MICROBIAL QUALITY AND RISK ASSESSMENT OF ALTERNATIVE SOURCES OF DRINKING WATER IMPACTED BY WASTE WATER: AN ANALYSIS OF NC TYPE 2 RECLAIMED WATER FOR POTABLE REUSE

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

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ABSTRACT

Emily Bailey: Microbial Quality and Risk Assessment of Alternative Sources of Drinking Water Impacted by Waste Water: An Analysis of NC Type 2 Reclaimed Water for Potable Reuse (Under the direction of Mark Sobsey)

Recent North Carolina reclaimed water legislation has proposed a new potable reuse scheme that involves the use of the combination of tertiary treated, dual disinfected reclaimed water with currently used drinking water sources of surface water in a ratio of at least 80% surface water and up to 20% reclaimed, followed by storage for a minimum of 5 days and treatment by conventional drinking water treatment processes. However, the tertiary treated, dual disinfected reclaimed water proposed by NC, for potable reuse and designated as type 2 has not been evaluated for microbial quality or examined in full-scale production scenarios.

The goal of this research was to collect real world data on type 2 reclaimed water by conducting field studies on the performance of NCT2 like reclaimed water producing treatment facilities, as well as to evaluate the risk of exposure to this water in potable reuse scenarios by conducting microbiological water quality analyses and then quantitative microbial risk assessments (QMRAs). Field samples of wastewater and water were collected over a one-year period from 4 NCT2RW producing facilities, along with sewage impacted surface waters considered candidates for the 80/20 combination as sources for drinking water production. Water samples were examined for the microbial indicators specified in the NC legislation and for representative pathogens of public health interest.

Based on microbial water quality analyses and QMRA analysis, there is evidence that the risks associated with either consumption associated with potable reuse or agricultural risks

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associated with exposure to raw fruits and vegetables, are not reduced below the annual risk level of 1 x 10^{-4} set by US EPA for drinking water. Relatively high concentrations of human enteric viruses, especially culturable enteric adenoviruses as well as microscopically detectable protozoan parasites were detectable in samples of NCT2RW that met the water quality requirements for the regulated fecal indicator microorganisms. These results have implications for the practical use of this type of reclaimed water as a source of drinking water and produce irrigation in the future, compared to its current use only for landscape irrigation.

To the Sobsey Lab

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LIST OF ABBREVIATIONS

CFU	Colony forming unit		
Ct	Cycle Threshold		
DALY	Disability Adjusted Life Year		
DNA	Deoxyribonucleic acid		
GEC	Genetic equivalent copies		
ICC-PCR	Integrated cell culture polymerase chain reaction		
ICC-qPCR	Integrated cell culture quantitative polymerase chain reaction		
ICC-RTqPCR	Integrated cell culture reverse transcriptase quantitative polymerase chain reaction		
IMS-FA	Immunomagnetic separation – fluorescence assay microscopy		
IU	Infectious unit		
L	Liter		
М	Meter		
MF	Membrane filtration		
mL	Milliliter		
MPN	Most probable number		
MPNIU	Most probable number of infectious units		
mRNA	Messenger ribonucleic acid		
NAS	National Academy of Science		
NC	North Carolina		
NC DENR	North Carolina Department of Environment and Natural Resources		
NCT2RW	North Carolina Type 2 Reclaimed Water		
OWASA	Orange Water and Sewer Authority		

PFU	Plaque forming unit
QMRA	Quantitative microbial risk assessment
qPCR	Quantitative polymerase chain reaction
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
RV	Rappaport-Vassilades broth
SMEWW	Standard Methods for the Examination of Water and Wastewater
SS	Salmonella-Shigella agar
Spp.	Species
μL	Microliter
US	United States
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1: OVERVIEW AND OBJECTIVES

1.1 Introduction and Background

As of September 2014, the State of North Carolina has approved tertiary treated, dual disinfected (with UV radiation and free chlorine as defaults) wastewater (called type 2 reclaimed water, NCT2RW) for both non-potable agricultural use and potable reuse. This research evaluated both NCT2RW and riverine surface waters currently used as drinking water sources for their health-related microbial quality by quantifying fecal indicator organisms mandated for water quality testing by NC law and compared them to concentrations of culturable pathogenic bacteria (*Salmonella* spp.), human enteric viruses detected by cell culture (adenoviruses) and molecular (adenoviruses and noroviruses) methods, and protozoan parasites (Cryptosporidium and Giardia) detected by immunofluorescent microscopy methods in each type of water. Public health interest is generally focused on pathogen content in treated wastewater used as reclaimed water for both potable and non-potable purposes, but safety decisions and management systems on microbial quality are often based on concentrations of fecal indicator organisms. Therefore, a secondary focus of this research was to evaluate the relationships between microbial indicator and pathogenic microorganisms in NCT2RW and ambient surface waters.

The State of North Carolina defines potable reuse as a combination of up to 20% NCT2RW with at least 80% surface source water with a 5-day storage time under unspecified conditions. Performance targets are defined as allowable concentrations of fecal indicator bacteria (*Escherichia coli*), viruses (coliphages) and a protozoan parasite surrogate (*Clostridium perfringens*) in the reclaimed water, reductions in log₁₀ concentrations of these fecal indicator

microbes as well as monitored monthly geometric mean levels for each of these indicator microorganisms. Log₁₀ reduction targets are 6 for bacteria, 5 for viruses, and 4 for protozoan parasite surrogates. The monthly geometric mean and daily maximum target concentrations for these fecal indicator bacteria, viruses, and the protozoan parasite surrogates in final effluent NCT2RW are summarized below.

- Monthly geometric mean *Escherichia coli* or fecal coliform levels of less than or equal to 3/100mL with a daily maximum of less than or equal to 25/100mL.
- Monthly geometric mean coliphage levels of less than or equal to 5/100mL with a daily maximum level of less than or equal to 25/100mL.
- Monthly geometric *C. perfringens* levels of less than or equal to 5/100mL with a daily maximum of less than or equal to 25/100mL.

After the 5-day storage time, the combined up to 20% NCT2RW and at least 80% surface source water blend is treated by traditional drinking water treatment processes. For conventional drinking water treatment processes, which typically involve the use of coagulation/flocculation followed by rapid granular media filtration and final disinfection, it is anticipated that this treatment will result in additional log₁₀ reductions. Both the United States Environmental Protection Agency (US EPA) and the World Health Organization (WHO) have defined performance targets for treated drinking water (US EPA, 2006;WHO, 2011); targets will be defined later in this report.

The microbial quality of reclaimed water produced by some reclaimed water treatment trains has been evaluated previously (Harwood et al., 2005; Rodriguez et al., 2009). However, the tertiary treated, dual disinfected reclaimed water proposed by the state of North Carolina for potable reuse and designated as type 2 has not been evaluated in full-scale production scenarios

nor has it been studied when blended with surface source waters and stored for 5 days. A previous pilot scale study evaluating the dual UV and chlorine disinfection system for tertiary treated sewage as type 2 reclaimed water proposed in NC, concluded that dual disinfection is effective for reducing concentrations of fecal indicator bacteria, viruses and protozoan parasite surrogates in producing high quality reclaimed water (Sobsey et al., 2005). The goal of this research is to expand on this initial study by conducting field studies on the performance of NCT2-like reclaimed water producing treatment facilities, as well as to evaluate the risk of exposure to this water in various potable reuse scenarios by conducting quantitative microbial risk assessments (QMRAs).

1.2 Research Objectives

This study aims to address the need for real-world data that documents the microbial quality and microbial reduction performance of NCT2 reclaimed water systems, by quantifying the levels of fecal indicators and key pathogens in raw sewage, final reclaimed water, and source surface water proposed for blending and potable reuse. The specific research objectives of this work are outlined below:

- To quantify indicator and pathogenic microorganisms in North Carolina Type 2-like Reclaimed Water (NCT2RW) and raw sewage from 5 representative wastewater treatment plants, 4 with dual disinfection (Type 2-like) and one with single barrier disinfection (Type 1-like). Candidate treatment plants include those local to the Research Triangle area of which four produce type 2-like reclaimed water and one produces Type 1-like reclaimed water.
- To quantify concentrations of microbial indicators and pathogens in actual or potential drinking water treatment plant influent waters, specifically run of river drinking water

sources representative of candidate source water influents for blending with NCT2RW. Candidate drinking water treatment plant source waters include those downstream of wastewater treatment plants as run of river drinking water treatment plants and those using influent waters from reservoirs impacted by municipal or industrial wastewater discharges.

- To evaluate the allowable 80/20 mix of surface water and Type 2-like reclaimed water approved for potable reuse in NC for the effects of storing this mixture for the required 5 days at various temperatures and with and without sunlight exposure, based on initial microbial indicator concentrations and their changes in concentrations after 5 days of storage under different environmental conditions.
- To use indicator and pathogen data, collected in aims 1-3, to conduct quantitative microbial risk assessment (QMRA) analyses for potable reuse of reclaimed water combined with surface water in an 80/20 mix and stored for 5 days, followed by conventional drinking water treatment, and compare risks to the US EPA acceptable microbial drinking water risk level of 10⁻⁴ infections/person/year based on data for key virus, bacteria and protozoan parasite pathogens.
- 1.3 Review of the Literature
- 1.3.1 Global Population and Water Resources

As the global population grows and stress on water resources continues to escalate, it becomes increasingly important to consider solutions and alternative water sources to prevent restrictions and overcome shortages. In the United Nations' <u>World Population Prospects: The 2012 Revision</u>, the world population is estimated to reach 8.1 billion by 2025 and 9.6 billion by 2050. In the United States specifically, between 1900 and 2000 the population grew from 76 million persons to 282 million persons, an increase of 240 percent (US Census, 2010). Also by

2015, urban populations are expected to grow by more than 2/3 from 3.9 billion to 6.3 billion people with the largest increases in Asia and Africa (UNDESA, 2014).

In order to address the expanding water supply needs for the growing population in the United States, the 20th century was a time of building water infrastructure, particularly dams and aqueducts (NAS, 2012). Large projects built on the Colorado River, and the Central Valley of California have provided water and power to support the rapidly growing population and increases in irrigated agriculture (NAS, 2012). Although some smaller water supply and storage projects are still being constructed, infrastructure advancement has decreased in recent years (Gliek et al., 2003).

The decline in infrastructure capacity expansion has come at a time of increased demand likely as a result of several causes: 1) a diminished number of rivers with appropriate flow for dam projects, 2) increased environmental concern over dam or other large water infrastructure projects, and 3) an increased understanding of water quality problems resulting from irrigated agriculture (NRC, 1989). Regional development and migration have also put stress on water sources, as large populations have migrated to warmer climates in California, Nevada, Arizona, and Florida.

Population growth, and movement is further complicated by the impact of climate change and variability, specifically with the effects of droughts and flooding (World Water Assessment Program 2009, IPCC 2014). Temperature increases will result in increased evapotranspiration and the use of additional water for irrigation of agriculture and landscaping; additionally, changes in precipitation patterns may diminish the ability of existing water infrastructure (such as reservoirs) to collect water (NRC, 2007). As considerable uncertainty remains about the

potential impact of climate change on water resource availability, it is necessary to re-examine the way in which water is acquired and used before problems with water supply arise. One response to reducing vulnerabilities to the impacts of climate change and climactic variability is increasing the sustainability of drinking water supplies; as such, one of the mechanisms proposed for this change is reducing or restricting water consumption and use. As the population of the United States continues to increase, water consumption for public supply use has also continued to increase; however, industry and irrigation use have decreased in recent decades (NAS, 2012). Reuse of municipal wastewater has also been proposed as a potential solution for augmenting drinking water supplies either by direct or indirect mechanisms. As per capita water use continues to increase, alternative water solutions such the reuse of municipal wastewaters have been proposed as solutions to augment the water supply. It is possible to produce large volumes of high quality drinking water from treated wastewater through various potable reuse schemes. In addition, potable reuse reduces the potential impacts of wastewater discharge on downstream marine or freshwater habitats. However, as the need for freshwater resources for drinking water supply and other beneficial uses increases, it is imperative that the currently used surface water and other freshwater resources, including those impacted by wastewater discharges, as well as the reclaimed water sources proposed for potable reuse be evaluated and examined for microbial quality and risk.

1.3.2 Wastewater Reclamation for Potable Purposes

Water reclamation for potable reuse has been proposed by communities both within the US and abroad. In many cases, this reclaimed water is used indirectly for purposes such as groundwater recharge or for agricultural or landscape irrigation purposes. Since the 1980s, the effluent from the Upper Occoquan Sewage Authority (UOSA) has been serving as a raw water supply for Washington, DC (Lauer and Rogers, 1996). This model of high quality effluent used

as source water for a reservoir has been repeated in Orange and Los Angeles counties, California and El Paso, Texas. However, at present, many states are currently working to expand their capacity for direct reuse of reclaimed wastewater, specifically drinking water purposes. With California leading the way on legislative standards, draft regulations for potable reuse projects have been proposed and a report and cost analysis was provided to the California legislature in December of 2016 (California Environmental Protection Agency, 2016). Since 2004, San Diego has operated an indirect potable reuse treatment facility, with the ultimate goal of conversion to direct potable reuse to produce one-third of the city's water supply in the future (Brandhuber et al., 2015). The state of Texas is also planning to expand its potable reuse infrastructure to 1% of all new water supplies by 2070 (Texas Water Development Board, 2015). In the City of Big Spring, TX, the state's first direct potable reuse facility is currently providing 2 million gallons per day that are blended with surface water; additionally, two other indirect potable reuse projects currently operating include the City of Abilene's Lake Fort Phantom Hill project and the North Texas Municipal Water District's constructed wetland project. Additionally, the city of Wichita Falls, TX participated in an emergency direct potable reuse production as a result of an extended drought in 2014, which provided 5 million gallons per day of disinfected wastewater effluent which was blended with raw surface water (Brandhuber et al., 2015). New Mexico has also been a leader in direct potable reuse projects, with its Village of Cloudcroft project that incorporates advanced treatment technologies (chloramination, reverse osmosis, and advanced oxidation) to provide up to 50% of the drinking water supply for the golf village (Brandhuber et al., 2015).

Globally, potable reuse schemes have also become more common as population pressures become a reality in many areas of the world. The city of Windhoek, Namibia has been reusing

wastewater since 1969; as of 2006, reclaimed water fulfilled 35% of the city's water demand (du Pisani, 2006). Though Namibia itself, and the city of Windhoek in particular, has few natural freshwater resources, reclaimed water has augmented water infrastructure to increase water supply access to a larger number of people.

Singapore has also taken significant strides to incorporate reclaimed water into the supply of available water. However, in Singapore, only a small portion, approximately 2.5%, of the country's domestic water usage comes from reclaimed water. This water is used for blending with source waters for potable reuse purposes or for industrial applications that require ultrapure water (Tortajada, 2007). In Singapore, as a result of historical water agreements there is increased focus on the purchase of water from Malaysia as well as the implementation of desalination as an alternative water source (Marks, 2006).

Though potable reuse, direct or indirect, has become more common globally, in the US it is still considered relatively uncommon. Communities that typically consider potable reuse are those that are either in areas of intense water stress or areas with large populations in need of expanded water supplies, such as Texas, California, or New Mexico. In the case of North Carolina, it is likely that potable reuse would occur in either large cities with stressed water resources or in areas with high water demand.

1.3.3 Microorganisms of Concern in Waste and Reclaimed Water and Treatment of Wastewater for Pathogen Reduction

As the source of reclaimed water is domestic raw sewage, the microorganisms of concern are generally those of human health interest that are present and persist through conventional treatment processes. The most common and persistent organisms of concern are presented in Table 1-1.

Class	Pathogen				
Bacteria	Aeromonas hydrophila				
	Salmonella spp.				
	Shigella spp.				
	Campylobacter spp.				
	Cyanobacteria				
	Helicobacter pylori				
	Legionella pneumophila				
	Pathogenic Escherichia coli				
	Mycobacterium spp.				
Virus	Human caliciviruses				
	Human rotaviruses				
	Enteroviruses				
	Hepatitis A Virus				
	Adenoviruses				
	Astroviruses				
	Coxsackieviruses				
	Reoviruses				
Protozoa	Acanthamoeba				
	Giardia lamblia				
	Cryptosporidium parvum and Cryptosporidium hominis				
	Entamoeba histolytica				
	Naegleria fowleri				
Helminth	Ascaris lumbricoides				
	Ancylostoma duodenale				

Table 1-1: Pathogens of concern in wastewater (from US EPA Contaminant Candidate List (CCL), US EPA, 2016)

Undisinfected secondary effluent typically contains relatively high levels of fecal indicator bacteria (fecal coliforms, and enterococci), with concentrations in the thousands per 100mL (Sobsey et al., 2005). Pathogen levels are typically much lower, typically in the range of 10s to 100s per 100 liters. For protozoan parasites, the main method of removal by wastewater treatment is physical removal by chemical pretreatment and filtration. Robertson et al., 2000 found that by using vital stains, a technique known for over estimating infectivity, 46% of effluent samples contained viable *C. parvum* oocysts, while another study (Harwood et al., 2005) using a cell culture infectivity method found that in chlorine treated reclaimed water, 25% of oocysts were infectious. However, it is important to note that UV radiation plays an important role in the inactivation of *C. parvum* and *G. lamblia* (oo)cycsts in water and wastewater (Shin et al., 2001; Linden et al., 2002) as some chemical disinfectants such as chlorine are not effective in the disinfection of these pathogens.

In typical raw sewage, \log_{10} reductions of *C. parvum* and *G. lamblia* are expected to be <1 \log_{10} by primary sedimentation and between 1-2 \log_{10} by biological treatment (Caccio et al., 2003). A UV dose of 1 mJ/cm² is expected to give >4 \log_{10} inactivation of *G. lambia* infectivity (Linden et al. 2002) while a 10 mJ/cm² dose is expected to give a 3 \log_{10} inactivation of *C. parvum* infectivity (Shin et al., 2001). For viruses, the expected \log_{10} reductions are <1 \log_{10} by primary sedimentation and about 1-2log₁₀ reductions by secondary biological treatment. Viruses, though inactivated by low doses of free chlorine, ozone and chlorine dioxide, are more resistant to disinfection processes than enteric bacteria (Sobsey, 1989). Other factors that impact virus disinfection include the presence of organic material and other particulate debris in wastewater, which increases the need for pretreatment to ensure virus inactivation. As the least resistant and persistent of the enteric pathogens, bacterial pathogens are expected to be reduced extensively by

wastewater treatment processes. Enteric bacteria are expected to be reduced by $<1 \log_{10}$ by primary treatment, $2 \log_{10}$ by secondary biological treatment and by $>4 \log_{10}$ by disinfection processes.

As the North Carolina legislation for potable reuse (NC DENR, 2014) proposes the combination of tertiary treated and then dual disinfected reclaimed water blended with surface water, followed by 5 days of storage and then drinking water treatment, it is also important to consider the impact of drinking water treatment. After the 5-day storage time, the combined up to 20% NCT2RW and at least 80% surface source water blend is treated by traditional drinking water treatment processes. In the United Sates, the US EPA's Safe Drinking Water Act establishes microbiological water treatment objectives. Expansions of the Safe Drinking Water Act, specifically the Long Term Enhanced Surface Water Treatment Rule (LTESWTR) and more recently the Long Term 2 Enhanced Surface Water Treatment Rule (LR2ESWTR), have increased the requirements for pathogen reduction in surface waters by requiring additional treatment in some circumstances. The LTESWTR has expanded protection to communities of less than 10,000 people and established a minimum 2 log₁₀ reduction for Cryptosporidium (US EPA, 2003); additionally, this rule established filter monitoring to minimize poor performance of individual units and explicitly considered unfiltered system watershed control provisions. The goal of the LT2ESWTR was to expand protection for high risk water sources, such as those who store treated water in open reservoirs by requiring water utilities to either cover their open reservoirs or to achieve additional log₁₀ reduction performance requirements (4 log₁₀ for virus, 3 log₁₀ for *Giardia lamblia*, and 2 log₁₀ for *Cryptosporidium*) (US EPA, 2006). These drinking water performance objectives provide a minimum performance target for water suppliers to treat water and produce microbiologically safe drinking water. In a recent review of studies evaluating

conventional drinking water treatment processes, characterized by coagulation/flocculation followed by rapid granular media filtration, Medema and Hijnen found that this treatment would result in \log_{10} reductions of approximately $2.1 \pm 0.8 \log_{10}$ in bacteria a $3.0 \pm 1.4 \log_{10}$ in viruses, and a $3.3 \pm 1.1 \log_{10}$ in protozoan parasites (Medema and Hijnen, 2007). In general, these values, when combined with disinfection processes, meet the \log_{10} performance guidelines set by the US EPA for surface waters treated by conventional treatment.

