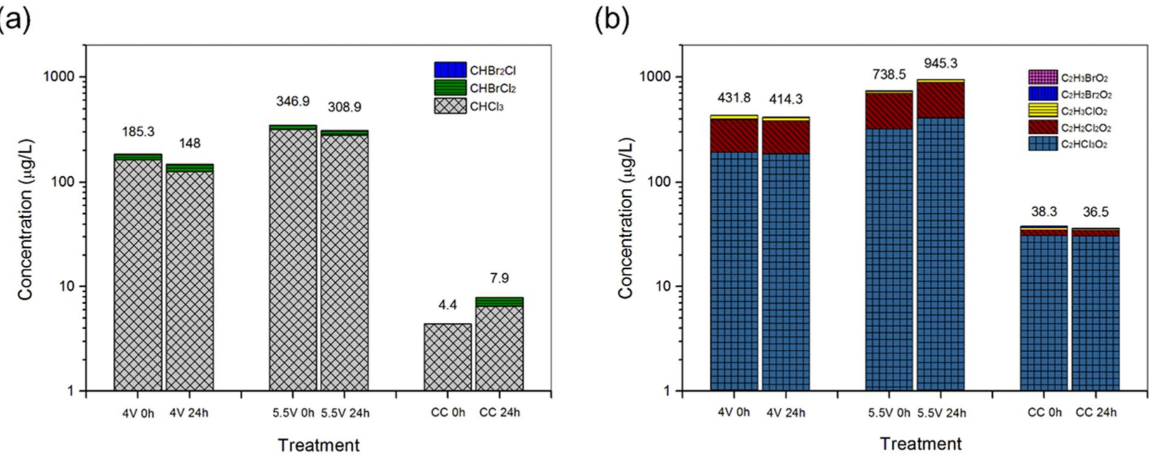

Figure 4-8 The formation of (a) THMs ($CHCl_3$, $CHBrCl_2$, and $CHBr_2Cl$) and (b) HAA_5 ($C_2HCl_3O_2$, $C_2H_2Cl_2O_2$, $C_2H_3ClO_2$, $C_2H_2Br_2O_2$, $C_2H_3BrO_2$) in toilet wastewater after electrochemical (EC) disinfection under applied cell voltage of 4V and 5.5V, and chemical chlorination (CC) disinfection using hypochlorite (5mg/L, as Cl_2). 0 h samples were collected immediately at the end of 1 h reaction, while 24 h samples were collected after a 24-h incubation time.

4.4 Discussion

4.4.1 Oxidant generation and energy requirements

For effective RCS production, the actual anodic potential needs to be higher than the chlorine evolution overpotential (E^0 $[Cl_2]/[Cl^-]$ = 1.36 V). Based on the current data, the applied cell voltage of 4V was necessary to overcome the ohmic resistance to have chlorine generation in the toilet wastewater (Figure 4-3a). The $[Cl_2]$ produced by EC reaction quickly reaches equilibrium with [HOCl] and $[ClO^-]$ in the bulk aqueous phase. At the same time, further oxidation of free chlorine ([HOCl] or $[ClO^-]$) to $[ClO_3^-]$ and $[ClO_4^-]$ can happen

considering the relatively lower reducing potential for the redox couples (E^0 [ClO_3^-]/[ClO^-] = 0.94 V, E^0 [ClO_4^-]/[ClO_3^-] = 1.23 V). Cho et al. (2014b) reported that, [ClO_3^-] was the main inorganic chlorinated byproduct in the electrolysis of municipal wastewater. Trace-levels of [ClO_2] may also be produced during the reaction, which is depending on the pH. Acidic solutions were reported to favor the formation of [ClO_2], while alkaline conditions support the production of [ClO_3^-] (Ghernaout et al., 2011). Although higher applied cell voltage can increase the [Cl_2] generation and maintain the total oxidants at a higher level in the bulk solution (Figure 4-3a), it may also increase the formation of [ClO_3^-] and other highly oxidized oxidants. These oxidants are toxic to plants even at low concentration, which may limit the potential uses of the treated water. Therefore, higher applied voltage or extended reaction time should be avoided once the disinfection goal is achieved. The oxidants generated during the electrolysis of MW samples are consistent with the characteristics of active electrodes. [·OH] and other ROS were not efficiently produced by the BiOx/TiO_2 anode as indicated by the low level of oxidants detected during the electrolysis of inert sample, MW1 (Figure 4-3b). As a consequence, no inactivation of MS2 was observed in MW1 (Figure 4-7a). The result indicates [Cl^-] is an indispensable component for the current EC disinfection system. The increase of chlorine evolution rate in MW2 and MW3 with the addition of the [·OH] scavenger *t*-BuOH conflicts with a previous report showing no significant changes in chlorine evolution levels were observed with active anode (Jeong et al., 2009). The possible reason could be the reaction of *t*-BuOH with the surface bounded [·OH], which in return enhanced the oxidation of [Cl^-] at the interface due to electron transfer.

Based on the current setting for microbial inactivation, the energy requirement of the WEC can be calculated using equation $\boldsymbol{\varepsilon} = \frac{E_{cell} \int i(t)}{V}$, where E_{cell} is the applied cell voltage, i is the current and V is the volume of reactor. Under the optimal condition (E_{cell} =4V, i = 0.125A), to achieve a 5-$\log_{10}$ reduction of the MS2 (conservative model microorganism), the reaction time was about 1 h and thus the energy consumption was estimated to be 2 Wh/L. Considering the ohmic losses and the power required for the circulation pumps and electronic controller, the actual energy consumption of the Caltech 20 L prototype WEC was estimated to be 13~15 Wh/L (260~300 Wh/reaction for 20L). The energy requirement indicates that the WEC can use inexpensive commercial PV-panels as the sole power source (e.g., Sonali 300W, Miami Gardens, FL). As there is no need for external power supply and supporting chemicals, the system is suitable for decentralized toilet wastewater disinfection.

4.4.2 Disinfection mechanisms

The death of bacteria during disinfection process can be generally explained by two types of damages. First, disinfectants can react with cell surface components causing cell membrane permeability changes or the malfunction of enzymatic transport systems. Second, impairments in the intracellular constituents, especially the loss of DNA integrity, can be introduced with or without obvious cell surface damages (Cho et al., 2010). Certain disinfectant may cause more significant damages to either the cell surface or internal components, but these two types of damages are not exclusive, which would also strongly depend on the Ct-value (disinfectant concentration reaction time) as well as the type of bacterial cells. During EC disinfection, *E. coli* and *Enterococcus* behaved differently,

especially in the beginning of the reaction when the concentration of oxidants was below 2 mg/L (0-5 min, 4V). Similar results were found in studies on traditional chlorination disinfection process (Tree et al., 2003). The different inactivation kinetics between the two indicator bacteria are likely related to the cell surface structure differences of the Gram-negative and Gram-positive bacteria, as at low chlorine concentration (< 0.5 mg/L, as Cl_2), the damages of chlorine were reported mainly to the cell surfaces (Phe et al., 2005). However, severe damage to bacterial genomes happened when the chlorine concentration exceed the threshold (free chlorine, between 1.5 and 3 mg/L as Cl_2), which can be assessed by the fluorescence intensity changes in FCM analysis, as damaged bacterial genomic DNA cannot be effectively stained by fluorescent dyes (Phe et al., 2005, Ramseier et al., 2011). In the current study, although both *E. coli* and *Enterococcus* became non-culturable after 30 min of EC reaction, significant fluorescent intensity decrease was only observed for *E. coli* cells (R2 to R3). No such changes were observed for *Enterococcus* cells (Figure 4-6). This result indicates that most of the *Enterococcus* cells still maintained their genomic DNA integrity. These cells could be already dead due to damages to cell surfaces, or they may be still viable but non-culturable. . Injured bacterial cells have been known to be able to repair certain damages when the environmental forcing factors are removed. Thus, the maintenance of a chlorine residual (e.g., 2 mg/L) in the treated water storage tank (Figure 4-1) will help reduce the regrowth of bacteria after EC treatment.

In addition to concerns about bacteria, pathogenic viruses raise even greater concern in toilet wastewater reuse. Current results confirmed that neither *E. coli* nor *Enterococcus* is an adequate indicator for virus inactivation during toilet wastewater disinfection. The fate

of human adenovirus (a double stranded DNA virus) was for the first time studied in an EC disinfection system. The rAd5 demonstrated higher resistance compared to both bacterial indicators (Figure 4-5a). Virus inactivation is more complicated than bacteria by the fact that highly related viruses can exhibit different disinfection kinetics when treated by same disinfection procedure. For example, human adenovirus serotype 40 (HAdV40) and HAdV41 were reported to be more susceptible to monochloramine disinfection than HAdV2 (Cromeans et al., 2010). These variable responses suggest that even minor variations in structural or genomic components can have a remarkable impact on virus resistance to inactivation. Studies have also shown that seeded laboratory-cultured viruses are less resistant to disinfection processes compared to their indigenous counterpart, because indigenous strains are often embedded in biofilm or attached to suspended solids, which may shield the virus against disinfectants (Tree et al., 2003). All these factors highlighted the importance of choosing a conservative virus surrogate in evaluating the disinfection capability of EC system. In this context, MS2 should be an ideal candidate as it demonstrated higher resistance than rAd5 in the current study and had been reported to be more resistant than poliovirus (Tree et al., 2003), coxsackieviurs (Tree et al., 2003), and hepatitis A virus (Casteel et al., 2008) to a variety of disinfectants. When a 5-$\log_{10}$ reduction of MS2 is achieved after EC disinfection, other bacteria and viruses in the toilet wastewater should also be effectively inactivated and the health risks associated with non-potable water reuse would be significantly reduced.