In addition to the required log₁₀ reductions, disinfection processes are commonly used to achieve additional log₁₀ reductions or credits for the removal of pathogens from surface waters. Drinking water treatment plants may use chemical treatment processes such as ozone or chlorine to disinfect surface waters that have been previously treated by conventional processes. Typically, a treatment plant will adjust either the residual disinfectant concentration "C" or the contact time for the disinfectant "T" to increase the overall CT value and provide additional credit for *Giardia* cyst and virus reductions as calculated by the LTESWTR (US EPA, 2003); it is expected that by increasing the CT value, viruses will be reduced by an additional 4 log₁₀ and *Giardia* cysts will be reduced an additional 3 log₁₀.

In contrast to EPA log₁₀ performance target monitoring, the World Health Organization (WHO) has an alternative system for evaluating the health risk associated with water containing pathogens. The World Health Organization's *Guidelines for Drinking Water Quality* (WHO, 2011) uses the Disability Adjusted Life Year (DALY) as a unit of measure for risk. The goal of a DALY is to calculate a value that considers both the probability of experiencing an illness or injury and the impact of the associated health effects (Havelaar and Melse, 2003). The WHO guidelines adopt a 10⁻⁶ DALY/person per year as a health risk management target. In general, this means if 1 virus is found in 100L of water, the overall log₁₀ reduction required would be 4

 \log_{10} Likewise if 10 viruses were found in 100L of water, the \log_{10} reduction would be 5 \log_{10} . The WHO recommends a minimum of a 4 \log_{10} reduction for enteric viruses in surface waters, depending on the surface water source. Similarly, a 3 \log_{10} reduction is required for *Giardia* and *Cryptosporidium*.

The potable reuse scheme proposed in North Carolina is a multi-barrier scheme having several treatment processes. Though more expensive wastewater treatment technologies, such as membrane filtration technologies (micro-, ultra-, nano and reverse osmosis filters), required by states such as California, are not required for the production of the reclaimed water in NC, the provision for a combination with at least 80% surface water, storage for 5 days as well as further treatment by conventional drinking water treatment processes, has been proposed as a means of providing adequate log₁₀ reductions of pathogens prior to direct potable reuse.

1.3.4 Epidemiological Studies of Wastewater Reuse Systems

Several epidemiological studies have been conducted with the goal of determining the human health effects from exposure to pathogens in reclaimed water. These studies have focused on health effects such as risk for workers who handle reclaimed water or individuals living adjacent to reclaimed water systems. The quality of water resources impacted by reuse systems has been evaluated in California, specifically the groundwater recharge systems.

A study examining the health impacts of a groundwater recharge system using reclaimed water was conducted in the Montebello Forebay area of Los Angeles County, CA. This project has been in place since 1962, and the study focused on evaluating water quality, percolation, the development of population exposure data, and the epidemiological investigation of potentially exposed population (Sloss et al., 1996). No adverse health impact was found on the area's groundwater or on consumers of the recharged groundwater. In 1986, the State of California appointed an independent panel of scientists to revaluate this work, and the panel concluded that

the risks associated with this reclaimed water project were minimal and likely equivalent to those predicted for the use of surface waters for drinking (Sloss et al., 1996). It is important to note that the reclaimed water used in California for groundwater recharge is of very high microbial quality, indicating that it is possible to achieve a low level of epidemiological risk with a high quality level of reclaimed water.

Another study was done in Mexico, with reclaimed water produced with a low to moderate level of biological treatment and no disinfection and used for agricultural irrigation. This epidemiological study examined the quality of the reclaimed water, seasonal effects, and wastewater storage (Blumenthal et al., 2001). Health effects analysis was conducted for Ascaris *lumbricodes* infection and diarrheal illness; data was collected from individuals by survey with 3 categories of exposure including: untreated wastewater, effluent from a storage reservoir (< or =1 nematode egg/L), or no wastewater irrigation (control group). This study found that direct exposure to untreated wastewater was associated with increased risk of A. lumbricoides infection and an increased risk of diarrhea (Blumenthal et al., 2001). The authors concluded that treatment in wastewater retention in a single reservoir (to a quality of 10^5 fecal coliforms/100mL, < or = 1eggl/L) does not significantly reduce risk, but that treatment in two reservoirs in series does reduce risks to non-detectable levels. As the burden of *Ascaris* infection and infections with other enteric pathogens in the US is much lower than in Mexico, the exposures of these two populations are likely different. However, from these results it is clear that modest treatment of wastewater to produce reclaimed water is not sufficient to adequately reduce risks from pathogens.

Studies in the US more specifically have examined human health risks from the agricultural reuse of reclaimed water. One such study, referred to as the Lubbock Infection

Surveillance study (Camann et al., 1985) focused on monitoring viral and bacterial infections associated with aerosol exposures in a rural community surrounding a spray injection site near Wilson, Texas. The reclaimed irrigation water was undisinfected trickling filter effluent that was used for spray irrigation; fecal coliforms, fecal streptococci, mycobacteria and coliphages were above ambient levels for at least 650 feet (200 meters) downwind. Geometric mean concentrations of enteroviruses recovered from 150 to 200 feet downwind were 0.05pfu/m³, which is a higher than observed at other spray irrigation sites in the US and Israel (Camann et al., 1988). Despite high detectable levels of indicator organisms and enteroviruses, no significant relationship was detected between the self reported acute illness and degree of aerosol exposure. However, among individuals with a higher degree of aerosol exposure, serological testing of blood samples indicated that the rate of viral infections was slightly higher.

As presented in the epidemiological studies above, it is clear that the quality and treatment of the reclaimed water is important in determining human health risk. Wastewater that has been treated to a greater extent, such as biological treatment, and disinfected, to achieve greater levels of pathogen reduction has had less human health effect in the past, as seen in the study in California. However, with new treatment technologies and new reuse schemes it is important to evaluate the quality of the microbiological reclaimed water before assessing the epidemiological risks and human health effects.

1.3.5 Current Guidelines on Wastewater Reuse

Currently, there are no comprehensive standards recommended for the production and quality monitoring of reclaimed water for direct potable reuse; however, in the United States, indirect potable reuse is considered in the 2012 Guidelines for Water Reuse (US EPA, 2012). These guidelines propose broad recommendations for indirect potable reuse requiring a combination of treatment processes, water quality criteria, monitoring requirements, and a 2

month retention time using an environmental buffer. In the Framework for Direct Potable Reuse, Tchobanoglos et al., 2015 review relevant US standards and summarize microbial and chemical criteria for direct potable reuse. A summary of US states and organizations requiring log₁₀ reductions in their reclaimed water standards is providing in Table 1-2, adapted from information in the Tchobanoglos et al., 2015 review. Regulations in California require that indirect potable reuse (a groundwater recharge application) achieve at least a 12 log₁₀ enteric virus reduction, a 10 log₁₀ *Giardia* cyst reduction, and a 10 log₁₀ *Cryptosporidium* oocyst reduction (California Department of Health Services, 2014). Standards established by the National Water Research Institute require similar log₁₀ reductions of 12 for enteric viruses, 10 for enteric protozoa (*Cryptosporidium* and *Giardia*) and 9 for total coliform bacteria.

In addition to California, Florida has also expanded capacity for groundwater recharge applications, with established standards for on-going fecal coliform testing with periodic pathogen monitoring for *Giardia* and *Cryptosporidium* (Florida Department of Environmental Protection (DEP), 1999). The fecal coliform limit for reclaimed water used for recharge of groundwater for indirect potable reuse is a monthly geometric mean of 4 CFU/100mL, while the suggested detection of *Cryptosporidium* and *Giardia* is 1(00)cyst per 100L. Guidelines for direct potable reuse have also been prepared by the state of New Mexico (NWRI, 2016) and by the Texas Water Development Board (TWDB, 2015). The Texas guidelines set log₁₀ reductions for wastewater treatment plant effluent, and not untreated wastewater, of 8 log₁₀ enteric virus removal, 6 log₁₀ *Giardia* removal, and 5.5 log₁₀ *Cryptosporidium* removal.

In terms of global monitoring of potable reuse, the WHO has proposed reclaimed water guidelines that involve monitoring fecal coliforms and intestinal nematodes with levels depending on the category of water use (type of crop irrigation) (WHO, 1989). Other countries,

particularly those in water stressed regions of the world, have been regulating and producing reclaimed water for many years. One important example is Windhoek, Namibia. As no international guidelines existed for potable reuse, a publication by Stander and Van Vuuren (1969) was referenced to establish chemical criteria, health hazards, and toxicity as indicators for pollution in treated wastewaters for early reuse in Namibia. These initial standards were later used in conjunction with the International Standards for Drinking Water (WHO, 1963). Australia also has guidelines for drinking water augmentation (NRMMC-EPHC-NHMRC, 2008); these guidelines focus on defining microbial safety in terms of a health outcome target (10⁻⁶ DALYs per person per year), and achieving safety through applying microbial performance targets (9.5 log₁₀ enteric virus, 8 log₁₀ *Cryptosporidium*, and 8.1 log₁₀ *Campylobacter* from untreated wastewater).

Similar to other regulations presented above, the North Carolina reclaimed water guidelines have specific log_{10} reduction targets. The North Carolina legislation specifies a 6 log_{10} reduction for *E. coli* or fecal coliform with a monthly geometric mean level of less than or equal to 3/100mL with a daily maximum of less than or equal to 25/100mL. For viruses, a 5 log_{10} reduction is required for coliphage virus with a monthly geometric mean of less than or equal to 5/100mL with a daily maximum level of less than or equal to 25/100mL. For protozoan parasite indicators, a 4 log_{10} reduction is required for *C. perfringens* with a monthly geometric mean less than 25/100mL.

Organization	Water Type	Bacteria	Virus	Protozoan Parasite
National Water Research Institute (NWRI)	Untreated Wastewater	9 log ₁₀ Total Coliform	12 log ₁₀ Enteric Virus	10 log ₁₀ Cryptosporidium and Giardia
California, USA	Untreated Wastewater	-	12 log ₁₀ Enteric Virus	10 log ₁₀ Cryptosporidium and Giardia
North Carolina, USA	Untreated Wastewater	6 log ₁₀ <i>E. coli</i> or Fecal Coliform	5 log ₁₀ Coliphage Viruses	4 log ₁₀ Clostridium perfringens
Australia	Untreated Wastewater	8.1 log ₁₀ Campylobacter	9.5 log ₁₀ Enteric Virus	8 log ₁₀ Cryptosporidium
Texas, USA	Wastewater Treatment Plant Effluent	-	8 log ₁₀ Enteric Virus	5.5 log ₁₀ Cryptosporidium 6 log ₁₀ Giardia

Table 1-2: Summary of log₁₀ reduction standards in reclaimed water legislation

1.3.6 Application of Quantitative Microbial Risk Assessment (QMRA) to Wastewater Reuse

Although reclaimed water is increasingly used for non-potable or indirect potable purposes, in the selection of treatment technologies for achieving indicator or pathogen reduction standards, the balance between cost and technical feasibility and health effects related to wastewater reuse is an important consideration. Some studies (Carr et al. 2004 and Westrell et al., 2004) have proposed the use of risk assessment as a means of establishing reclaimed water guidelines. Quantitative microbial risk assessment (QMRA) is a systematic way to evaluate scientific information in order to consider health impacts (NRC, 2009). This risk-based approach is relevant to reuse applications as it allows stakeholders such as water utilities, and communities considering reuse systems to evaluate the risks under various treatment and exposure scenarios.

The four main steps included in quantitate microbial risk assessment are 1) hazard identification/problem formation, 2) exposure assessment, 3) dose-response assessment, and 4) risk characterization (Haas et al., 2014; WHO, 2016) In the hazard identification, the microbial agents of interest are identified and defined within the scope of the QMRA (Haas et al., 2014). Of particular interest are the exposure pathways, and the relevant detection methods for the exposure pathways. The goal of exposure assessment is to determine the frequency and magnitude of exposure to the pathogens by the pathways defined during the hazard identification (WHO, 2016). In this step, both the quantitative data on the concentration and survival of pathogens in water sources as well as data on human exposure is necessary to inform the exposure assessment. The aim of a dose-response assessment is a mathematical characterization of the relationship between the dose of a microorganism and probability of infection, disease, or death in the exposed population (Haas et al., 2014). Typically, dose-response models are based on experimental data; however, human dose-response models are difficult to obtain for pathogenic organisms, resulting in the use of historic studies for dose-response models of some pathogens. The final step in QMRA is risk characterization and it involves the combination of information from the previous steps into an assessment of the probability of adverse health effects in the exposed population. The probability of adverse health effect is then compared to a health-based target.

US EPA recommends a 1×10^{-4} risk of infection per person per year threshold for drinking water; however some researchers (Haas et al., 1996) have suggested that this threshold may be too conservative as the incidence of waterborne illness is likely several million cases per year. A more recent study by Colford et al., 2006 has estimated the average number cases of acute gastrointestinal illness attributable to the public drinking water supply in the United States

to be 4.26-11.69 million cases per year. DeFelice et al., 2015 found that the average acute gastrointestinal illness risk attributable to community water supplies in North Carolina was 4.1×10^{-4} .

Other studies (Rose et al., 1996; Ryu et al., 2007) have evaluated the risks from exposure to reclaimed water used for non-potable or indirect potable uses, and specifically reclaimed waters treated using a single disinfection step, typically chlorine or UV only. These studies have typically concluded that the health risk of exposure to pathogens by these routes, which do not include drinking in most cases, are lower than the 1×10^{-4} threshold. Rose et al., 1996 found that the protozoan parasite risk associated with a single exposure to 100mL of reclaimed water resulted in a probability of infection between 10^{-6} and 10^{-8} for landscape irrigation, while Ryu et al., 2007 found that for multiple exposure routes (landscape irrigation for golf courses, playgrounds, and for recreational impoundments), the probability of infection for *Cryptosporidium* and *Giardia* was still between the range of 10^{-6} and 10^{-8} .

Current literature does not adequately evaluate the microbiological risk from reclaimed water sources used for direct potable purposes. Though microbial quality and health risks have been previously assessed, the results from these previous studies have not assessed tertiary treated water involving dual UV irradiation and chlorine disinfection that is currently proposed for potable reuse (Asano et al., 2007). Additionally, many of the studies conducted previously evaluated technologies, such as pre-treatment by chemical coagulation or rapid granular medium filtration, which are not required by most states to produce the proposed high quality reclaimed water (NAS, 2012; Rose et al., 1996; Ryu et al., 2007). New requirements from California, and guidelines from NWRI require health based log₁₀ reductions for the production of reclaimed water for potable reuse; however, to date, one study (Sobsey et al., 2005) has evaluated the dual
disinfection system for the production of high quality reclaimed water in North Carolina. The systems in use in Texas, California, and Florida require the use of single disinfection wastewater treatment systems followed by drinking water treatment involving more advanced treatment methods, such as ultrafiltration or reverse osmosis. The Sobsey et al. study was done on a pilot scale and indicated that this type of disinfection scheme was effective in removing indicator microbes for the production of high quality reclaimed water (Sobsey et al., 2005). Despite this, the dual disinfection system proposed for use in North Carolina has not been examined on a larger scale and has not been evaluated for the presence of pathogens in water treated by this method. Additional study is needed to investigate both the presence of indicator and pathogenic microbes in the dual disinfected high quality reclaimed water allowed under North Carolina regulations and to provide information on the microbial public health risks of potable reuse.

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CHAPTER 2: MICROBIAL QUALITY AND LOG₁₀ REDUCTION PERFORMANCE OF NORTH CAROLINA TYPE 2 RECLAIMED WATER (NCT2RW)—LIKE PRODUCING RECLAIMED WATER FACILITIES

2.1 Introduction

With increasing pressure from population growth and climatic variability, there is interest in alternative water sources to augment the drinking water supply (NAS, 2012). Treated wastewater is increasingly used for either non-potable purposes, such as agriculture, or as source water for drinking water supplies, either unplanned as upstream wastewater discharges reaching water supply intakes or by purposeful use of engineered water reuse systems (NCDENR, 2011). Potable reuse involves the use of treated wastewater as either a supplement to the drinking water supply (indirect reuse) or as the water supply itself (direct reuse).

In North Carolina, tertiary treated, dual disinfected reclaimed water has been proposed as a source for potable reuse. The proposed dual disinfection system is characterized by the use of two disinfection steps, typically UV radiation and chlorine, followed by combination with surface water at a ratio of no more than 20% reclaimed water to at least 80% surface water currently used as drinking water sources, a 5-day storage time of this blend, and then conventional drinking water treatment processes.

To meet the growing water need, NC passed revised reclaimed water regulations (subchapter 02U – Reclaimed Water) (NC DENR, 2011) to expand reclaimed water uses, particularly by establishing a higher quality "Type 2" reclaimed water (NCT2RW) with expanded uses. Design criteria for wastewater treatment to meet NCT2RW requirements must achieve log₁₀ reductions of 6 for *E. coli*, 5 for coliphages and 4 for *Clostridium perfringens*.

Effluent quality for NCT2RW must meet a geometric mean concentration of 3/100 ml for *E. coli* and 5/100 ml for both coliphages and *Clostridium perfringens*, with daily maxima of 25/100 ml for each of these three target microbes. To meet these microbial (pathogen) log₁₀ reduction and microbial quality requirements for NCT2RW, treatment facilities must use dual disinfection systems containing UV disinfection and chlorination or equivalent dual disinfection processes to control pathogens.

The microbial quality of reclaimed water produced by some treatment processes has been evaluated previously (Harwood et al., 2005; Rodríguez et al., 2009). However, the tertiary treated, dual disinfected reclaimed water proposed by the state of North Carolina for potable reuse and designated as type 2 has not been evaluated in a full-scale production scenario. A previous pilot scale study evaluating the dual UV and chlorine disinfection system for tertiary treated sewage as type 2 reclaimed water indicated that dual disinfection is effective for fecal indicator bacteria, viruses and protozoan parasite surrogates in producing high quality reclaimed water (Sobsey et al., 2005). In this study, I aim to address the need for real-world data documenting the microbial quality and microbial reduction performance of NCT2 reclaimed water systems.

My goals in this research are to evaluate the log₁₀ reductions achieved by NCT2RW producing water reclamation facilities for: 1) the indicator organisms specified by NC state law, which include *E. coli*, coliphage viruses, and *Clostridium perfringens*; and, 2) the pathogenic microorganisms for each microbial class, specifically *Salmonella* spp. bacteria, human enteric viruses (Adenoviruses, and Noroviruses), and protozoan parasites (*Cryptosporidium* and *Giardia*). An additional goal is to evaluate and compare the log₁₀ concentrations of pathogens in

raw sewage and reclaimed water, particularly as they relate to the NC standards for production of potable water.

2.2 Methods

2.2.1 Water Samples

Raw sewage and reclaimed water samples were collected bi-monthly for 1 year, during and after storm events, as grab samples using approved techniques (Standard Methods for the Examination of Water and Wastewater; SMEWW) from 4 different water reclamation facilities located in central North Carolina, resulting in 22 reclaimed water samples. Each of the water reclamation facilities produces NC Type 2 reclaimed water, which is characterized by both tertiary treatment and dual disinfection (typically treatment by UV radiation and chlorine disinfection).

2.2.2 Sample Processing and Concentration Methods

Samples were split into two volumes upon arrival at the laboratory, one sample (larger volume) for pathogen analysis and a second sample for indicator analysis. Raw sewage samples were collected from wastewater treatment plants in 300mL volumes and split into a 200mL sample for pathogen analysis and a 100mL sample for indicator and *Salmonella* spp. culture analyses. Reclaimed water samples were collected in 12L sample volumes and split into a 10L sample volume for pathogen analysis and a 2L volume for indicator analysis. Samples processed for culture of indicator organisms were not concentrated before analysis.

Primary concentration for reclaimed water samples was performed using hollow fiber ultrafiltration following the protocol described in Hill et al. (2007) and Polaczyk et al. (2008): primary concentration for raw sewage samples was achieved by low speed centrifugation involving an initial centrifugation at 1500 x g and pellet separation for IMS-FA and pellet elution. Secondary concentration for viruses was performed via polyethelyeneglycol (PEG-8000)

precipitation by the method described in Yamamoto et al. (1970), while secondary concentration and purification for protozoan parasites was achieved by immunomagnetic separation (IMS) and immunofluorescent (oo)cyst examination. Figure 2-1 shows the reclaimed water sample processing procedures.