The comparative experiments between EC and CC disinfection clearly demonstrated the advantage of EC disinfection for virus disinfection in toilet wastewater. In [NH_4^+] free MW1,

a 5-log_{10} reduction of MS2 was achieved at free chlorine level of 1.5 mg/L within 5 min. The result showed that MS2 was in fact very sensitive to free chlorine. Wigginton et al. (2012) studied the damages of free chlorine to MS2 and found it is a non-discriminative oxidant causing the losses of functions like genome-mediated replication and protein-meditated injection. The current results indicate that the chlorine species (free or combined chlorine) were the decisive factor controlling the virus disinfection efficacy. Compared to free chlorine, monochloramine is a much weaker disinfectant, which is only effective for bacterial inactivation, but not effective for viruses (Figure 4-5b). When free [Cl_2] to [NH_4^+] ratio (weight ratio Cl_2:NH_3-N) was less than 5, the conversion of free chlorine to monochloramine can be completed within seconds under optimal conditions (pH=8.4; 25 ^oC) (Kirmeyer et al., 2004). This explained the limited virus reduction observed during CC disinfection in the toilet wastewater and MW3. For CC disinfection, free chlorine residual can be created by adding extra amount of [NaClO] to bring the system past the breakpoint (weight ratio Cl_2:NH_3-N>9:1). However, this may not be practical for raw toilet wastewater disinfection, as the total chlorine residual would be too high for any reuse applications (> 500 mg/L, as Cl_2). In contrast, free chlorine was always present during toilet wastewater electrolysis even with the present of free ammonia, because it was continuously produced on the surface of anode. Free chlorine may react with viruses before it was converted to chloramines. In addition, a local pH decrease at the anode surface occurs due to the production of [H^+] through [O_2] evolution. Lower pH can significantly reduce the chlorine-ammonia reaction rate. Acidic conditions also favor the formation of neutral [HClO], a more effective disinfectant than the negatively charged [ClO^-]. Lastly, ammonia was converted to [NO_3^-] and [N_2] during EC reaction, and thus the [Cl_2] to [NH_4^+] ratio was increasing with

reaction time. Besides free chlorine, [ClO_2] may also contribute to the MS2 inactivation in EC disinfection. [ClO_2] have been shown to cause the degradation of viral proteins and thus inhibit the host-cell recognition and binding (Wigginton et al., 2012). However, considering the low concentration of [ClO_2] during the reaction, its virucidal effect may be limited.

4.4.3 DBPs Formation

Previous studies have shown that the formation and distribution of DBPs in chlorinated waters are dependent on water source (levels of organic precursors), contact time, pH, and the bromide concentration (Hua and Reckhow 2008, Hua et al., 2006). In the present study, the DBPs formation observed after EC and CC disinfection can be mainly explained by the difference of chlorine species present in the systems. Although the measured total chlorine residuals were the same in both systems (EC 4V *v.s.* CC), as discussed before, free chlorine was constantly present in the EC system during toilet wastewater electrolysis. It may react with organic matter to form DPBs before it was converted to chloramines. However, in CC disinfection, the high reaction rate between free chlorine and ammonia indicates that the competition reactions between free chlorine and organic matter were suppressed. This postulation is supported by previous studies showing that fewer THMs, HAA_5, and total organic halogen (TOX) were generated in chloramination process (combined chlorine) than those in chlorination process (free chlorine) (Hua and Reckhow 2008). The presence of [NH_4^+] also affected the ratio between THMs and HAA_5. A high concentration of ammonia level (e.g., >5 mg/L as N) was reported to inhibit the production of THMs, while lower ammonia concentration (<0.5 mg/L as N) favored the THMs production in treated wastewater effluent (De Leer et al., 1990). Currently, there are no guidelines related to

DPBs levels in wastewater reuse. In this study, the concentrations of THMs and HAA_5 detected after EC disinfection were generally within the range of those reported in chorine disinfected secondary wastewater effluent (Bougeard et al., 2010, Krasner et al., 2009) or swimming pool waters (Lee et al., 2010). The result indicates the EC treated toilet wastewater should be safe for non-potable reuse applications from the aspect of DBPs.

4.5 Conclusions

- EC disinfection using WEC can effectively inactivate both viruses and bacteria in toilet wastewater without adding any supporting electrolytes. The system can be developed into a commercial viable self-sufficient solar-powered mobile toilet for decentralized wastewater treatment.
- Viruses were more resistant than bacteria in both EC and CC disinfection. A 5-$\log_{10}$ reduction of MS2 (the conservative model microorganism) in toilet wastewater can be achieved by EC disinfection at applied cell voltage of 4V in 1 h, while CC disinfection is not effective for virus inactivation in toilet wastewater.
- RCS are the main disinfectants produced by the active bismuth-doped TiO_2 anode. Ammonia can significantly reduce the disinfection efficiency by converting free chlorine to chloramines. The high inactivation rate of viruses with EC disinfection can be explained by the coexistence of free chlorine and free ammonia during EC reaction.
- Higher applied cell voltage and longer reaction time will generate more organic DBPs (THMs and HAA_5). Most of the DBPs are formed during the EC reaction rather than the after treatment incubation period.

- The WEC treated toilet wastewater is safe for non-potable reuse, such as toilet flushing and agricultural irrigation.

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