Briefly, 10L of reclaimed water was spiked with a commercially available positive internal control that was uniquely fluorescently labeled *Giardia* and *Cryptosporidium* (oo)cysts (BTF Precise Microbiology, Inc., Pittsburgh, PA) and filtered through the Fresenius Optiflux F250NR hollowfiber ultrafilter. Water samples were concentrated to produce a retentate liquid of approximately 100-200mL volumes, and ultrafilters were backflushed with a solution containing 0.5% Tween 80, 0.01% Sodium polyphosphate (NAPP) (Sigma-Aldrich, cat# 305553-25G), and 0.001% Antifoam Y. The backflush liquid was added to the retentate liquid to produce a total concentrate volume of approximately 200-250mLs. Next, reclaimed water samples were centrifuged at 1,500 x g for 30 minutes at 4°C to separate out protozoan parasites, the pellet from this centrifugation was eluted using 0.5M pH7.5 threonine for 1 hour at room temperature with a mixing speed of 60RPM. The eluted mixture was then re-centrifuged at 1,500 x g, and the supernatant was combined with the supernatant collected from the previous centrifuge step, while the pellet was processed by immunomagnetic separation (IMS) and then examined for Cryptosporidium and Giardia by fluorescence microscopy. Similarly, for raw sewage samples, a 200mL sample was centrifuged at 1,500 x g for 30 minutes and pellet from this centrifugation was eluted using 0.5M pH 7.5 threonine for 1 hour at room temperature with a mixing speed of 60RPM. After a second centrifugation at 1,500 x g, the pellet was then processed by IMS for Cryptosporidium and Giardia. IMS and fluorescence microscopy steps are described in section 2.2.9. Virus processing continued for both raw sewage and reclaimed water samples for

combined supernatant samples with an additional centrifugation step at 5,000 x g at 4°C for 30 minutes. The pellet from this centrifugation was also eluted with 0.5M pH 7.5 threonine for 1 hour at 60RPM, re-centrifuged at 5,000 x g, and then combined with the supernatant from the previous step. A secondary concentration was then performed on the total supernatant using 10% PEG-8000, 0.5M NaCl, with an overnight incubation at 4°C. After incubation, the samples were then centrifuged at 5000 x g at 4°C for 30 minutes, the supernatant was discarded and the pellet was eluted with PBS-Tween (US EPA, 2012) to a volume of 4mLs.



Figure 2-1: Diagram of reclaimed water sample processing for indicator and pathogenic microorganisms

2.2.3 Quantification of Bacterial Indicators

Concentrations of *E.coli* and *Enterococcus* spp. were determined by defined substrate Most Probable Number (MPN) methods using the Quantitray 2000 system with Colilert and Enterolert media, respectively. Manufacturer instructions were followed and MPN values were determined using the manufacturer's MPN tables. Samples were processed in duplicate, diluted in phosphate buffered saline (PBS) (US EPA, 2012) and incubated at 37°C for *E. coli* and 41.0°C for *Enterococcus* spp. for 24±4h; replicate MPN values were averaged to obtain the average MPN/100mL. The lower limits of detection for the Colilert and Enterolert methods are 1 MPN unit of *E. coli* or *Enterococcus* per 100mL sample volume.

2.2.4 Quantification of Coliphage Viruses

Somatic and F+/Male-specific coliphage concentrations were determined using US EPA Method 1602, the single agar layer method (US EPA, 2001). Samples were diluted using phosphate buffered saline (PBS) (US EPA, 2012). In this study, F+/Male-specific coliphage analysis was conducted using the *E. coli* Famp as the *E. coli* host, with *E. coli* CN13 as the somatic coliphage host, and with *E. coli* CB390 as a total coliphage host. Guzmán et al. (2008) proposed that *E. coli* CB390 can be used for the simultaneous detection of both F+/Male-specific and somatic coliphages. The limit of detection for the SAL method is 1 plaque forming unit (PFU) per 100mL.

2.2.5 Quantification of *Clostridium perfringens* Vegetative Cells and Spores as Protozoan Parasite Surrogates

Concentrations of *C. perfringens* were determined using standard membrane filter (MF) methods modified from those originally developed for US EPA by Bisson and Cabelli (1980) using CP ChromoSelect Agar (Sigma-Aldrich, St. Louis, MO). Briefly, the agar base was prepared by adding 6.28 grams/100 mL deionized water, bringing the mixture to a boil on a hot plate and then removing to cool and keep molten at 55-60 degrees C. Once tempered to 55 degrees, 0.04 grams of D-Cycloserine were added per 100 mL of molten agar medium base. Supplemented medium was dispensed in 5-mL volumes in 60 mm diameter sterile, polystyrene petri dishes, which were then stored at 4°C until use. Samples were prepared by pasteurizing 100mL volumes of reclaimed water or 100mL of diluted raw sewage at 60°C for 30 minutes. Samples were diluted in phosphate buffered saline (PBS) (US EPA, 2012). Membrane filtration

plates were incubated in anaerobic jars at 45° C for $24\pm4h$, post-incubation, anaerobic jars were opened for at least 1 hour prior to counting to allow the characteristic color change to occur for *C. perfringens* colonies, which were then counted and recorded. The limit of detection for the MF method using CP ChromoSelect Agar is 1 colony forming unit (CFU) per 100mL.

2.2.6 Detection and Quantification of Salmonella Bacteria

Salmonella spp. concentrations were determined by a modification of the method described by both Hill and Sobsey (2001) and Krometis et al. (2010). Briefly, triplicate volumes of buffered peptone at pH 7.2 water were inoculated with three different sample volumes (for Reclaimed Water 300mL, 30mL, and 3mL; for Raw Sewage 1mL, 0.1mL, and 0.01mL) and incubated at 37°C for 24±4h as a pre enrichment step. After incubation, 10% of the enrichment culture volume was transferred into a volume of selective Rappaport-Vassilades (RV) broth and incubated at 41°C for 24±4h. After incubation, 10µL volumes of the RV broth were then streaked on to Salmonella-Shigella (SS) agar plates and incubated at 37°C for 24±4h. Presumptive black positive colonies were identified and then confirmed as Salmonella-positive using the Triple Sugar Iron Agar slant biochemical test. A Salmonella-positive reaction was defined as a tube that fermented glucose and reduced sulfur. The presence of one or more *Salmonella* MPN concentrations were then determined using a 3 replicate, 3 dilution volume MPN table to calculate MPN volumes per 100mL.

2.2.7 Detection and Quantification of Enteric Viruses

2.2.7.1 Virus Concentration and Nucleic Acid Extraction

Enteric viruses in reclaimed water and raw sewage were concentrated and processed as described above to obtain concentrated samples. Representative bacteriophages were used as positive controls for virus recovery in sample processing at each step in order to evaluate the efficiency of the virus recovery methods used. Specifically the Genogroup IV F+ RNA coliphage SP was used as a processing positive control for Norovirus and the Salmonella bacteriophage PRD1 was used as a positive control for Adenovirus. PRD1 was provided by Dennis Bamford at the University of Helsinki, and SP was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). PRD1 was chosen because of its similarities with adenovirus, specifically its morphological characteristics (size and shape); SP was chosen because it is a single stranded, non-enveloped RNA virus similar to Noroviruses. Positive control bacteriophages were added to 10L reclaimed water samples before hollow fiber ultrafiltration and to 200mL raw sewage samples before low speed centrifugation in order to follow as many processing steps as possible. Table 2-1 shows a summary of processing control recovery efficiency. Nucleic acids were extracted simultaneously by the method described by Rodríguez et al. (2012), with 100 μ L volumes of concentrated sample. Briefly, 100 μ L of Guanidinium thiocyanate (GuSCN) lysis buffer and concentrated sampled were vortex-mixed together and incubated at room temperature for 10 minutes. Then, 200mL of 100% ethanol was vortex-mixed together with the sample and lysis buffer. The entire solution was then centrifuged for 1 minute at 14,000 x g for 1 minute in a high bind RNA mini column (OMEGA BIOTEK, Norcross, GA). The waste effluent was discarded, and 500μ L of 75% ethanol was added and the column was centrifuged at 14,000 x g for 1 minute two more times. The mini column was then centrifuged an additional time at 14,000 x g and then placed in a new 1.5mL collection tube. Then, 50μ L of RNAse free water (Sigma-Aldrich) was added to the column membrane and after 1 minute, the column was again spun at 14,000 x g. Purified nucleic acids were then collected and used for qPCR or RT-qPCR.

2.2.7.2 Standard Curve Generation

Adenovirus standard curves were prepared using a stock of known concentration of Adenovirus 2 by the method described by Wu et al. (2011). Norovirus standard curves were generated using Quantitative Norovirus GII from the American Type Culture Collection (ATCC®, Manassas, VA, Product # VR-3235SDTM). Samples were serially diluted in phosphate buffered saline (PBS, US EPA, 2012), standard curves were generated using 100 to 108 copies of adenovirus, and with 100 to 105 copies of Norovirus. Positive control viruses were used for both adenovirus and Norovirus experiments. Adenovirus 2 (ATCC VR-846) was used as a positive control for qPCR experiments, and positive control Norovirus GII was provided by the lab of Dr. Ralph Baric (UNC Chapel Hill).

2.2.7.3 Prevention of PCR Carryover Contamination

Standard precautions were taken to prevent PCR contamination, including the use of dedicated laboratory spaces, pipettes, and barrier-filtered pipette tips. Two negative controls, containing no nucleic acid, were included in each run, and no indications of (RT-)PCR contamination were detected for any of the virus nucleic acids that were analyzed. Samples of positive control DNA and RNA were prepared in a separate room and never taken into the PCR set-up area.

2.2.7.4 Real-Time PCR Assay

Both the norovirus (RT-qPCR) and the adenovirus (qPCR) assays were performed using the QuantiTect Probe PCR Kit (Qiagen, CA) using a BioRad CFX96 Touch Real Time PCR System in a 96 well format. Primers and probes used in Real-Time PCR are described in Table 2-2. Norovirus protocols were performed as described by Loisy et al. (2005). Briefly, the norovirus reaction mixture contained 2µL of extracted RNA, 200nM of GII primers and probe, 1.25U RNAse Inhibitor (Applied Biosystems, France), and 0.25µL Qiagen RT Enzyme (Qiagen, CA).

Norovirus PCR conditions were as follows: reverse transcription for 30 minutes at 50°C, denaturation for 5 minutes at 95°C, and then 45 cycles of amplification with denaturation at 95°C for 15s and annealing and extension at 60°C for 1 minute. Protocols for SP, the Norovirus processing control were as described in Friedman et al. (2011). Briefly, the SP reaction mixture contained 2µL of extracted RNA, 10µM of the forward and reverse primers, 5µM of the probe, with a final volume of 25uL. The PCR conditions for SP required reverse transcription for 30 minutes at 50°C, denaturation for 15 minutes at 95°C, and then 45 cycles of amplification with denaturation at 95°C for 1s, annealing at 56°C for 30s and extension at 76°C for 1 30s. Adenovirus protocols were as described by Jothikumar et al. (2005). Briefly, the adenovirus reaction mixture contained 2µL of extracted DNA, 50uM of the forward and reverse primers, 5uM of the JJVXP probe, with a final volume of 25uL. The PCR conditions for adenovirus involved denaturation for 15 minutes at 95°C, followed by 45 cycles with denaturation for 10s at 95°C, annealing for 30s at 55°C, and elongation for 15s at 72°C. Protocols for PRD1, the adenovirus processing control were as described in Dika et al. (2015). Briefly, the PRD1 reaction mixture contained 5µL of extracted DNA, 900nM of the forward and reverse primers, 225mM of the probe, with a final volume of 25uL. The PCR conditions for PRD1 involved denaturation for 3 minutes at 95°C, followed by 45 cycles with denaturation for 30s at 95°C, annealing for 30s at 55°C, and elongation for 1 minute at 72°C.

2.2.8 Adenovirus Integrated Cell Culture Polymerase Chain Reaction (ICC-PCR)

2.2.8.1 Cell Culture Infectivity Assay and mRNA Extraction

Cell culture infectivity assays were performed as described by Rodríguez et al. (2013) and Polston et al. (2014). Briefly, HEK 293 cells were grown in 25cm² tissue culture flask with Eagle's Minimal Essential Medium (EMEM) (Gibco/Invitrogen, Carlsbad, California) and supplemented with 10% Fetal Bovine Serum (FBS) (Gibco/Invitrogen). Cells were incubated for 4-5 days at 37°C until at least 80% confluence was attained. After confluence was reached, a 1.5mL inoculum was produced by diluting 350μ L of concentrated adenovirus sample using 1050 complete MEM medium without serum and containing 10µg kanamycin, 50µg gentamicin, and 20µg nystatin per mL. After 1 hour of incubation the inoculum was removed, 6mL of complete MEM medium with 2% bovine serum was added to each flask and cell cultures were incubated for 4-5 days at 37°C. After incubation, the cell culture medium of each separate 25cm^2 tissue culture flask was disrupted and removed using 1mL pH 7.5 phosphate buffered saline (PBS) by vigorous pipetting up and down. Cells were transferred to a 1.5mL microfuge tube and centrifuged at full speed (16,000 x g) for two minutes at 4°C. The Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) was used to extract nucleic acids from the cell culture monolayers following the method described by Rodríguez et al. (2014). Briefly, cells were resuspended using RLT lysis buffer (provided in the Qiagen kit) and homogenized using QIAshredder minicolumns (Qiagen, Valencia, CA). Nucleic acid extraction was then performed using the RNeasy Kit and the final purified nucleic acids were collected in 50uL of nucleic acid free water.

2.2.8.2 Real-Time ICC-qRT-PCR

The adenovirus (qPCR) assays were performed using the QuantiTect Probe PCR Kit (Qiagen, CA) using a Bio-Rad CFX96 Touch Real Time PCR System in a 96 well format. Primers and probes used for the detection of mRNA are the same as those used for the detection of DNA described in Table 2-2. Protocols were performed as described by Rodríguez et al. (2013). Briefly, the adenovirus reaction mixture contained 2µL of extracted mRNA, 0.5µM of forward and reverse primers and probe, 1.25U RNAse Inhibitor (Applied Biosystems, France), and 0.25µL Qiagen RT Enzyme (Qiagen, CA). Adenovirus PCR conditions were as follows: reverse transcription for 30 minutes at 50°C, denaturation for 15 minutes at 94°C, and then 45 cycles of amplification with denaturation at 94°C for 15s and annealing for 30seconds at 58°C and extension at 72°C for 15 seconds.

2.2.8.3 Quality Assurance and Control

As described in section 2.2.7.4, standard precautions were taken to prevent PCR contamination. Positive control reference viruses (Adenovirus 2) were used for infectivity assays and a positive DNA control (the adenovirus viral hexon gene), as described by Rodríguez et al. (2013) were run parallel to each set of qPCR. The titer of the adenovirus 2 viral stock as infectious units (MPNIU) was determined using end point dilution. Briefly, adenovirus stock was diluted serially ten-fold in PBS, with three replicates per dilution, in 6-well plates containing HEK 293 monolayers with incubation in complete MEM medium at 37°C in a 5% CO₂ incubator. The viral hexon gene was detected after RNA extraction after up to 5 days post infection by the RT-PCR methods described above. Two negative phosphate buffered saline (PBS) control reactions were included in each ICC-qPCR run, and no indications of contamination were detected. The cycle threshold (C₁) is the cycle at which a significant increase in fluorescence occurs; a sample with a C₁ value below 43, with no evidence of amplification in the negative controls (threshold not reached after 45 cycles) was considered positive.

	Average Recovery Efficiencies (%)						
	<u>R</u>	Raw Sewage (n	=22)	Reclaimed Water (n=22)			
Microbe	Average Percent Recovery	Standard Deviation	Range (%)	Average Percent Recovery	Standard Deviation	Range (%)	
Adenovirus A-F (PRD1)	100.54%	7.63%	(86, 118)	109.36%	13.65%	(86, 139)	
Norovirus GII (SP)	99.98%	5.14%	(92, 121)	104.64%	11.92%	(90, 127)	
Cryptosporidium (ColorSeed)	84.40%	10.22%	(70, 101)	82.50%	6.47%	(71, 89)	
Giardia (ColorSeed)	73.06%	18.07%	(40, 103)	63.50%	12.20%	(44, 85)	

Table 2-1: Summary of percent recovery data for processing control organisms

	Oligonucleotide	Oligonucleotide			
Assay	type	name	Sequence (5'- 3')	Position	Orientation
Norovirus GII					
	Forward Primer	QNIF2da ^a	ATGTTCAGRTGGATGAGRTTCTCWGA	5012–5037	+
	Reverse Primer	COG2Rb ^b	TCGACGCCATCTTCATTCACA	5080-5100	-
	Probe	QNIFSa ^a	FAM-AGCACGTGGGAGGGCGATCG-BHQ	5042-5061	+
SP					
	Forward Primer	IV Forward ^d	CGGYCAYCCGTCGTGGAAG	2941-2959	+
	Reverse Primer	IV Reverse ^d	AGT GAC TGC TTT ATT YGA AGT GCG	3082-3059	-
	Probe	IV Probe ^d	FAM-CCT GTC CGC AGG ATG TWA CCA AAC-BHQ	2964–2987	+
Adenovirus A-F					
	Forward Primer	JTVXFc ^c	GGACGCCTCGGAGTACCTGAG	18895–18915	+
	Reverse Primer	JTVXRc ^c	ACIGTGGGGTTTCTGAACTTGTT	18990–18968	-
	Probe	JTVXPc ^c	FAM-CTGGTGCAGTTCGCCCGTGCCA-BHQ	18923–18944	+
PRD1					
	Forward Primer	PRD1F ^e	AAACTTGACCCGAAAACGTG	9546-9565	+
	Reverse Primer	PRD1R ^e	CGGTACGGCTGGTGAAGTAT	9728-9747	-
	Probe	PRD1P ^e	FAM-ATGGTAACGTGGGCTTTGTC-BHQ	9658-9677	+

Table 2-2: Primers and probes used in Real-Time PCR

^a Norovirus GII forward primer and probe as described by Loisy et al., 2005 ^b Norovirus GII reverse primer as described by Kageyama et al., 2003 ^c Adenovirus groups A-F primers and probe as described by Jothikumar et al., 2005 ^d SP primers and probe described by Friedman et al., 2011 ^e PRD1 primers and probe described by Dika et al., 2015

2.2.9 Protozoan Parasite Detection and Quantification

Cryptosporidium spp. and Giardia spp. were recovered and quantified in raw sewage and reclaimed water by modifications of EPA Method 1623. Primary concentration for reclaimed water samples was performed using modifications of hollow fiber ultrafiltration and elution protocol described by Hill et al. (2007) and Polaczyk et al. (2008), with Fresenius Optiflux F250NR hollow fiber ultrafilters (dialyzers). Briefly, 10L of reclaimed water was spiked with a commercially available positive internal control that was uniquely fluorescently labeled *Giardia* and Cryptosporidium (oo)cysts (BTF Precise Microbiology, Inc., Pittsburgh, PA), and filtered through the Fresenius Optiflux F250NR hollowfiber ultrafilter. Water samples were concentrated to produce a retentate liquid of approximately 100-200mL volumes, and ultrafilters were backflushed with a solution containing 0.5% Tween 80, 0.01% Sodium polyphosphate (NAPP) (Sigma-Aldrich, cat# 305553-25G), and 0.001% Antifoam Y. The backflush liquid was added to the retentate liquid to produce a total concentrate volume of approximately 200-250mLs. Next, reclaimed water samples were centrifuged at 1,500 x g for 30 minutes at 4° C to separate out protozoan parasites, the pellet from this centrifugation was eluted using 0.5M pH7.5 threonine for 1 hour at room temperature with a mixing speed of 60RPM. The eluted mixture was then recentrifuged at $1,500 \times g$, and the supernatant was combined with the supernatant collected from the previous centrifuge step for human enteric virus detection, while the pellet was processed by immunomagnetic separation (IMS). Similarly, for raw sewage samples, a 200mL sample was centrifuged at 1,500 x g for 30 minutes and the pellet from this centrifugation was eluted using 0.5M pH7.5 threonine for 1 hour at room temperature with a mixing speed of 60RPM. After a second centrifugation at 1,500 x g, the pellet was then processed using IMS. Immunomagnetic separation was performed using the Dynabeads Cryptosporidium/Giadia combo kit (cat#:73012, Invitrogen, Carlsbad, CA) as per manufacturers instructions. Briefly, a 0.5mL pellet was

examined by combining 1mL of the provided 10X SL-buffer A and 10X SL-buffer B in a flat sided tube and then adding 100µL each of the Dynabeads Cryptosporidium and Giardia and incubating for 1 hour on a rotating mixer at 18RPM. The tube was then sequentially placed in the Dynabeads Magnetic Particle Concentrator (MPC-1) and concentrated to a 1.5mL sample volume, which was then placed in the MPC-M, the supernatant was eluted, and the pellet was rinsed with 50uL of 0.1N HCl after removing the MPC-M magnet. After a 10 minute incubation, the magnet was replaced, and the sample was transferred to a microscope slide containing 5uL of 1.0N NaOH. Microbe slides were stained and processed using the Meriflour kit (Waterborne, Inc., New Orleans, LA), and slides were examined using a Leitz Orthoplan 2 fluorescent microscope. For raw sewage samples, primary concentration was done by a simple centrifugation method and for both samples primary concentration was followed by further concentration and purification by immunomagnetic separation (IMS) and then direct immunofluorescent microscopic (oo)cyst enumeration using the Merifluor kit (Waterborne, Inc., New Orleans, LA) along with a commercially available positive internal control that is uniquely fluorescently labeled *Giardia* and *Cryptosporidium* (oo)cysts (BTF Precise Microbiology, Inc., Pittsburgh, PA) (US EPA, 2012). The limit of detection, based on the volume of initial sample processed, for protozoan parasite recovery is 0.5 (oo)cysts for raw sewage and 0.01 (oo)cysts for reclaimed water. A summary of processing control recovery efficiency is provided in Table 2-1. 2.2.10 Statistical Analysis

Microbial recovery efficiencies of positive control microbes were determined by calculating the number of microbes recovered after an experiment (concentration multiplied by sample volume) and multiplying that value by the number of each positive control microbe present in the sample (concentration multiplied by sample volume) before the experiment and then multiplying that value by 100. Initial concentrations of positive control microbes were

determined by analyzing an initial spiked water sample using qPCR or RT-qPCR methods for viral control organisms and counts of (oo)cysts by flow cytometry for protozoan parasite control organisms. Log₁₀ reduction values were calculated by subtracting the log₁₀ concentration of microorganism in NCT2RW from the log₁₀ concentration in influent raw sewage. Most probable number calculations were performed using either the FDA Bacteriological Analytical Manual calculator (FDA, 2006) or the IDEXX MPN Generator for Quanti-Tray 2000 System (IDEXX Laboratories, Westbrook, Maine). Data were analyzed using Excel 2011 (Microsoft, Redmond, WA) and Graph Pad Prism 7 (Graph Pad, San Diego, CA).

2.3 Results

The results presented here represent data from multiple samplings (n=22) from four water reclamation facilities over a one-year period that represent the concentrations of fecal indicator microorganisms and representative bacterial (*Salmonella* spp), viral (adenovirus and norovirus) and protozoan (Cryptosporidium and Giardia) pathogens in the influent (raw sewage) and the tertiary treated, dual disinfected, North Carolina Type 2 reclaimed water (NCT2RW), which has been proposed for potable reuse. It is important to note that all fecal indicator microbes, Salmonella bacteria and adenoviruses were analyzed by culture or infectivity methods, noroviruses and adenoviruses were analyzed by nucleic acid amplification methods, RT-qPCR and qPCR, respectively, and the protozoan parasites Cryptosporidium and Giardia were analyzed by immunofluorescent microscopy methods. In the cases of adenoviruses, it is therefore possible to compare their detection and quantification in NCT2RW by both infectivity (combined cell culture-qPCR) methods and direct qPCR methods.

2.3.1 Microbial Concentrations in Raw Sewage and NCT2RW

The concentrations of fecal indicator microorganisms and pathogens in influent raw wastewater and in tertiary treated, dual disinfected NCT2RW are summarized in Table 2-3. The lower limit of microbial detection (described in the materials and methods) was substituted as measured values in samples in which the target microorganism was not detected; this was most frequently an issue in the NCT2RW samples, particularly for fecal indicator microorganisms. 2.3.1.1 Raw Sewage Concentrations

As summarized in Table 2-3, the concentrations of the fecal indicator bacteria *E. coli* were the microbes detected most frequently in the influent raw wastewater samples, with an average concentration of 2.63 x 10⁶ MPN per 100mL with a standard deviation of 1.91, followed by *Enterococcus* spp. with a mean of 3.90 x 10⁵ MPN per 100mL and a standard deviation of 1.66. Concentrations of *Clostridium perfringens* bacteria, the surrogate for protozoan parasites, averaged 1.95 x 10⁴ ± 2.34 CFU/100 mL for spores and 3.09 x 10⁴ ± 2.39 CFU/100 mL for spores and vegetative cells. Mean concentrations of coliphages were 1.62 x 10⁴ ± 2.29, 9.33 x 10³ ± 2.34 and 2.75 x 10⁴ ± 2.09 PFU per 100mL for somatic, F+, and total coliphages, respectively.

Average *Salmonella* spp. concentrations in raw wastewater, detected by a culture-based MPN assay, were on average $1.23 \times 10^4 \pm 9.8$ MPN/100mL. This value was about 1-2 orders of magnitude lower than the concentrations of the fecal indicator bacteria *E. coli* at 2.63 x 10^6 MPN/100 mL and enterococci at of 3.90 x $10^5/100$ mL, as would be expected for the relationship between concentrations of FIBs and bacterial pathogens.

It should be noted that enteric virus concentrations are as genome copies and not concentrations of infectious viruses, as raw wastewater samples were analyzed only by direct real time q-PCR for adenoviruses and direct RT-qPCR for noroviruses. Additionally, concentrations of *Cryptosporidium* and *Giardia* (oo)cysts are the total number of immuno-

microscopically visible (oo)cysts present in each sample, and not infectious (oo)cysts. Mean concentrations for Norovirus GII were $3.09 \times 10^3 \pm 5.01$ GEC per 100mL and for Adenovirus Group A-F were $3.72 \times 10^4 \pm 8.13$ GEC per 100mL, or about an order of magnitude greater for the latter than the former. It is noteworthy that mean concentrations of adenoviruses and noroviruses (based on gene equivalent copies) were similar to those of coliphages (as fecal indicator viruses) measured by culture infectivity in host cells, with mean concentrations of 1.6 x 10^4 , 9.3 x 10^3 and 2.75 x 10^4 PFU per 100mL for somatic, F+, and total coliphages, respectively.

Concentrations of *Cryptosporidium* oocysts constituted a mean of 37.15 ± 2.25 oocysts/100 mL and ranged from 7 to 94 per 100mL and *Giardia* cysts comprised a mean of 30.2 ± 2.40 cysts/100 mL and ranged from 6 to 111 cysts per 100mL. These concentrations of *Cryprosporidium* and *Giardia* in raw sewage (based on immunofluorescent microscopy counts) are lower than those for the protozoan parasite surrogate *C. perfringens*, for which mean concentrations based on culture were $3.09 \times 10^4 \pm 2.39$ CFU/100 mL for vegetative cells and spores and $1.95 \times 10^4 \pm 2.34$ CFU/100 mL for spores only, a concentration difference of nearly 3 orders of magnitude.

2.3.1.2 NCT2RW Concentrations

As expected, the microbial concentrations in the tertiary treated, dual disinfected (NCT2) reclaimed water were much lower than those in raw wastewater (Table 2-3). In some cases the concentrations were at or below the lower detection limit, particularly for the fecal indicator microorganisms; the number of positive reclaimed water samples for each microorganism is displayed in Table 2-3. In contrast, for many of the pathogens, detectable levels were present in the reclaimed water. Mean concentrations of *E. coli* were 1.12 ± 1.51 MPN/100 mL and ranged from <1.0 to 6.90 MPN per 100mL, with 20 out of 22 samples at the lower detection limit. *Enterococcus* concentrations were at the detection limit (<1 MPN per 100mL) for all 22 samples.

Concentrations of somatic coliphage constituted a mean of 1.17 ± 1.51 PFU/100 mL and ranged from <1.0 to 4.0 PFU/100 mL with 19 samples of 22 at the lower detection limit. Concentrations of F+/ male-specific coliphages comprised a mean of 1.29 ± 2.14 PFU/100 mL and ranged from <1 to 15.13 PFU per 100mL, with 20 of 22 samples at the lower detection limit. The average concentrations of total coliphages were 1.62 ± 2.34 PFU/100 mL and concentrations ranged from and <1 to 15.14 PFU/100mL, with 16 of 22 samples at the lower detection limit. Mean concentrations of *C. perfringens* were 1.14 ± 1.70 for vegetative cells and spores with a range of <1.0 to 10 with 19 samples of 22 at the lower detection limit. Concentrations of *C. perfringens* spores were an average of 1.10 ± 1.29 and ranged from <1.0 to 2.69, with 20 of 22 at the lower detection limit.

The ICC-qPCR infectivity assay was used to detect adenoviruses in NCT2 reclaimed waters samples. The average viral infectivity was 6.79×10^1 MPNIU per 100mL with a range of 8.05×10^0 to 1.84×10^2 ; the total number of samples positive for infectious adenovirus was 7 out of 22 total reclaimed water samples analyzed. The equivalent volume of undiluted NCT2RW analyzed was 0.875L per sample. Compared to the infectious adenoviruses, the concentrations of coliphages detected in reclaimed water samples were 1.17 ± 1.51 PFU/100 mL, 1.29 ± 2.14 PFU/100 mL, and 1.62 ± 2.34 PFU/100 mL for somatic, F+ and total coliphages (respectively) or one to two orders of magnitude lower than adenovirus levels. Levels of adenovirus detected by qPCR were about an order of magnitude higher than those detected by infectivity assay, with average concentrations of $5.24 \times 10^2 \pm 36$ GEC per 100mL and 17 of 22 samples of NCT2RW has detectable adenoviruses by qPCR. Table 2-4 presents the average viral infectivity (mRNA-IU) for each wastewater reclamation facility along with the average viral genomes (GEC) detected by qPCR. This table also shows the number of positive samples, by wastewater

treatment plant, for both ICC-qPCR and qPCR, as well as the ratio between genome copes (GEC) and infectious units. Based on this table, approximately half of the samples positive by qPCR were positive for infectious units. The average ratio of GEC to infectious units was 204/1.

Salmonella spp. was detected at average concentrations of $0.13 \pm 2.82/100$ mL with 2 of 22 samples of NCT2RW as positive. When compared to concentrations of *E. coli* and *Enterococcus* in NCT2RW, with average concentrations of 1.12 ± 1.51 MPN/100 mL and <1/100mL respectively, *Salmonella* spp. concentrations were approximately 1 to 2 orders of magnitude lower.

Average *Cryptosporidium* concentrations were 0.22 ± 2.29 oocysts/100mL with all 22 samples above the detection limit. Average *Giardia* concentrations were 0.08 ± 2.40 cysts/100mL with 2 of 22 samples at the detection limit. When compared with average concentrations of *C. perfringens* and *C. perfringens* spores, with average concentrations of 1.14 \pm 1.70 and 1.10 \pm 1.29 (respectively), the concentrations of *Cryptosporidium* and *Giardia* were approximately 1 order of magnitude lower.

2.3.2 Log₁₀ Reductions of Fecal Indicators and Pathogens

The North Carolina reclaimed water legislation specifies log_{10} reduction performance requirements for NCT2RW, including reduction requirements of 6 log_{10} for *E. coli*, 5 log_{10} for coliphages and 4 log_{10} for *Clostridium perfringens*. Figures 2-2, 2-3, and 2-4 show box and whisker plots of the median log_{10} reduction values of pathogens and indicators; the asterisks in these figures indicate an upper detection limit value. In other words, the log_{10} reduction was calculated based on the value detected in raw sewage (influent) and the detection limit value in the reclaimed water sample. This upper detection limit value plays an important role in determining the log_{10} reduction, as the actual log_{10} reduction may be higher than presented in this figure. However, concentrations in the influent raw sewage were not sufficiently high and the lower detection limit levels in the reclaimed water (based on the 100 mL sample volumes analyzed) were not sufficiently low to estimate more reliably the true log₁₀ reduction values.

Figure 2-2 displays box and whisker plots for the bacterial indicators (*E. coli* and enterococci) and *Salmonella* spp. Based on this figure, the highest median log_{10} reductions are seen for *E. coli* and enterococci, followed by *Salmonella* spp.; this result is expected because these microorganisms were at the highest concentrations in the influent wastewater samples. The median log_{10} reductions of *E. coli*, *Enterococcus* spp., and *Salmonella* spp., were >6.34, >5.61, and 4.45, respectively. Although the upper and lower quartile ranges for these three microorganisms do not overlap in Figure 2-2, it is clear that the maximum value for *Salmonella* bacteria is within the minimum and maximum value for both *E. coli* and *Enterococcus*.

Figure 2-3 displays box and whisker plots for the log₁₀ reductions of enteric viruses and indicator organisms. The median log₁₀ reductions for somatic, F+, and total coliphages were 4.15, 3.90, 4.22, respectively. As these log₁₀ reduction values are censored based on the detected concentrations of coliphages in raw sewage, the actual log₁₀ reduction value could be higher than the values calculated here. For the enteric viruses, much like the other pathogens detected, the log₁₀ reductions were lower and more variable than those for the fecal indicator viruses. For Norovirus GII, the median log₁₀ reduction was 3.48 and for Adenovirus groups A-F the average reduction was 1.29. In contrast to the upper limit detection value issue seen with many of the indicators such as coliphages and bacteria, for Adenovirus, high concentrations (>10³ GEC per 100mL) were present in both the influent and NCT2RW samples, resulting in the log₁₀ reductions being very small in some samples. When the log₁₀ reduction of adenovirus is calculated using only infectious adenoviruses, the average log₁₀ reduction is still relatively low 2.83. Based on Figure 2-3, wastewater treatment achieves a minimum of 1 to 2 log₁₀ greater

reductions for indicator viruses. Norovirus log_{10} reductions were the greatest of the enteric virus log_{10} reductions; however, as with coliphage viruses, these reductions were based on upper limit detection values, as no Norovirus was detected in most reclaimed water samples.

Figure 2-4 displays the box and whisker plots of log₁₀ reductions of protozoan parasites and surrogates. Based on this figure, the median log₁₀ concentrations of *C. perfringens* spores only and spores plus vegetative cells, as bacterial indicator surrogates for protozoan parasites, were 4.28 and 4.51, respectively. The median log₁₀ reduction for *Cryptosporidium* oocysts was 2.20, and the median log₁₀ reduction for *Giardia* was 2.64. The low log₁₀ reductions for *Cryptosporidium* and *Giardia* are based on not only the low levels of (oo)cysts in the influent raw wastewater samples (at about 100 (oo)cysts per 100 mL) but also on the detection of measurable concentration of (oo)cysts in the NCT2RW samples. From Figure 2-4, the log₁₀ reduction minimum and maximum values, as well as the median values, are similar between *C. perfringens* spores and vegetative cells and likewise, they are similar for *Cryptosporidium* and *Giardia*. The log₁₀ reduction difference between protozoan indicators and pathogens is approximately 2 log₁₀.

		Raw Sewage (n=22)			Reclaimed Water (n=22)			-
Microbe	Units	Average Log ₁₀ Concentration	Standard Deviation	Range	Average Log ₁₀ Concentration	Standard Deviation	Range	Number of positive samples
E. coli		6.42	0.28	(5.99, 7.28)	0.05	0.18	(0.00, 0.84)	2/22
Enterococcus	MPN/100mL	5.59	0.22	(5.06, 6.09)	ND*	ND	ND	0/22
Salmonella spp.		4.08	0.99	(2.88, 5.88)	-0.86	0.45	(-1, 0.99)	2/22
Somatic Coliphage		4.21	0.35	(3.60, 4.88)	0.07	0.18	(0, 0.60)	3/22
F+ Coliphage	PFU/100mL	3.97	0.36	(3.38, 4.75)	0.11	0.33	(0.00, 0.33)	2/22
Total Coliphage		4.44	0.32	(3.70, 4.93)	0.21	0.37	(0, 1.18)	6/22
Norovirus GII		3.49	0.7	(2.28, 5.99)	0.24	0.77	(0.00, 2.72)	2/22
Adenovirus A-F	GEC/100mL	4.57	0.92	(2.44, 5.98)	2.72	1.56	(0.00, 4.69)	17/22
C. perfringens (Spores)		4.29	0.37	(3.22, 4.82)	0.04	0.11	(0.00, 0.43)	2/22
C. perfringens (Total)	CFU/100mL	4.49	0.38	(3.05, 4.93)	0.06	0.23	(0.00, 1.00)	3/22
Cryptosporidium		1.57	0.35	(0.81, 1.98)	-0.78	0.36	(-1.52, -0.20)	22/22
Giardia	(oo)cycsts/100mL	1.48	0.38	(0.65, 2.05)	-1.22	0.38	(-2, -0.62)	20/22

Table 2-3: Summary data of all indicator and pathogenic organisms for 22 raw sewage and reclaimed water samples

*ND is a Non-Detect Sample

Location ^a	Average Viral Infectivity (mRNA -IU) ^b	Average Viral Genomes (genome copies, GEC)	Samples positive by ICC-qPCR	Samples Positive by qPCR	Ratio (GEC/IU)
А	3.32E+01	5.67E+03	2	4	171/1
В	2.01E+01	1.11E+04	2	5	552/1
С	1.56E+02	3.89E+03	1	3	25/1
D	6.26E+01	4.44E+03	2	5	71/1
Total	6.79E+01	6.27E+03	7	17	204/1

Table 2-4: Comparison of estimated concentrations of human adenovirus by cell culture/mRNA qRT-PCR and direct qPCR

^a Reclaimed water samples were collected from 4 water reclamation facilities producing tertiary treated dual disinfected reclaimed water.

^b Adenovirus infectivity (mRNA) was determined using a cell culture/mRNA qRT-PCR assay. The estimation of concentrations of human adenovirus from sewage samples was determined semi-quantitatively using a calibration curve obtained with adenovirus 2.



* Indicates upper detection limit value. Log₁₀ reduction is calculated based on value detected in raw sewage and detection limit value in reclaimed water

Figure 2-2: Box and whisker plots of average log_{10} reductions per 100mL for pathogenic bacteria and indicators. The box portion of the box and whisker plot is characterized by the midline, which is the median value of the dataset as well as the two hinges, which are the upper and lower quartiles of the data set, while the whiskers are the minimum and maximum values of the dataset, excluding any outliers. Any outliers are plotted as individual points outside the whiskers; however there were no outliers detected in this analysis.



* Indicates upper detection limit value. Log₁₀ reduction is calculated based on value detected in raw sewage and a detection limit value in reclaimed water

Figure 2-3: Box and whisker plots of average log_{10} reductions per 100mL for enteric viruses and indicators. The box portion of the box and whisker plot is characterized by the midline, which is the median value of the dataset as well as the two hinges, which are the upper and lower quartiles of the data set. The whiskers are the minimum and maximum values of the dataset, excluding any outliers. Any outliers are plotted as individual points outside the whiskers; however there were no outliers detected in this analysis.



Figure 2-4: Box and whisker plots of average log_{10} reductions per 100mL for protozoan parasites and surrogates. The box portion of the box and whisker plot is characterized by the midline, which is the median value of the dataset as well as the two hinges, which are the upper and lower quartiles of the data set, while the whiskers are the minimum and maximum values of the dataset, excluding any outliers. Any outliers are plotted as individual points outside the whiskers; however there were no outliers detected in this analysis.

2.4 Discussion

From my results, it is not clear that NCT2RW meets the log_{10} reduction performance requirements as established by the state of North Carolina for potable reuse. The measured log_{10} reductions for bacterial indicators differed, with greater than 6 log_{10} reductions for E. coli, thus exceeding the specified 6 log_{10} reduction target of the State of NC. However, the FIBs of enterococci and *C. perfringens* were consistently below this reduction target. Measured reductions for *Salmonella* spp. were even lower than the log_{10} reductions for bacterial indicators. For viral indicators, the log_{10} reductions for somatic, F+, and total coliphages averaged 4 to 4.5 log_{10} , a value nearly 1 log_{10} less than the state mandated 5 log_{10} reduction level. For pathogenic enteric viruses the log_{10} reductions were consistently lower than measured indicator virus log_{10} reductions for both adenovirus and noroviruses, with gene copies of both organisms remaining detectable in reclaimed water samples. The log_{10} reduction of infectious adenovirus samples was higher when compared with the reduction of total gene copies, but was still below the state mandated reduction target of 5 log_{10} for viruses. Reclaimed water treatment did achieve a 4 log_{10} reduction for *C. perfringens* as a protozoan parasite surrogate in most reclaimed water samples, but protozoan parasites were still present at low but detectable levels in nearly all samples of tertiary treated reclaimed water, resulting in low log_{10} reductions that were less than the 4 log_{10} reduction target.

Current practice involves the use of indicator organisms to evaluate the microbial quality of reclaimed water; specifically in North Carolina, the organisms specified by the Type 2 reclaimed water legislation are *E. coli*, coliphage viruses, and *C. perfringens*. Though indicator organisms are generally assumed to have a predictive relationship with pathogens in water samples, this relationship is considered to be imperfect (Havelaar et al., 1993; Simpson et al., 2003). As evident in the data presented here, the treatment effect seen with indicator organisms is not always the same as that seen with pathogens. This difference in log₁₀ reductions may be a result of the differences in detection methods. The way in which the treatment performance effect is traditionally evaluated (log₁₀ reductions) also may not always be the most representative way of displaying treatment efficacy data because it does not consider lower and upper detection limit values. As such, one of my goals in this research was to present log₁₀ concentrations and log₁₀ reductions for both indicators and pathogens from NC Type 2 producing water reclamation facilities.

Log₁₀ reductions are a common measure of treatment efficacy (Rose et al., 1996; Rose et al., 2001), but do not always give an accurate picture of treatment effect. For example, in my
results, some of the organisms (particularly the indicator organisms) are limited by an insufficient upper limit detection value in that the log₁₀ reduction is calculated based on the value detected in raw sewage and the exceedance of the lower detection limit value in reclaimed water. The actual log₁₀ reduction based on treatment may be higher than observed; however, values are limited based on the measurable values in the influent raw sewage and the lower detection limit values in the reclaimed water. As these values are not entirely representative of the treatment effect, I have presented the log₁₀ concentrations for both the influent and NCT2RW samples (Table 2-3).

Another important consideration is the relationship between pathogen infectivity and direct genome or physical particle recovery values, specifically for the enteric viruses and the protozoan parasites. For Norovirus and for the protozoan parasites, log₁₀ reductions are based on the total number of virus particles and the total number of (oo)cysts in each sample rather than the number of infectious units. I found *Cryptosporidium* and *Giardia* at low levels in all reclaimed water samples, but I did not assess their infectivity. Harwood et al. (2005), who also evaluated reclaimed water (single disinfection only), found that in approximately 25% of disinfected effluent samples infectious *C. parvum* was present. When I assessed infectivity for adenoviruses, the concentrations of gene copies (DNA) as detected by real time q-PCR differed from the concentrations of infectious viruses (mRNA) as detected by integrated cell culture RT-qPCR. Nearly 32% of all type 2 reclaimed water samples had detectable infectious adenovirus after dual disinfection treatment. With such a large proportion of samples containing infectious viruses, it is important to consider the risk of infection from exposure to this type of reclaimed water, particularly from potable reuse scenarios.

Additional study is required to determine if the \log_{10} performance targets for the indicator organisms are met in NCT2RW samples. In some cases, especially for enterococci and coliphages, raw sewage concentrations were too low and the lower detection limits in reclaimed water were too high to quantify the \log_{10} reduction performance targets of the State of North Carolina. More study is also needed to determine whether the pathogens I detected, specifically the protozoan parasites, are viable and infectious. However, from my results it is clear that the current treatment scheme is not effective in removing or inactivating infectious adenovirus despite the complete inactivation of indicator viruses based on their limits of detectability. I used standard techniques to detect indicator bacteria, coliphage viruses, and protozoan parasite surrogates, but detected very few to no indicator organisms in the reclaimed water samples. To address this issue, either larger samples of reclaimed water or alternative detection methods are required to better quantify and evaluate the \log_{10} reduction performance of NCT2RW systems. In addition, alternate treatment options, such as advanced membrane treatment, could be considered for potable reuse options if the log_{10} reduction performance targets proposed by the state of NC are required but are not being met by the currently recommended treatment scheme.

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CHAPTER 3: EVALUATION OF THE RELATIONSHIP BETWEEN FECAL INDICATORS AND PATHOGENS IN SEWAGE IMPACTED SURFACE WATERS PROPOSED FOR COMBINATION WITH RECLAIMED WATER FOR POTABLE REUSE IN NORTH CAROLINA

3.1 Introduction

Rivers and other surface waters are widely used as a resource for drinking water production and for various recreational activities; however, increases in population has put pressure on freshwater resources as well as contributed to the risks associated with freshwater quality (Jacob et al., 2015). In many areas, freshwater used for drinking water production is impacted by upstream wastewater inputs, resulting in *de facto* reuse for drinking water supplies as well as for primary contact recreation. As interest grows in the various beneficial uses of treated wastewater as reclaimed water, including its potable reuse for drinking water supply, there is a need to evaluate its microbial quality and the risks associated with such *de facto* reuse (NAS, 2012).

Pathogens present in fecal waste and wastewaters are of concern because treated sewage effluent discharged to water resources used for beneficial purposes downstream have the potential for health related microbial risks (NAS, 2012). Specific microorganisms of concern may include pathogenic bacteria such as *Salmonella* spp., enteric viruses such as Noroviruses and Adenoviruses and protozoan parasites such as *Cryptosporidium parvum*, and *Giardia lamblia*. Traditional monitoring of the microbial quality of water and wastewater for legislated state or Federal regulations is rarely based on the analysis of pathogens and instead involves the monitoring of fecal indicator microorganisms, which are typically non-pathogenic microorganisms known to be associated with fecal contamination. In North Carolina, potable reuse has been proposed as a combination of at least 80% surface water with up to 20% tertiary treated, dual disinfected reclaimed water, which is stored for 5 days then treated using conventional drinking water treatment methods. The state of North Carolina has set standards for both intake surface water and for the reclaimed water produced by wastewater utilities, using indicator microorganisms to monitor indirectly for the potential pathogens of concern. In North Carolina, run of river (or flowing stream) systems used as source water for drinking water supply must have \leq 300-50 fecal coliforms or *E. coli* per 100mL depending on off stream storage (0.5 to 4 hours) (NC DENR, 1996). Source waters must also have a minimum 5 days of off stream pre-treatment/storage to maintain raw water quality and avoid plant influent water variations. Reclaimed water standards for Type 2 reclaimed water allowed for use as source water for the drinking water supply specify log₁₀ reduction targets of 6 log₁₀ for *E. coli* bacteria, 5 log₁₀ for coliphage viruses, and 4 log₁₀ for *Clostridium perfringens* as a surrogate for protozoan pathogens.

Despite continued reliance on indicator microorganisms to indirectly monitor pathogen occurrence in environmental waters used for beneficial purposes, it has been demonstrated that fecal indicator bacteria (FIB) (and in particular coliform bacteria) do not always reflect the presence and concentrations of all classes of pathogens in water or wastewater due to the relatively susceptibility of most FIB to chemical disinfection (Miescier and Cabelli, 1982). Assessments of coliform bacteria have also failed to correlate with the presence of protozoan parasites, specifically *Cryptosporidium* (Bonadonna et al., 2002), and enteric viruses (Havelaar et al., 1993). The use of alternative indicator microorganisms has been proposed to indicate the presence and concentrations of fecal pathogens in environmental, drinking and wastewaters, specifically FIB such as *Enterococcus* spp. (Miescier and Cabelli, 1982) and *C. perfringens*

(Fujioka and Shizumura, 1985; Payment and Franco, 1993), and fecal indicator viruses such as coliphages (Debartolomeis and Cabelli, 1991; Gantzer et al., 1998).

Only a few studies have been conducted to evaluate reuse waters and ambient surface waters for the relationships of candidate fecal indicators with pathogens (Rose et al., 1996; Harwood et al., 2005). Rose et al. (2001) has suggested that ambient surface waters used for direct combination with reclaimed waters was of lower microbiological quality than treated wastewaters. Additionally, Harwood et al. (2005) has suggested that in reclaimed waters there is no statistically significant relationship between traditionally monitored indicator organisms and Cryptosporidium and Giardia (00)cysts or enteric viruses, indicating the need for a suite of indicator viruses in the evaluation of treated wastewaters. Studies of fecal indicators and enteric pathogens in sewage impacted surface waters have indicated that the relationship is complex and variable (Borrego et al., 1987; Wilkes et al., 2009). Borrego et al. has suggested that the relationship between indicators and pathogens is complicated by temperature and the source of contamination, while Wilkes et al. 2009 has proposed that indicator/pathogen relationships are overall weak, seasonally dependent, site specific, but primarily positive. There is therefore a need for further study on the occurrence and concentrations of both alternative fecal indicators and various enteric pathogens in such waters to gain additional understanding as to their relationships. My goal in this study was to quantify both fecal indicator microorganisms and pathogens in run of river and sewage impacted surface waters proposed for or otherwise candidates for potable reuse in North Carolina and to examine the predictive relationships between these two groups by several statistical methods, including binary logistic regression.

3.2 Methods

3.2.1 Sample Collection and Storage

I collected surface water samples from 2 run of river drinking water treatment plants and 2 sewage impacted reservoir drinking water treatment plants in central North Carolina. The facilities included: (1) the Hillsborough Drinking Water Treatment Plant, using the Eno River; (2) the Cary/Apex Drinking Water Treatment Plant, using Jordan Lake; (3) the E.M. Johnson Water Treatment Plant, using Falls Lake; and (4/5) the Smithfield Water Treatment Plant using both the local reservoir (4) and the Neuse River (5). Surface waters were collected as grab samples from various sampling points in sterile bottles, and kept chilled in coolers with ice during transport to Chapel Hill. Samples collected from treatment plants with reservoirs (Cary/Apex, and E.M. Johnson) were collected from the water treatment plant intake structure. Run of river treatment plant samples and the Smithfield Reservoir samples were collected approximately 2 meters from shore and approximately 1 meter below the surface of the water. The samples were stored at 4°C upon arrival at the laboratory.

3.2.2 Sample Processing and Microbial Detection

Surface water samples were collected as 16L sample volumes and split into a 12L sample volume for pathogen analysis and a 4L volume for indicator analysis. Samples were processed and concentrated according to the procedures described in section 2.2.2, with the addition of an initial centrifugation of 1500 x g for 30 minutes step applied to the enteric virus concentration method in order to remove sediment and other solids before hollow fiber ultrafiltration. If the supernatant turbidity was greater than 4 NTU (a turbidity appropriate for hollow fiber ultrafiltration), the surface water was centrifuged again at 5000 x g for an additional 30 minutes. Viruses in the centrifuged sediment were recovered by elution at 60RPM with 5 parts 0.5M, pH 7.5 Threonine to 1 part surface water solids for 1 hour, added back to the concentrated

supernatant for further processing and analysis, following the method of Sheih et al. (1997). Sample processing and concentration steps for surface water are summarized in Figure 3-1. Methods for the detection of pathogenic and indicator organisms are as described in section 2.2.3-2.2.8.

3.2.3 Statistical Analysis

In order to evaluate the relationship between indicator organisms and pathogens in surface water samples, the detected concentrations were first adjusted by sample volume and then log transformed and analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). An ANOVA regression analysis was performed using a Tukey post test with log_{10} concentration data, which uses group means to compare differences among surface water samples. Specifically, the mean \log_{10} concentration of each indicator organism was compared with each of the other indicator organisms by class and with the relevant pathogen detected. To evaluate the correlation between indicators and pathogens in these samples, Pearson's test was used or the relevant nonparametric tests for data not normally distributed were used. The purpose of this test is to measure the linear dependence or correlation between two variables by using linear regression tools. In this analysis, indicator organisms were evaluated for their correlative relationship to other indicators and pathogens. Additionally, a binary logistic model was used to test the hypothesis as to whether indicator organism concentrations were predictive of the presence or absence of pathogens in surface water, as described by Harwood et al. (2005). Briefly, this method involved the use of continuous independent variables with non-detectable values being reported as a value of 0. The data for indicator organisms (total coliforms, E. coli, *C. perfringens*, and coliphages) was then converted into a string of binary variables that represented the presence or absence of each indicator. The ability of the indicator data string to predict the presence of each pathogen (Cryptosporidium, Giardia, Salmonella spp., adenoviruses

and noroviruses) was assessed separately and also for all viruses (Adenovirus groups A-F, Norovirus GII, and combined as an enteric viruses category). Results were expressed as the percentage of samples correctly classified into the "pathogen present" and "pathogen absent" categories. Binary logistical modeling was conducted using SPSS Version 24 (IBM Corporation, Armonk, NY).



Figure 3-1: Diagram of surface water sample processing for indicator and pathogenic microorganisms

3.3 Results

The results presented here represent 22 seasonally representative multiple samplings from four run-of-river or minimally stored source waters of drinking water treatment facilities. The focus is on the concentrations of microorganisms in these sewage-impacted surface water sites, either downstream of a wastewater discharge or a reservoir impacted by wastewater effluent input.

3.3.1 Microbial Concentrations in Surface Water

Average log₁₀ concentrations of indicator organisms in sewage impacted surface waters are summarized in Figure 3-1. The lower limits of detection, as described in sections 2.2.3 – 2.2.8, were used as measured values for samples in which the microorganism was not detected in the sample volume analyzed. Norovirus was not detected in any of the surface water samples, was below 1 RT-qPCR gene copies per 12 L of sample water analyzed and will not be included in this analysis. Table 3-1 displays the results of statistical comparison for log₁₀ concentration data for fecal indicators, Salmonella bacterial pathogens, adenoviruses and Norovirus as viral pathogens, and *Cryptosporidium* and *Giardia* as protozoan parasite pathogens. The median log₁₀ concentrations of each microorganism detected in surface waters are presented in Figure 3-2. Site specific information on average log₁₀ microbial concentration for each organism is provided in Figures 3-3, 3-4, and 3-5.

Total coliform concentrations were the highest of the microbial indicator levels in the surface waters, with an average concentration of 7.9 x 10^3 MPN (most probable number) per 100mL, followed by *E. coli* and *Enterococcus* spp. with average concentrations of 2.12 x 10^2 MPN per 100mL and 1.98 x 10^1 MPN per 100mL, respectively. As shown in Table 3-1, there was a statistically significant difference between the log₁₀ concentration of total coliforms and *E. coli* (p-value <0.0001) and between the concentration of Enterococci and total coliforms (p-value

<0.0001); however, there was no statistically significant difference between *E. coli* and Enterococci. Concentrations of *Clostridium perfringens* bacteria, the surrogate for protozoan parasites, averaged 7.17 x 10¹ CFU/100mL for the bacterial spores, and 7.99 x 10¹ CFU/100mL for both spores and vegetative cells. There was no statistically significant difference between the log₁₀ concentrations of vegetative plus spores or spore only *Clostridium perfringens* concentrations (p-value = 0.997).

Average *Salmonella* spp. concentrations in surface waters, detected by a culture based MPN assay, were on average 3.68×10^2 MPN per 100mL with a range of 1.0×10^{-1} to 8×10^3 MPN per 100mL. Based on ANOVA using the Turkey post test, *Salmonella* spp. concentrations were significantly different from the concentrations of total coliforms (p-value <0.0001), *E. coli* (p-value: 0.0021), and *Enterococcus* spp. (p-value: 0.011). As indicator organisms are expected to be detected at higher concentrations than pathogens, these relationships were not unexpected. Indicator bacteria were detected in concentrations approximately 10-fold greater than *Salmonella* spp. for total coliforms, *E. coli*, and *Enterococcus* and 10-fold less for *Clostridium perfringens* spores and spores plus vegetative cells.

Based on Figure 3-3, the highest concentrations of bacteria were detected in surface water samples collected from the Neuse River at Smithfield, followed by the Eno River at Hillsborough. Concentrations of bacteria were higher in river water samples than in reservoir samples, and higher in the Smithfield reservoir when compared to Jordan and Falls Lake. *E. coli* was detected at highest concentrations at an average of 4.17×10^1 MPN per 100mL in the Neuse river, 2.51×10^1 MPN in the Eno river, 3.16×10^0 MPN in the Smithfield Reservoir, and 2.43×10^0 and 1.56×10^0 MPN in Falls and Jordan Lakes, respectively. Similar levels of Enterococci were detected in surface water samples, with average levels of 3.89×10^1 MPN per 100mL in the Neuse river, $5.14 \ge 10^{\circ}$ MPN in the Eno River, $5.90 \ge 10^{\circ}$ MPN in the Smithfield Reservoir, $2.14 \ge 10^{\circ}$ in Falls Lake and no *Enterococcus* spp. was detected in Jordan Lake. There was a statistically significant difference between the log_{10} concentration of *E. coli* detected in Neuse River water samples and Jordan Lake (p-value: 0.0454). Very low levels of *Salmonella* spp. were detected in surface water samples - the average concentrations were 0.38 MPN per 100mL in the Neuse River, 0.17 MPN in the Eno River, 0.42 MPN in the Smithfield Reservoir, and 0.24 and 0.38 MPN in Falls and Jordan Lakes, respectively. Despite apparent differences in concentrations between river and reservoir samples, there was no statistically significant difference water samples.

Average concentrations of coliphage indicator viruses were 2.44×10^{1} PFU (plaque forming units) per 100mL, 1.5×10^{0} CFU per 100mL, and 2.48×10^{1} PFU per 100mLfor somatic, F+ and total coliphages respectively. Therefore, concentrations of somatic coliphages were greater than those of F+ coliphages by 16-fold on average. As shown in Table 3-1 there was no statistically significant difference between the log₁₀ concentrations of somatic and total coliphage, but there was a statistically significant difference between somatic and F+ coliphage (p-value:0.0007) and between total and F+ coliphage (p-value:0.0004). The average log₁₀ concentration of Adenovirus A-F detected in surface water samples was 1.44×10^{4} GEC per 100mL. It should be noted that the concentrations of enteric virus concentrations are genome copies, as samples were analyzed by real time q-PCR for adenoviruses and RT-qPCR for noroviruses. There was a statistically significant difference between the log₁₀ concentration of Adenovirus A-F detected in surface water samples and each type of coliphage and RT-qPCR for Adenovirus A-F detected in surface water samples and each type of coliphage and RT-qPCR is a sample of the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage virus (p-value for the surface water samples and each type of coliphage

<0.0001). Coliphage viruses were detected at levels up to 1000-fold lower than Adenovirus in surface water samples.

Based on Figure 3-4 and the site specific analysis of detected viruses, Adenovirus A-F was detected at higher concentrations than the coliphage indicator viruses; the highest concentrations were detected in the Eno River at Hillsborough, with an average concentration of 6.71×10^2 GEC per 100mL, and in the Smithfield Reservoir, where the average concentration was 1.89×10^2 GEC per 100mL. Somatic and total coliphages were detected at similar levels at all sites, with the highest average concentrations detected in the Neuse River at Smithfield, where the average concentration of total coliphages was 5.85×10^{1} PFU per 100mL and the average concentration of somatic coliphages was 1.20×10^{1} PFU per 100mL, followed by the Smithfield Reservoir, where the average concentration of total coliphages was 4.95 x 10¹ PFU per 100mL and the average concentration of somatic coliphages was 1.23×10^{1} PFU per 100mL. F+ coliphages were detected least frequently, and at the lowest concentrations, with average concentrations below 1.77×10^{0} PFU per 100mL for all samples and all sites. Despite apparent differences in concentrations between river and reservoir sampling sites, there was no statistically significant difference between the \log_{10} concentrations of somatic, F+, total or adenoviruses detected at either of these sampling sites.

The US EPA Recreational Water Quality Guidelines recommend that to reduce illness below 36/1000 individuals, the concentrations of *Enterococcus* and *E. coli* in ambient surface waters should be at or below a level of 35 and 100 per 100mL respectively. For this dataset, 4 samples exceeded this recommendation for *Enterococcus* while 2 samples exceeded the level for *E. coli*. Furthermore, when *E. coli* levels exceeded the 100 per 100mL target, there was a higher detectability of adenovirus and *Salmonella* spp. in these samples. Similarly, for *Enterococcus*,

when levels exceeded the 35 per 100mL level, there was a higher detectability of *Salmonella* in these samples.

As with enteric viruses, the presence and concentrations of *Cryptosporidium* and *Giardia* (oo)cysts are not in units of infectivity or culturability but instead in microscopically detectable immunofluorescent particles of characteristic size and shape. They are the total number of (oo)cysts present in each sample and are not necessarily infectious (oo)cysts; the cited numbers of (oo)cysts do not reflect viability based on either staining reactions using DAPI/PI or infectivity using cell culture for Cryptosporidium, due to a lack of the time and resources needed to conduct such additional testing on these samples. Average Cryptosporidium concentrations were 1.18 oocysts per 100mL with a range of 0.10 to 7.02 oocyts per 100mL and Giardia concentrations were on average 0.26 cysts per 100mL with a range from 0.10 to 1.52 cysts per 100mL. As displayed in Table 3-1, there was a statistically significant difference between the log₁₀ concentration of *Cryptosporidium* and total *C. perfringens* (p-value<0.0001) and spores of C. perfringens (p-value<0.0001), as well as between Giardia and total C. perfringens (pvalue<0.0001) and spores of C. perfringens (p-value<0.0001). Additionally, there was a statistically significant difference between the \log_{10} concentration of *Cryptosporidium* and Giardia (p-value: 0.0083). On average, C. perfringens was detected at levels 10-fold greater than *Cryptosporidium* and 100-fold greater than *Giardia*.

Based on Figure 3-5, *C. perfringens* spores and spores plus vegetative cells were detected at a greater frequency than *Cryptosporidium* and *Giardia* for each sampling site. The highest concentrations of *C. perfringens* spores and vegetative cells were detected in the Neuse River at Smithfield with average concentrations of 1.81×10^2 CFU per 100mL for spores and 1.74×10^2 CFU per 100mL for spores plus vegetative cells. A similar trend occurred for *Cryptosporidium*

and *Giardia*, with average concentrations of 2.29 oocysts per 100mL and 0.57 cysts per 100mL (respectively) in the Neuse River at Smithfield. The lowest concentrations of both *C. perfringens* and the protozoan parasites were detected in the reservoir sites (Smithfield Reservoirs, Jordan Lake, and Falls Lake). There was a statistically significant difference between the log₁₀ concentration of *Cryptosporidium* detected in the Neuse River and with the log₁₀ concentration of *Cryptosporidium* detected in each of the other surface water sampling sites, including the Eno River (p-value: 0.0203), Jordan Lake (p-value:0.0007), Falls Lake (p-value: 0.0060), and the Smithfield Reservoir (p-value:0.0153).

Concentration Analysis						
Organism 1	Organism 2	Post-Test	Mean Difference	P-value	Ν	Significant?
Total Coliforms	E. coli	Turkey	2.169	< 0.0001	22	Y
E. coli	Enterococcus	Turkey	0.1962	0.5057	22	Ν
Enterococcus Salmonella	Total Coliforms	Turkey	2.365	< 0.0001	22	Y
	Total Coliforms	Turkey	-3.201	< 0.0001	22	Y
	E. coli	Turkey	-1.033	0.0021	22	Y
	Enterococcus	Turkey	-0.8364	0.011	22	Y
Somatic Coliphage	F+ Coliphage	Turkey	0.7869	0.0007	22	Y
F+ Coliphage	Total Coliphage	Turkey	-0.8143	0.0004	22	Y
Total Coliphage Adenovirus A-F	Somatic Coliphage	Turkey	-0.02738	0.999	22	Ν
	Somatic Coliphage	Turkey	2.795	< 0.0001	22	Y
	F+ Coliphage	Turkey	3.582	< 0.0001	22	Y
	Total Coliphage	Turkey	2.767	< 0.0001	22	Y
Total C. perfringens	C. perfringens spores	Turkey	0.03687	0.9972	22	Ν
Cryptosporidium	Total C. perfringens	Turkey	-1.743	< 0.0001	22	Y
	C. perfringens spores	Turkey	-1.78	< 0.0001	22	Y
Giardia	Total C. perfringens	Turkey	-2.348	< 0.0001	22	Y
	C. perfringens spores	Turkey	-2.385	< 0.0001	22	Y
Cryptosporidium	Giardia	Turkey	0.6049	0.0083	22	Y

Table 3-1: ANOVA results for log₁₀ concentration comparison

3.3.2 Correlations between Pathogens and Indicators

Surface water data were analyzed by treatment plant and as a pooled data set (all facilities) to determine if the concentrations of the indicators (total coliforms, *E. coli*, *Enterococcus* spp., pasteurized and unpasteurized *C. perfringens*, somatic, F+, and total coliphages) were correlated with each other or with the concentrations of the pathogens (*Salmonella* spp., *Cryptosporidium, Giardia*, and Adenovirus A-F. Norovirus GII was not included in the analysis because it was not detected in any of the surface water samples.

No significant correlations were found in the analysis of results by facility, likely due to small sample size. However, significant correlations were found between pooled data sets of the log₁₀ concentrations of *Salmonella* spp. and total coliforms (Spearman's $r_s = 0.513$; P = 0.015) and between Adenovirus groups A-F and F+ coliphages (Spearman's $r_s = -0.430$; P = 0.047). Figure 3-6 displays the correlation analysis between *Salmonella* spp. and total coliform bacteria. Significant correlations were also observed between the concentrations of indicator organisms in the pooled data sets; specifically, these included the correlation between the concentrations of *Enterococcus* spp. and *E. coli* (Spearman's $r_s = 0.6829$, P = 0.0005), somatic and F+ coliphages (Spearman's $r_s = 0.9484$, P = <0.0001), F+ and total coliphages (Spearman's $r_s = 0.5783$, P = 0.0048), and pasteurized and unpasteurized *C. perfringens* (Spearman's $r_s = 0.9795$, P = <0.0001).

Adenoviruses were found above detection limits in 41% of the surface water samples (n=22); coliphage viruses co-occurred with adenovirus in 78% of these samples for total coliphages, and 67% for somatic coliphages. There was no adenovirus co-occurrence for F+ coliphages. *Cryptosporidium* oocysts were present and above the detection limits in 86% of samples and co-occurred with both pasteurized and unpasteurized *C perfringens* in 100% of

samples examined. Similarly, *Giardia* cysts were detectable in 81% of samples and there was a co-occurrence of 100% with both pasteurized and unpasteurized *C. perfringens*.

Binary logistic regression analysis was used to test the hypothesis that indicator organisms were correlated with the presence or absence of pathogens in sewage impacted surface waters. The data for the detected pathogens (Cryptosporidium, Giardia, Salmonella spp., and adenoviruses) were converted to binary data, either pathogen present (1) or pathogen absent (0), and compared to the detected concentrations of their respective fecal indicators (total coliforms, E. coli, and enterococci as bacteria indicators, C. perfringens, and the different coliphages (somatic, male-specific/F+ and total) as virus indicators) and evaluated for the relationships between and among the two groups of microorganisms based on presence or absence in samples. Nagelkerke's *R*-square, which ranges from 0.0 to 1.0, indicates the strength of the association; stronger associations have values closer to 1.0. An indicator-pathogen combination that displayed a moderate correlation was F+ coliphages and adenovirus presence/absence, with an Rsquare of 0.476. A much stronger association was seen between *E. coli* and *enterococcus* spp. as fecal indicator bacteria (R-square = 0.706), and between pasteurized and unpasteurized C. *perfringens* as protozoan parasite indicators (R-square = 0.774). These associations between pathogens and their corresponding fecal indicators, or between fecal indicators of the same microbial group (e.g., the different FIB), is to be expected if they are meeting the key criteria of a suitable fecal indicator. In addition, some were detected using the same analytical method with variations in either the culture media or the treatment of the sample (Colilert and Enterolert by defined substrate analysis in multiwell plates), and the use of the same agar (CP Chromoselect agar by membrane filtration and culture analysis of pasteurized and unpasteurized samples, respectively, for *C. perfringens*).

Figure 3-3 displays the results of this binary logistic regression analysis. True positives were positive for fecal indicators and pathogens, true negatives were samples negative for both fecal indicators and pathogens, false positives were positive for the indicator but negative for the pathogen, and false negatives were positive for the pathogen and negative for the indicator. The sum of each of these categories is 100% for each indicator-pathogen grouping. For many of the fecal indicator organisms evaluated here, especially the fecal indicator bacteria for Salmonella and the *Clostridium perfringens* for the two protozoan parasites, Cryptosporidium and Giardia, there is a high true positive rate, typically of about 50% or more for the FIBs and about 70% or more for the C. perfringens, indicating that the pathogen and the indicator were both present and co-occurred in the surface water. However, there is often not a correspondingly high true negative rate for many of these indicators, including C. perfringens and the FIBs. For the viruses, the fecal indicator viruses (somatic, male-specific/F+ and total coliphages) gave true positive and true negative rates that were in the range of about 20-35% and 10-35%, respectively. However, there were also relatively high rates of false positives (about 20 to 40%) and sometimes false negatives (about 40% for both male-specific/F+ and total coliphages). There were no true positives for Adenovirus A-F detected using the male-specific/F+ coliphage indicator. The implications of these rates of true positives and negatives will be discussed further in section 3.4. 3.3.3 Comparison of the Microbiological Quality of Reclaimed Water and Surface Water

As the state of North Carolina has proposed the blending of 80% surface water with up to 20% reclaimed water as one of the steps in producing finished water for potable reuse, it is important to evaluate the microbiological quality of the two water types. In reclaimed water samples, *E. coli* and enterococci were detected on average at levels of 1.12 MPN per 100mL and 0 MPN per 100mL while in surface water these bacteria were detected at levels of 2.12×10^2 MPN per 100mL and 1.98 x 10¹ MPN per 100mL, respectively. This is an approximately 100

fold higher concentration of *E. coli* in surface waters. For the pathogenic bacteria, *Salmonella* spp. was detected at concentrations of 0.14 MPN per 100mL and 3.68 x 10^2 MPN per 100mL in reclaimed and surface waters, respectively. As with *E. coli*, there is also a higher proportion of pathogenic bacteria in the examined surface waters; based on the results presented here, there was a 100-fold greater concentration of *Salmonella* spp. in surface waters than in reclaimed waters. However, the difference in concentrations of *Salmonella* spp. detected in surface waters and reclaimed waters was not statistically significant, with a p-value of 0.8149.

For indicator viruses, very low levels were detected in reclaimed water samples at concentrations of 1.17, 1.29, and 1.62 PFU per 100mL for somatic, F+, and total coliphages, respectively. In surface waters, the average concentrations of these viruses were 2.44 x 10^1 PFU for somatic coliphages, 1.5 x 10^0 for F+ coliphages, and 2.48 x 10^1 for total coliphages. This is approximately a 10 fold difference between surface (higher concentrations) and reclaimed waters (lower concentrations) for somatic and total coliphages. However, there were similar levels of detection for F+ coliphages in these two waters. No noroviruses were detected in surface waters, however, in reclaimed waters, the average concentrations were 1.73 GEC per 100mL. Adenoviruses were detected at high levels in both water types with average concentrations in reclaimed water of 5.26×10^2 GEC per100mL and concentrations in surface water of 1.44×10^4 GEC per 100mL; this difference in concentration was statistically significant (p-value: 0.0105).

As with the other indicator organisms, low levels of *C. perfringens* were detected in reclaimed water samples, with average concentrations of 1.10 PFU per 100mL and 1.15 PFU per 100mL for spores and vegetative cells plus spores, respectively. In surface waters, concentrations of *C. perfringens* were 7.17 x 10^1 CFU/100mL for spores, and 7.99 x 10^1 CFU/100mL for vegetative cells plus spores, an increase of approximately 10 fold over reclaimed water samples.

For *Cryptosporidium* and *Giardia*, average concentrations in reclaimed water were 0.17 oocysts per 100mL and 0.06 cycsts per 100mL, while average concentrations in surface water were 1.18 oocysts per 100mL and 0.26 cysts per 100mL. Again, similar to the other microorganisms examined, the concentrations of protozoan parasites in surface water were approximately 10 fold greater than the concentrations in reclaimed water. For *Cryptosporidium*, the difference in log₁₀ concentrations in surface and reclaimed water was statistically significant (p-value: 0.0038), but for *Giardia*, the difference was not statistically significant (p-value: 0.0916).



Microorganism

*Norovirus GII was not detected

Figure 3-2: Median log₁₀ concentrations per 100mL of indicator and pathogenic organisms in 22 surface water samples from 4 drinking water treatment plants and 5 sources of surface water. Detection limits were used as concentrations for parameters that were not detectable.



Figure 3-3: Site specific average log_{10} concentrations per 100mL of bacterial indicators and pathogens



Figure 3-4: Site specific average log₁₀ concentrations per 100mL of viral indicators and pathogens



Figure 3-5: Site specific average log_{10} concentrations per 100mL of protozoan parasite surrogates and pathogens



Figure 3-6: Correlation analysis of relationship between *Salmonella* spp. and total coliform bacteria



Figure 3-7: Associations between detection of indicators and combination of indicators and pathogens detected in sewage impacted surface waters. Percentages were calculated from the total sample number (n=22).

3.4 Discussion

The current surface water quality monitoring approach targets indicator organisms, specifically total or fecal coliforms in surface water, in either a single daily grab sample or as a composite sample. In North Carolina, the suggested targets for drinking water intake sources are \leq 300-50 fecal coliforms or *E. coli* per 100mL depending on off stream storage (0.5 to 4 hours) (NC DENR, 1996). Additionally, the US EPA has specific requirements for the treatment of surface waters based on the reduction of *Cryptosporidium* and viruses in these sources. The most recent rule, the Long Term 2 Enhanced Surface Water Treatment Rule (US EPA, 2006), established a minimum 2 log₁₀ reduction of *Cryptosporidium*, with a requirement of filter monitoring to minimize the effects of poor performance. The goal of this rule was to expand protection for high risk surface water sources, such as those that may store water in open reservoirs, by requiring water utilities either to cover open reservoirs or to achieve additional log₁₀ reduction performance requirements (4 log₁₀ for virus, 3 log₁₀ for *Giardia lamblia*, and 2 log₁₀ for *Cryptosporidium*) (US EPA, 2006).

Although indicator microorganisms are typically used as predictors of fecal contamination and therefore are considered indirectly representative of pathogen content in water, this relationship is imperfect (Havelaar et al., 1993; Simpson et al., 2003; Harwood et al., 2005). One of my goals in this research was to evaluate the relationship between indicator microorganisms and pathogens they are intended to represent in surface waters to determine any predictive relationship between the two categories, fecal indicators and pathogens, in this type of water. As the detection of indicator microorganisms is typically the only microbiological testing performed by most drinking water treatment facilities, the link between and representativeness of pathogens and their indicators is an important consideration for water supply system and water supply regulators, including public health regulators of water supplies.

I found that pathogens were detectable in nearly all samples of sewage impacted surface water analyzed. *Salmonella* spp. was found in 91% of all samples at concentrations ranging from 0.1 to 1.2 MPN/100mL, Adenoviruses (detected based on the presence and concentrations of their nucleic acids) were found in 41% of all samples at concentrations ranging from 1 to 3.60 x 10⁴ GECs/100mL. *Cryptosporidium* and *Giardia* as detected by immunofluorescent microscopy were found in 100% and 81% of all samples, respectively. Total coliforms, *E. coli*, and *Enterococcus* were detected in 95%, 64%, and 50% samples (respectively), while somatic, F+ and total coliphage viruses were detected in 77%, 32% and 77% of samples, respectively. *C. perfringens* spores and vegetative cells plus spores were detected in 91% of all samples. As pathogens and indicators were detected in different volumes of surface water, it is likely that this

larger sample volume impacted the detectability of pathogens in surface waters. A larger sample volume and sample size is desirable and likely improved my ability to detect pathogens.

Bacteriophages have been previously suggested as indicators for enteric viruses (Havelaar et al., 1993; Turner and Lewis, 1995) because of their similar morphological characteristics and survival characteristics. I found a weak but statistically significant relationship between the presence or absence of adenoviruses and F+ coliphages by binary logistic regression but this was a negative relationship with adenoviruses present when F+ coliphages were absent. Additionally, the log_{10} concentration of adenoviruses was also negatively correlated with the log₁₀ concentration of F+ coliphages by Spearman's correlation analysis. However, it is important to note that low levels of F+ coliphages were detected in the surface water samples, while relatively high levels of genome equivalent copies (GEC) of adenovirus were detected. Despite the apparent correlations, levels of the indicator organisms (F+ coliphages) were neither higher than nor positively associated with the pathogen in this case. This is not an ideal quality of an indicator organism and is not necessarily protective of human health for surface water systems. As adenoviruses were detected by qPCR methods, an important factor in the evaluation of this indicator-pathogen relationship is the infectivity of these pathogenic viruses as well as their survival in surface waters.

As US EPA Method 1623 does not allow for the determination of (oo)cyst infectivity or the detection of human specific (oo)cysts, these are important limitations to my study, especially the lack of infectivity data on the protozoan parasites (US EPA, 2012). In this study, *Cryptosporidium* and *Giardia* were found at low levels by immunoflorescent microscopy, in nearly all surface water samples, but infectivity was not assessed due to lack of time and additional resources needed to process these surface water samples. Consequently, an important

limitation of my work is the lack of infectivity data on human pathogens and the inability to reliably predict human health risk based on the detection of these organisms in surface waters. Although the presence of pathogens in surface water is of concern, it is difficult to evaluate the human health risk posed by these microorganisms in the absence of infectivity data for them.

Indicator presence or absence was not consistently predictive of pathogen presence or absence by binary logistic regression, and my results indicated a high number of false negative or false positive values for one of the indicator pathogen combinations, specifically the adenovirus/F+ coliphage relationship. Those indicators that were detected more frequently, such as F+ coliphages, showed a higher frequency of false positives (pathogens absent, indictors present). This result is not necessarily undesirable because the goal of an indicator is to trigger an alert for pathogen presence, rather than for pathogens to be present at equal or greater numbers than the indicator. The pathogens detected less frequently, such as *Salmonella* spp., showed a higher frequency of true positives (pathogens present, indicators present); as the Salmonella was detected at concentrations on average 100-fold lower than the indicator organism, this represents an ideal indicator organism. FIB occurrence was not predictive of Salmonella spp. presence by binary logistic regression, but *Salmonella* spp. was statistically significantly correlated with the concentrations of total coliform by Spearman's correlation analysis. My results suggest that there may not be one "ideal" indicator for the prediction of survival or presence of pathogens in surface water; however, I did find evidence that the \log_{10} concentrations of indicator organisms are often correlated with pathogen concentrations.

Although individual indicator organisms and pathogens were weakly correlated or uncorrelated by binary logistic regression, there is some evidence that log₁₀ concentrations of indicator organisms are correlated with log₁₀ concentrations of pathogens in surface water. My

results indicate that enteric pathogens, including Salmonella bacteria, human enteric viruses such as Adenoviruses and the protozoan parasites *Cryptosporidium* and *Giardia*, are often present at detectable concentrations in surface waters that may be used as drinking water sources. In the comparison of reclaimed water to surface waters presented here, it is clear that the quality of surface waters is not of the same microbiological quality as NC Type 2-like reclaimed water. For nearly every microorganism examined, concentrations in surface water were at least 10 fold greater in the surface waters samples. Additional studies are needed to evaluate more thoroughly and rigorously the relationships between the fecal indicators and the enteric pathogens in these waters. Important consideration should be given to infectivity and culturability of protozoan parasites and enteric viruses in order to evaluate more accurately the human health risk from these pathogens. Additionally, my study only included a small number of samples (n=22), from a limited number of sample sites (n=5). Therefore, future work should therefore expand on both the number of samples and the surface water sources to provide a more representative selection of the range of conditions that occur in surface water sources used for drinking water.

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CHAPTER 4: EFFECTS OF 5 DAYS OF STORAGE OF SURVIVAL ON OF INDICATOR ORGANISMS IN AN 80/20 MIX OF SURFACE WATER AND NC TYPE 2 RECLAIMED WATER UNDER DIFFERENT ENVIRONMENT CONDITIONS

4.1 Introduction

Many ambient surface sources of drinking water in North Carolina and elsewhere are impacted by upstream wastewater sources and are practicing unplanned *de facto* wastewater reuse (NAS, 2012). Drinking water sources are categorized using a system that considerers hydrological conditions (lake, reservoir, stream), impacts from pollution sources, benefits from sedimentation, and potential die-off or dilution of pathogens in ambient water over time and space. Run of river (or flowing stream) systems in North Carolina also must have \leq 300-50 fecal coliforms or *E. coli* per 100mL, depending on the duration of off-stream storage (0.5 to 4 hours) prior to subsequent steps in the processes to produce drinking water by further treatment (NC DENR, 1996). Source waters must also have a minimum 5 days of off-stream pretreatment/storage to maintain raw water quality and avoid plant influent water variations. The basis of this time and distance requirement is unknown and potentially questionable due to the diversity and variability of microbial pathogens present in surface water and wastewater as well as potential differences in their survival under various conditions, such as water quality, temperature, sunlight, etc. (Auer et al., 1993; Astrom et al., 2007; Medema et al., 1997; 2003).

Recent North Carolina reclaimed water legislation has proposed a new potable reuse scheme that involves the combination of tertiary treated dual disinfected reclaimed water with currently used drinking water sources of surface water in a ratio of at least 80% surface water and up to 20% reclaimed water, followed by storage for a minimum of 5 days and then treatment by conventional drinking water treatment processes (NC DENR 2011, 2014). Currently, no studies have been conducted evaluating the survival of microorganisms in the NC approved 80/20 blend of surface and reclaimed water over the 5-day storage period. However, previous researchers have examined the survival of pathogens and indicators in marine water, groundwater and surface water.

Microbial survival is impacted not only by water type and quality but also by temperature, turbidity, sunlight exposure and other factors. Studies conducted in groundwater have indicated that microorganisms are generally stable in this type of water, with low log₁₀ reductions (<2) over relatively long periods of time (15 days or longer) (Keswick et al., 1982, Bitton et al., 1983). For example, Hepatitis A virus, poliovirus 1 and echovirus 1, survived well $(<1 \log_{10} \text{ inactivation})$ for at least 12 weeks in groundwater, wastewater and soil suspensions at 5°C (Sobsey et al., 1986). At 25°C HAV survived generally longer than poliovirus and echovirus, with 1-2 log₁₀ inactivation of HAV and 3-4 log₁₀ inactivation of poliovirus and echovirus in 12 weeks. From a review of the available literature on the decay rates of poliovirus 1, phage T7, and E. coli, Bitton et al. (1983) found that these microorganisms survived longer in ground water compared to marine water or surface water. Studies have also been conducted evaluating the survival of indicator organisms in sediments and indicated that with the increase in turbidity and potential for settling of microorganisms and other organic debris there is an increase in the variability of both the detection and the survival of the organisms over time (Gerba et al., 1975; Anderson et al., 2005). Gerba et al. (1975) found that the major factors influencing the survival of indicator organisms in soil are salt concentration, pH, organic matter, and electronegativity and that viruses survive at least as long as pathogenic bacteria in soil.

Anderson et al. (2005) found that decay rates (culturable counts over time) were influenced by strain of bacteria and that fecal coliforms decay rates were lower than those of enterococci.

Additionally, studies have been performed to examine the survival of microorganisms exposed to sunlight, which has been suggested as one of the main mechanisms of microbial decay in shallow waters. Many microbial survival studies have been conducted in seawater (Davies-Colley and Bell, 1994; Sinton et al., 1999), and have suggested that sunlight inactivation rates for indicator organism can be ranked (from greatest to least) as fecal coliforms > enterococci > F-RNA phages > somatic coliphages. However, equivalent survival data is not available for freshwater sources (Sinton et al., 2002). Most sunlight experiments performed using surface water were conducted in the field where solar radiation was not measured (deWet et al., 1995; Hernández-Delgado and Toranzos et al., 1995; Springthorpe et al., 1995), and many of these experiments indicated that E. coli varied with nutrient concentration (Springthorpe et al., 1995) or with lab versus field conditions (deWet et al., 1995) or with laboratory microcosms (Flint, 1987; McFeters and Stuart, 1972). Microcosm experiments typically considered the longer term (up to 250 days) effects of microbial storage in river water with temperature variations (Flint, 1987); in general, experiments found that survival was temperature dependent, with survival greater at 4°C (McFeters and Stuart, 1972).

Many of the factors specified above, such as the type of microbe, water type, water quality, turbidity, temperature, and sunlight, have the potential to influence both the detection and the survival of microorganisms over the state-mandated 5 day storage period for reclaimed water designated for potable reuse in NC. There is therefore a critical need to evaluate the survival of key microorganisms under specific conditions for these variables. My goal in this study is to evaluate the proposed 80/20 blend of surface water and reclaimed water approved for

potable reuse in NC for the effects on indicator microbe die-off during its storage for the required 5 days at various temperatures, mixing speeds, and both with and without sunlight exposure. A secondary goal is to evaluate the survival of naturally occurring fecal indicator bacteria in surface waters compared to survival in the same kind of water of mixed communities of the same kind of fecal indicator microbes from sewage that were first propagated in the laboratory and then seeded into the test water.

4.2 Methods

4.2.1 Sample Collection

Grab samples of tertiary treated, dual disinfected North Carolina "Type 2" reclaimed water (NCT2RW) were collected from the Orange County Wastewater Treatment plant in Chapel Hill, NC. Surface water was also collected as grab samples from the Cary/Apex Drinking Water Treatment Plant in Cary, NC. Samples were transported to the laboratory on ice and stored for less than 1 week at 4°C until combined at the approved 80% surface water to 20% reclaimed water volume ratio.

4.2.2 Test Microorganisms

Five indicator microorganisms relevant to the NC legislation for reclaimed water were propagated from raw sewage. These organisms included *E. coli, Enterococcus* spp., F+/malespecific coliphages, somatic coliphages, and *Clostridium perfringens* spores. Each microorganism was propagated from a sample of diluted raw sewage using selective culture media or selective *E. coli* hosts (for viruses). Raw sewage samples were diluted in phosphate buffered saline and plated on selective agar media to yield individual colonies or plaque forming unit (for viruses). A sample of diluted raw sewage was pasteurized for 30 minutes at 60°C to select for *C. perfringens* spores prior to plating on selective media. Selective media included, Bio-Rad Rapid *E. coli* 2 agar, mEnterococcus agar, *E. coli* Famp (for F+/male specific
coliphages), and *E. coli* CN13 (for somatic coliphages), both propagated in Tryptic Soy Broth and CP ChromoSelect Agar (for *C. perfringens*). Characteristic colonies or plaque forming units were then inoculated into broth culture, TSB for *E. coli, Enterococcus,* somatic and F+ coliphages, and Duncan-Strong Broth for *C. perfringens* and grown overnight at 37°C for *E. coli, Enterococcus,* somatic and F+ coliphages and at 44.5°C for *C. perfringens*. Each organism was then aliquotted in 20% glycerol and stored at -80°C for future use.

4.2.3 Microbial Survival Experiments in Blended Water

Each of the propagated microorganisms were spiked simultaneously into 100mLs of the 80/20 mix of surface water and reclaimed water at concentrations of approximately $10^6 - 10^8$ in order to track a minimum of a 4-log₁₀ reduction in each microorganism evaluated for survival using combinations of the following conditions: both 4° C and 20° C; at various mixing speeds (0,60, and 120 RPM); periods of 0, 3, and 5 days. Concentrations of microorganisms were quantified over the 5-day test period using the spot-plate titer assay as described in Beck et al. (2009), using selective agars or *E. coli* hosts (for coliphage viruses) as described above. Briefly, 150mm plates were prepared using the selective agars, Bio-Rad Rapid E. coli 2 agar, mEnterococcus agar, E. coli Famp (for F+/male specific coliphages), E. coli CN13 (for somatic coliphages), and CP ChromoSelect Agar (for *C. perfringens*), and the samples were diluted using phosphate buffered saline (US EPA, 2012). The samples were spotted onto plates in 10uL volumes with 5 replicate spots per dilution. Spot titer plates were incubated at 37° C for $24 \pm 4h$ for E. coli, F+ and somatic coliphages and 48 hours for Enterococcus spp. CP Chromoselect spot plates for C. perfringens were incubated in anaerobic jars at 44.5°C for $24 \pm 4h$. Each experiment was conducted in a dark room and samples were covered with wrapping paper during sampling and immediately returned to the dark room. Concentrations of bacteria and viruses were expressed as colony forming units (CFU/100 mL) or plaque forming units (PFU/100mL).

4.2.4 Sunlight Study on Microbial Survival in Water

To evaluate the impact of sunlight on the survival of indicator organisms in the 80/20 mixture of surface and reclaimed water, the propagated organisms were also evaluated when exposed to natural sunlight. For these sunlight experiments, organisms were spiked into 100mLs of the 80/20 mix, placed into clear polyethylene bags and set in the sun for approximately 4 hours (10am-2pm). The polyethylene bags were 16.5cm long by 8.2cm wide and the water depth was 0.3cm; the thickness of the polyethylene plastic was 101.6µm. The average turbidity of the 80/20 mix during experiments was 7.1NTU. Bags were laid flat on an ice pack to control temperature; samples were not allowed to reach temperatures greater than 20°C. Mixing occurred only at time of sample collection; 1mL samples were collected at 0, 15, 30, 45, 90, 120, 180, and 240 minutes. Samples were placed on ice immediately after collection. Spot titer plates were prepared as described in section 3.2.3; samples were diluted in phosphate buffered saline and plated in less than 1 hour from the time of original sampling. Plates were incubated as described in section 4.2.3.



Figure 4-1: Set-up of sunlight survival experiments

4.2.5 Natural Bacteria

As laboratory propagated fecal indicator bacteria may not model the behavior of natural fecal indicator bacteria in the environment, an experiment was also conducted with fecal indicator bacteria naturally occurring in the surface waters. As not all of the fecal indicator microorganisms were present at high enough concentrations to track their survival over time, this experiment was conducted with analysis for only total coliform bacteria, E. coli and *Enterococcus* spp. As with the previous survival experiments using laboratory propagated microorganisms, 2L volume samples of Jordan Lake water were evaluated at 20°C, at various mixing speeds (0,60, and 120 RPM) and at sampling times of 0, 3, and 5 days. Each experiment was conducted in a dark room in sealed containers opened only for sampling purposes. Samples were covered with wrapping paper during sampling and immediately returned to the dark room. Concentrations of test microorganisms were quantified over the 5-day period using a standard membrane filtration technique (Standard Methods for the Examination of Water and Wastewater, SMEWW) with plating on a selective agar medium for total coliforms and *E. coli*, Bio-Rad Rapid E. coli 2 agar, and the selective mEnterococcus agar for Enterococcus bacteria. Membrane filtration plates were incubated at 37° C for $24 \pm 4h$ for total coliforms and *E. coli* and 48 hours for *Enterococcus* spp. Concentrations of bacteria and viruses were expressed as colony forming units (CFU/100 mL) or plaque forming units (PFU/100mL).

4.2.6 Statistical Analysis

The concentrations and changes in concentrations of regulated microbial indicators in samples containing 20% mixtures of NCT2RW plus 80% surface source waters at time = 0, 3 and 5 days of storage at the specified conditions of temperature and mixing were compared using Microsoft Excel (Microsoft Corporation, Redmond, WA) and GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). An ANOVA regression analysis was performed using a Tukey

post test with log₁₀ (N₁/N₀) survival concentration data; this analysis uses group means to compare differences among samples. Specifically, the mean log₁₀ survival fraction of each indicator organism was compared with a no change (0) scenario as well as with the other conditions evaluated, specifically with the mixing conditions (0, 60 and 120RPM), and temperatures (4 and 20°C). For the sunlight study, the UV radiation was measured as watts per meter squared per minute and summed to give a cumulative UV dose curve over time. UV radiation was measured in 1 minute increments using a Total UV Radiometer (Eppley Lab, Newport, RI, Model TUVR). For these experiments, the log₁₀ (N₁/N₀) survival concentration data was calculated based on the survival of microorganisms exposed to sunlight. The Chick Watson (Log linear) model was then used to calculate the time needed to achieve a 4-log₁₀ reduction with the observed cumulative UV dose. Goodness of fit was evaluated using the coefficient of determination (R²), if the model was fit at a level of 0.5 or greater, no additional modeling was conducted.

4.3 Results

4.3.1 Survival of Fecal Indicator Microorganisms in 80/20 Water Blend over 5 Days

The average \log_{10} survival data for the lab grown indicator organisms examined here (*E*. coli, *Enterococcus* spp., *C. perfringens*, F+ coliphage, and somatic coliphage) are summarized by temperature and by mixing speed in Table 4-1. A positive $\log_{10} (N_t/N_0)$ value corresponds to an increase in microbe concentration over the 5 day storage period, while a negative $\log_{10} (N_t/N_0)$ value represents a decrease in microbe concentration.

The starting concentration of *E. coli* was 6.8 x 10^6 CFU per 100mL. At 4°C and 0 RPM mixing speed, average $\log_{10} (N_t/N_0)$ at 5 days was -0.94. These results were statistically significantly different from no change ($\log_{10} (N_t/N_0)=0$, p<0.0001). For samples mixed at 60 RPM at a temperature of 4°C the average $\log_{10} (N_t/N_0)$ was -1.17 at 5 days and was statistically

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significantly different from no change (p<0.0001), but not significantly different from the 0 RPM mixing speed (p=0.0598). For samples mixed at 120 RPM at 4°C, the average log_{10} (N_t/N₀) was - 1.61 at 5 days. These results were statistically significantly different from no change (p<0.0001) and from the 0 RM mixing speed (p<0.0001) and the 60 RPM mixing speed (p=0.0019).

For *E. coli* survival examined at temperatures of 20°C and a 0 RPM mixing speed, the average log_{10} (N_t/N₀) at 5 days was 1.40; this positive value indicates an increase in *E. coli* at this temperature and mixing speed. These results were statistically different from no change (p<0.0001). At 60 RPM, the average log_{10} (N_t/N₀) at 5 days was 1.87, a value also statistically significantly different from no change (p<0.0001) and statistically different from the 0 RPM mixing speed at this temperature (p=0.0071). At the 120 RPM mixing speed at 20°C, the average log_{10} (N_t/N₀) value was 2.11. This value was also statistically different from no change (p<0.0001), and 0 RPM (p=0.0001), but not statistically different from the log_{10} (N_t/N₀) 60 RPM mixing speed values (p=0.2954). For *E. coli*, all mixing speeds were statistically different (p<0.0001) for the two temperatures evaluated.

The starting concentration of *Enterococcus* spp. was 3.8×10^6 CFU per 100mL, and at 4°C and 0 RPM mixing speed, average $\log_{10} (N_t/N_0)$ value at 5 days was 0.00; this result was not statistically significantly different from no change (p>0.99). For samples mixed at 60 RPM, the average $\log_{10} (N_t/N_0)$ value at 5 days was 0.12, this value was also not statistically significantly different from no change (p=0.9008), or from the 0 RPM mixing speed (p=0.9162). At 120 RPM, the average $\log_{10} (N_t/N_0)$ value was 0.33, there was no statistically significant difference between this value and no change (p=0.0739), 0 RPM (p=0.0800) or 60 RPM mixing speeds (p=0.4430).

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For *Enterococcus* spp. survival at 20°C, the average log_{10} (N_t/N₀) value at the 0 RPM mixing speed was 2.43, indicating an increase in concentration; there was a statistically significant difference between this value and no change (p<0.0001). At the 60 RPM mixing speed, the average log_{10} (N_t/N₀) value was 2.23; there was also a statistically significant difference (an increase in concentration) between this value and no change (p<0.0001), but not between this value and the 0 RPM mixing speed (p=0.4926). At 20°C and 120 RPM, the average log_{10} (N_t/N₀) value was 2.25; there was a statistically significant difference (an increase in concentration) between this value and no change (p<0.0001), but not between this value was 2.25; there was a statistically significant difference (an increase in concentration) between this value and no change (p<0.0001), but not between this value and 0 RPM (p=0.6120), or between this value and 60 RPM (p>0.9999). In the comparison of mixing speed at the two temperatures, there was a statistically significant difference between each mixing speed (0, 60, and 120) for both temperatures evaluated (p<0.0001).

The starting concentration of somatic coliphages was 7.6 x 10^7 PFU per 100mL. At 4°C and 0 RPM mixing speed, average $\log_{10} (N_t/N_0)$ at 5 days was 0.22, indicating an increase in concentration. These results were statistically significantly different from no change (p=0.0002). For samples mixed at 60 RPM at a temperature of 4°C the average $\log_{10} (N_t/N_0)$ was 0.31 at 5 days, also an increase in concentration and was statistically significantly different from no change (p<0.0001), but not significantly different from the 0 RPM mixing speed (p=0.1927). For samples mixed at 120 RPM at 4°C, the average $\log_{10} (N_t/N_0)$ was 0.22 at 5 days, indicating an increase in concentration. These results were statistically different from no change (p=0.0002) but not from the 0 RM mixing speed (p>0.999) and the 60 RPM mixing speed (p=0.2006).

For somatic coliphage survival examined at a temperature of 20°C and a 0 RPM mixing speed, the average $\log_{10} (N_t/N_0)$ at 5 days was -0.69. These results were statistically different from no change (p<0.0001). At 60 RPM, the average $\log_{10} (N_t/N_0)$ at 5 days was -0.68, a value

also statistically significantly different from no change (p<0.0001) but not statistically different from the 0 RPM mixing speed at this temperature (p>0.9999). At the 120 RPM mixing speed at 20°C, the average $\log_{10} (N_t/N_0)$ value was -0.69. This value was also statistically different from no change (p<0.0001), but not statistically different from the $\log_{10} (N_t/N_0)$ 0 RPM or 60 RPM mixing speed values (p >0.9999). For somatic coliphages, all mixing speeds were statistically different (p<0.0001) for the two temperatures evaluated.

The starting concentration of F+ coliphages was 4.20 x 10⁶ PFU per 100mL, and at 4°C and 0 RPM mixing speed, average log_{10} (N_t/N₀) value at 5 days was -0.93; this result was statistically different from no change (p=0.0013). For samples mixed at 60 RPM, the average log_{10} (N_t/N₀) value at 5 days was -0.73, this value was also statistically significantly different from no change (p=0.0099), but not from the 0 RPM mixing speed (p=0.9043). At 120 RPM, the average log_{10} (N_t/N₀) value was -1.01, there was a statistically significant difference between this value and no change (p=0.0005), and 0 RPM (p=0.0246), but not the 60 RPM mixing speed (p=0.6603).

For F+ coliphage spp. survival at 20°C, the average log_{10} (N_t/N₀) value at the 0 RPM mixing speed was -1.78; there was a statistically significant difference between this value and no change (p<0.0001). At the 60 RPM mixing speed, the average log_{10} (N_t/N₀) value was -2.01; there was also a statistically significant difference between this value and no change (p<0.0001), but not between this value and the 0 RPM mixing speed (p=0.8112). At 20°C and 120 RPM, the average log_{10} (N_t/N₀) value was -1.57; there was a statistically significant difference between this value and no change (p<0.0001), but not between this value and 0 RPM (p=0.8791), or between this value and 60 RPM (p=0.2059). In the comparison of mixing speed at the two temperatures, there was a statistically significant difference between each mixing speed (0, 60, and 120) for

both temperatures evaluated (p<0.0001), except between the 4°C and 20°C at 120RPM mixing speed, for which there was a not quite significant difference (p=0.0605).

For *Clostridium perfringens*, the starting concentration was 4.03 x 10^7 CFU per 100mL. At 4°C and 0 RPM mixing speed, average log_{10} (N_t/N₀) at 5 days was -2.09. These results were statistically significantly different from no change (p=0.0012). For samples mixed at 60 RPM at a temperature of 4°C the average log_{10} (N_t/N₀) was -1.80 at 5 days and was statistically significantly different from no change (p=0.0044), but not significantly different from the 0 RPM mixing speed (p=0.9856). For samples mixed at 120 RPM at 4°C, the average log_{10} (N_t/N₀) was -1.89 at 5 days. These results were statistically different from no change (p=0.0030) but not from the 0 RM mixing speed (p=0.9977) and the 60 RPM mixing speed (p>0.9999).

For *C. perfringens* survival examined at a temperature of 20°C and a 0 RPM mixing speed, the average log_{10} (N_t/N₀) at 5 days was -1.61. These results were statistically different from no change (p=0.0112). At 60 RPM, the average log_{10} (N_t/N₀) at 5 days was -1.70, a value also statistically significantly different from no change (p=0.0073) but not statistically different from the 0 RPM mixing speed at this temperature (p>0.9999). At the 120 RPM mixing speed at 20°C, the average log_{10} (N_t/N₀) value was -1.62. This value was also statistically different from no change (p=0.0104), and the log_{10} (N_t/N₀) 0 RPM or 60 RPM mixing speed values (p >0.9999). For *C. perfringens*, all mixing speeds were not statistically different (p>0.85) for the two temperatures evaluated.

	4°C						20°C					
	0 RPM		60 RPM		120 RPM		0 RPM		60 RPM		120 RPM	
	Log ₁₀ (Nt/N ₀)*	SD^+	Log ₁₀ (N _t /N ₀)	SD	Log ₁₀ (N _t /N ₀)	SD	Log ₁₀ (N _t /N ₀)	SD	Log ₁₀ (Nt/N0)	SD	Log ₁₀ (N _t /N ₀)	SD
E. coli	-0.94	0.11	-1.17	0.06	-1.61	0.09	1.40	0.17	1.87	0.06	2.11	0.14
Enterococcus spp.	0.00	0.06	0.12	0.01	0.33	0.04	2.43	0.01	2.23	0.08	2.25	0.25
C. perfringens	-2.09	0.21	-1.80	0.05	-1.89	0.02	-1.61	0.09	-1.70	0.90	-1.62	0.38
F+ Coliphage	-0.93	0.16	-0.73	0.16	-1.01	0.06	-1.78	0.34	-2.01	0.16	-1.57	0.06
Somatic Coliphage	0.22	0.04	0.31	0.03	0.22	0.03	-0.69	0.04	-0.68	0.05	-0.69	0.03

Table 4-1: Survival data for indicator microorganisms spiked in to 80/20 blend of water stored for 5 days with mixing speeds of 0, 60 and 120 rpm and temperatures of 4 and 20 °C

*Log₁₀ (N_t/N_0) is the average log₁₀ reduction over the 5 day experiment +SD is standard deviation

4.3.2 Survival of Indicator Organisms Exposed to Sunlight

Figure 4-2 displays the log₁₀ survival of the 5 lab grown indicator organisms spiked into the 80/20 surface water/reclaimed water mixture versus the cumulative UV dose over time. Each point on the graph represents the average survival of 3 trials plated in triplicate versus UV dose at a specific time point (0, 15, 30, 45, 90, 120, 180, and 240 minutes). The temperature for these experiments was controlled, and samples did not reach temperatures above 20°C. Table 4-2 describes the best fit parameters for the log linear Chick-Watson model.

Based on these results, *E. coli* and *Enterococcus* spp. are the microorganisms declining most rapidly in the 80/20 blend, with decay constants of k = -0.0058 1/ watts*h per m² and k = -0.0025 1/ watts*h per m², respectively. There was no statistically significant difference between the rate of decay of *E. coli* and *Enterococcus* spp. (p=0.6185) and the estimated UV dose to achieve a 4-log₁₀ reduction for *E. coli* and *Enterococcus* spp. are 692 Watts*h per m² and 1587 W*h/m² respectively.

As *C. perfringens* was not adequately modeled by the Chick-Watson model (\mathbb{R}^2 value of 0.48), the decay of this organism was also modeled by the One-Hit Two Population Model. This alternative model, which proposes a first phase of microorganism inactivation with a quicker rate of decay (*k*) followed by a second, slower phase, was a better fit to the *C. perfringens* data, with an \mathbb{R}^2 value of 0.63, and an initial *k* value of 0.003 and a secondary *k* value of 0.0029. The initial *k* value indicates that the initial phase of decay was faster than the secondary phase. There was no statistically significant difference between the UV dose required to inactivate *C. perfringens* and *E. coli* (p=0.2785), or *Enterococcus* spp. (p=0.9534).

As with the fecal indicator bacteria, the survival of the coliphage viruses was a good fit to the Chick-Watson model (\mathbb{R}^2 values of 0.97). By this model, the decay rates for F+ coliphages and somatic coliphages were k = -0.0014 and k = -0.0017 respectively and the UV doses

required to achieve a 4 \log_{10} reduction were 2909 W/m² for F+ coliphage and 2327 W/m² for somatic coliphage. Compared to fecal indicator bacteria, there was not a statistically significant difference between the survival *E. coli* and somatic (p=0.1501) but there was a statistically significant difference between *E. coli* and F+ coliphages (p=0.0009) when exposed to sunlight. Similarly, there was also not a statistically significant difference between the survival of enterococci and somatic (p=0.7878) but there was a statistically significant difference between enterococci and F+ coliphages (p=0.0065). There was also a statistically significant difference between the survival of the two types of coliphage viruses (p=0.0360).



Figure 4-2: Survival of sewage propagated organisms exposed to sunlight $log_{10}(N_t/N_o)$ vs. cumulative UV dose over time, 3 trials plotted per time point, shown with one standard deviation.

Organism	Equation	k	R^2	UV Dose to Achieve 4 log ₁₀ Reduction (W*H/m ²)
E. coli	Y = -0.005779x + 0.4789	-0.0058	0.9778	692
Enterococcus spp.	Y = -0.002519x + 0.7229	-0.0025	0.9323	1587
Cl. perfringens	Y = -0.000922x - 3.444	-0.0009	0.4676	4342
F+ Coliphage	Y = -0.001375x - 0.1117	-0.0014	0.9695	2909
Somatic Coliphage	Y = -0.001719x - 0.5647	-0.0017	0.9651	2327

Table 4-2: Chick-Watson model parameters for sunlight survival data

4.3.3 Survival of Natural Bacteria in Surface Waters

Figure 4-3 displays the survival of indigenous populations of total coliforms, *E. coli* and *Enterococcus* spp. in surface waters at 20°C over a period of 5 days. Each data point represents the average of 3 trials plated in triplicate. The starting concentration of total coliforms was 1.33 x 10^4 CFU per 100mL. At 20°C and 0 RPM mixing speed, average log_{10} (N_t/N₀) at 5 days was - 0.83. There was a statistically significant difference between these results and no change (log_{10} (N_t/N₀)=0, p=0.0114). For samples mixed at 60 RPM at a temperature of 20°C, the average log_{10} (N_t/N₀) was -0.59 at 5 days and was not quite statistically significantly different from no change (p=0.0614), or from the 0 RPM mixing speed (p=0.6273). For samples mixed at 120 RPM at 20°C, the average log_{10} (N_t/N₀) was -0.87 at 5 days. These results were statistically different from no change (p=0.0082) but not statistically different from the 0 RPM (p=0.9938) and the 60 RPM mixing speed (p=0.4891).

For *E. coli* survival examined at a temperature of 20°C and a 0 RPM mixing speed, starting concentrations were 6.67 x 10^{0} CFU per 100mL and the average $\log_{10} (N_{t}/N_{0})$ at 5 days was -0.46. These results were not statistically different from no change (p=0.3870). At 60 RPM, the average $\log_{10} (N_{t}/N_{0})$ at 5 days was -0.58, a value also not statistically significantly different from no change (p=0.2248) and also not statistically different from the 0 RPM mixing speed at this temperature (p=0.9713). At the 120 RPM mixing speed at 20°C, the average $\log_{10} (N_t/N_0)$ value was -0.16. This value was not statistically different from no change (p=0.9656), and the $\log_{10} (N_t/N_0)$ 0 RPM (p=0.6266) or the 60 RPM mixing speed values (p=0.4004). At all mixing speeds, the natural *E. coli* were significantly different from the sewage propagated *E. coli* also mixed at 0, 60 and 120 RPM at 20°C (p<0.0001). However, for sewage propagated organisms mixed at 4°C, there was no significant difference between the survival of natural *E. coli* mixed at 0 RPM and sewage propagated bacteria mixed at 0 or 60 RPM mixing speeds (p-values:0.3645; 0.0798). There was also no significant difference between the survival of naturally occurring *E. coli* mixed at 60 RPM and sewage propagated bacteria mixed at 0 RPM, 4°C (p=0.6390) or at 60 RPM (0.1791).

The starting concentration of *Enterococcus* spp. in natural waters was 3.67 x 10¹ CFU per 100mL. At 20°C and 0 RPM mixing speed, average $log_{10} (N_t/N_0)$ at 5 days was -1.94. There was a statistically significant difference between these results and no change $(log_{10} (N_t/N_0)=0, p<0.0001)$. For samples mixed at 60 RPM at a temperature of 20°C the average $log_{10} (N_t/N_0)$ was -1.94 at 5 days and was statistically significantly different from no change (p<0.0001) but not different from the 0 RPM mixing speed (p>0.9999). For samples mixed at 120 RPM at 20°C, the average $log_{10} (N_t/N_0)$ was -1.88 at 5 days. These results were statistically different from no change (p<0.0001) but not statistically different from the 0 and the 60 RPM mixing speed (p = 0.9790). Much like the comparison for *E. coli*, for *Enterococcus* spp., at all mixing speeds the natural *E. coli* were significantly different from the sewage propagated Enterococci also mixed at 0, 60 and 120 RPM at 20°C (p<0.0001). Similarly at 4°C, at all mixing speeds there was a

statistically significant difference between the naturally occurring *Enterococcus* spp. mixed at 20°C and the sewage propagated organisms mixed at 20°C.



Figure 4-3: Survival of bacteria in natural surface waters at 0, 60, and 120 RPM and 20°C, 3 trials plotted per time point, shown with one standard deviation.

4.4 Discussion

Based on my results, there was a statistically significant difference between the log_{10} (N_t/N₀) value at 5 days and a log_{10} (N_t/N₀) of no change (log_{10} (N_t/N₀)=0) for all organisms examined under all conditions except sewage propagated *Enterococcus* examined at 4°C and naturally occurring *E. coli* examined at 20°C. However, there was no statistically significant difference between mixing conditions at either temperature. This suggests that the concentrations of the organisms change over the course of the 5 day storage period. For sewage propagated organisms stored at 4°C, concentrations of *E. coli*, *C. perfringens*, and F+ coliphage decreased over the 5 day period; however, there were slight increases of enterococci and somatic coliphages. Additionally, at the 20°C storage temperature for these organisms, there were much larger (about 2 log_{10}) increases in concentration for *E. coli* and *Enterococcus* spp., while *C*.

perfringens, F+ and somatic coliphages decreased in concentration. Potential reasons for this increase include the regrowth of bacteria due to the initial propagation and the disaggregation of aggregated bacteria. Despite the regrowth of *E. coli* and enterococci in the sewage propagated bacteria experiments, there was no regrowth, or growth, of these organisms or of the total coliform bacteria in the natural bacteria experiments. This suggests that it is not the tendency of natural bacteria to grow at 20°C, but that this was an artifact of the propagation or the disaggregation of the bacteria used in the spiking experiment. Although the regrowth of total coliform bacteria has been well documented (Butterfield, 1933; LeChevallier, 1990; Hammes et al., 2010), I observed no increases in total coliform bacteria. In the natural bacteria experiments, there was no significant difference between no change (log₁₀ (N_t/N₀)=0) and the survival of the natural total coliform bacteria or the *E. coli*, but there was a significant difference in the survival of *Enterococcus* spp. over the 5 day period. Much like the sewage propagated bacteria experiments, there was no significant difference between mixing speeds.

Previous studies have documented the survival of indicator organisms in reclaimed water; however, none has been conducted on this mix of surface water and reclaimed water for potable reuse storage. Bitton et al. found that in groundwater *E. coli* and f2 coliphage declined at rates of 0.0066 and 0.059 hr⁻¹ respectively. Other researchers have suggested that changes in the concentrations of bacteria in freshwater, including the potential for growth, may be a result of inhibitory substances (Klein and Alexander, 1986), or due to the activities of predatory and lytic organisms (Flint, 1987; González et al., 1990). However, for the factors evaluated in this study, there was no statistically significant effect related to the mixing speed, or temperature on the survival of sewage propagated or naturally occurring microorganisms over the 5 day period. Additionally, in comparing the naturally occurring microorganism to the sewage propagated

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microorganisms, there was no significant difference between sewage propagated *E. coli* stored at 4° C.

In the evaluation of sunlight inactivation, there was no significant difference between the log_{10} (N_t/N₀) values over time for many of the microorganisms examined here. Specifically, there was no difference between the inactivation of *E. coli* and *Enterococcus* over the 5 day period, or between *C. perfringens* and *E. coli* or enterococci. However, there was a significant difference between the inactivation of F+ and somatic coliphages. In my study, F+ coliphages appear to be more resistant to disinfection by sunlight than are somatic coliphages in the mix of reclaimed and surface water. This same pattern of F+ coliphage resistance to sunlight in freshwater was also found by Sinton et al. in 2002. Other researchers have evaluated the survival of microorganisms exposed to UV light (Gutiérrez-Alfaro et al., 2015) and have found *C. perfringens* to be the most resistant to sunlight disinfection. Much like my results, Gutiérrez-Alfaro et al. concluded that increased exposure times were required to achieve increased log₁₀ reductions for protozoan parasite surrogates like *C. perfringens*. Hijnen et al. (2006) also found that environmental spores (such as the strains used here) have increased UV resistance and may require additional UV treatment to achieve the log₁₀ reductions necessary for wastewater treatment.

My results suggest that the 5 day storage period proposed to be used as a component of potable reuse treatment does have an impact on the survival of indicator microorganisms. Based on my statistical analyses of survival concentrations, there was a significant difference between the concentrations of all organisms stored over the 5 day period at all temperatures and at all mixing speeds with the exception of *E. coli* (natural and sewage propagated). At a temperature of 20° C, there was a log_{10} reduction of approximately 1 log_{10} for all microorganisms, with the exception of *E. coli* and *Enterococcus* spp. (a 1 log_{10} reduction was achieved for enterococci in

the natural bacteria experiments). This suggests a $1 \log_{10}$ reduction credit would be appropriate for a 5 day storage system. Higher \log_{10} reduction credits could be achieved with documented reductions using increased treatment during storage; treatment conditions could include sunlight inactivation, increased temperature, etc. These results have practical implications for wastewater utilities considering potable reuse schemes, particularly those that require a 5 day storage period.

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CHAPTER 5: QUANTITATIVE MICROBIAL RISK ASSESSMENT OF NORTH CAROLINA TYPE 2 RECLAIMED WATER FOR POTABLE REUSE APPLICATIONS

5.1 Introduction

Treated wastewater for potable reuse presents an opportunity to expand currently available water resources while addressing water scarcity issues related to growing water demand (NAS, 2012). Currently, the reuse of treated wastewater is mostly limited to agricultural reuse, with an estimated 50 countries using reclaimed wastewater for this purpose (Jiméz, 2006). However, there is growing interest in potable reuse, and in the State of North Carolina, there are new potable reuse guidelines allowing for a percentage of tertiary treated, dual disinfected reclaimed water to be combined with surface source waters at a ratio of up to 20%, followed by five-day storage and conventional drinking water treatment (NC DENR, 2011; 2014).

As microbial risks are still of concern in reclaimed waters, the World Health Organization (WHO) has recommended the use of quantitative microbial risk assessment (QMRA) to assess the additional disease burden from wastewater reuse (WHO, 2006). QMRA is an analytical tool used to estimate the health risks resulting from exposure to microorganisms in water, food, soil, or air (Peterson et al., 2006; Haas et al., 2014).

I selected five representative pathogens from the three groups (bacteria, virus, and protozoan parasites) of microorganisms addressed in the NC legislation (NC DENR, 2014) on reclaimed water. Although the NC reclaimed water legislation uses indicator microorganisms to manage risks, my goal in this risk assessment was to select representative pathogens from each group to model the risk to potential consumers for various exposure scenarios. The selected pathogens from each group were *Salmonella* spp. bacteria, Adenovirus groups A-F, Norovirus

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GII, and the protozoan pathogens *Cryptosporidium* spp. and *Giardia* spp. Risks from enteric pathogens including bacteria, viruses, and protozoan parasites in drinking water have been previously evaluated in a number of studies (Regli et al., 1991; Asano et al., 1992; Ryu et al., 2007; Jacob et al., 2015; DeFelice et al., 2015). However, the NC proposed treatment scheme of dual disinfection, followed by five days of storage and conventional drinking water treatment has not been examined by QMRA.

5.2 Methods

5.2.1 Study Design and Sampling

Reclaimed water samples were collected bi-monthly for one year, during and after storm events in order to monitor worst case scenario microbial events as grab samples using approved techniques (Standard Methods for the Examination of Water and Wastewater; SMEWW) from four different water reclamation facilities located in central North Carolina, resulting in 22 reclaimed water samples. The wastewater treatment facilities were (1) the Neuse River Resource Recovery Facility, (2) the Utley Creek Water Reclamation Facility, (3) the Cary Water Reclamation Facility, and (4) the Orange Water and Sewer Authority. North Carolina Type 2 reclaimed water is characterized by tertiary physical and biological treatment (typically, primary sedimentation, secondary biological treatment and direct granular media filtration) followed by dual disinfection (typically by UV radiation and chlorine disinfection). Reclaimed water samples were collected in 12L volumes, transported on ice to the laboratory and stored at 4°C until analyzed.

Surface water samples were collected from two run-of-river drinking water treatment plants and 2 sewage impacted reservoir drinking water treatment plants also in central North Carolina. These facilities were (1) the Hillsborough Drinking Water Treatment Plant, using the Eno River; (2) the Cary/Apex Drinking Water Treatment Plant, using Jordan Lake; (3) the E.M. Johnson Water Treatment Plant, using Falls Lake; and (4/5) the Smithfield Water Treatment Plant using both the local reservoir (4) and the Neuse River (5). Surface waters were collected as grab samples from various sampling points in sterile bottles, and kept chilled in coolers with ice during transport to Chapel Hill. Samples collected from treatmetn plants with reservoirs (Cary/Apex, and E.M. Johnson) were collected from the water treatment plant intake structure. Run of river treamtment plant samples and the Smithfield Reservoir samples were collected approximately 2 meters from shore and approximately 1 meter below the surface of the water. The samples were stored at 4°C upon arrival at the laboratory.

5.2.2 Pathogen Recovery

A total of 22 reclaimed water and 22 surface water samples were assayed for Salmonella spp., Cryptosporidium spp., Giardia spp., Norovirus GII, and Adenovirus A-F by methods described in section 2.2. Salmonella spp. were analyzed in 1000 mL volumes of reclaimed and water samples by an established MPN method (Krometis et al., 2010) using triplicate sample volumes of 300 mL, 30 and 3 mL that were pre-enriched, initially in peptone water, followed by selective enrichment in RV broth and streak plating for colony isolation on SS agar to detect presumptive Salmonella spp. colonies. Presumptive colonies were biochemically confirmed using triple sugar iron agar. Concentrations were reported as MPN per 100mL. Enteric viruses and protozoan parasites were analyzed in 10L volumes of reclaimed water and 12L volumes of surface water by hollow fiber ultrafiltration and elution using the procedure described in Hill et al. (2007) and Polaczyk et al. (2008) with Fresenius Optiflux F250NR hollow fiber ultrafilters (dialyzers). Enteric viruses were further concentrated by polyethelyeneglycol (PEG-8000) precipitation by the method described in Yamamoto et al. (1970), while secondary concentration and purification for protozoan parasites was done by immunomagnetic separation (IMS) and immunoflorucescent (oo)cyst examination. Concentrations of (oo)cysts were reported as

(oo)cysts per 100mL, and concentrations of enteric viruses were reported as genome equivalent copies (GEC) per 100mL. An integrated cell culture-polymerase chain reaction assay (ICC-PCR) was used to detect infectious Adenovirus as described by Rodríguez et al. (2013) and Polston et al. (2014) and as further described in section 2.2.8. The survival of microorganisms in the 80/20 blend of sewage impacted surface water and reclaimed water was examined in Chapter 4 and the resulting log₁₀ survival of indicator microorganisms was used to model the survival of pathogens over a 5 day storage period for exposures 4 and 5.

5.2.3 Statistical Analysis

Data were entered into a Microsoft Excel (Microsoft Cooperation, Redmond, WA) spreadsheet and calculations for risk and Monte Carlo simulations were performed using Analytica 4.6 (Lumina Decision Systems, Los Gatos, CA), with random variables sampled 10,000 times for each analysis. Details on the components of the risk assessment model, assumptions, recovery efficiencies, etc., will be presented in detail in the sections below. The full model is displayed in Figure 5-1. Table 5-3 presents a step-wise example calculation for the potential risks from potable reuse consumption. Uncertainty analyses were conducted for microorganisms in each water type by evaluating the rank order correlation in uncertainty with variables used in the model. The results of this analysis are presented in section 5.6.



Figure 5-1: Analytica diagram of potable reuse model

5.3 Exposure Assessment

The focus of the exposure assessment is the estimation of the likelihood of an individual or a population to be exposed to the identified hazard as well as the estimation of the dose that is likely to be ingested. Based on the concentrations, recovery efficiencies, viability, and exposure scenarios, the average dose (N) of the pathogens of interest was calculated using the following equation:

$$N = C x R^{-1} x I x V$$

In this equation, *N* is the dose or number of organisms (viruses, bacteria, or (oo)cysts) ingested by a person through reclaimed water, surface water, or a combination of the two; C is the concentration of pathogens (organisms/L), R is the recovery efficiency of the detection method, I is the fraction of detected pathogens capable of causing infection, V is the exposure scenario volume (L).

5.3.1 Concentrations of Pathogens (C)

As risk approximations are best made based on unbiased estimates of the true mean concentration, my goal in this study was to model the behavior of pathogen concentrations in various water types. The concentration data from 4 NC type 2- like reclaimed water producing treatment plants were aggregated for analysis purposes. In order to model pathogen concentrations, previous researchers (Ginneken and Oron, 2000; Hamilton et al., 2006) have fitted a normal distribution to log data. This method was used to model the concentrations for reclaimed, surface and blended waters for all pathogens as data were found to be lognormally distributed. The lognormal distribution for concentration was then multiplied by a correction factor in exposure analysis if pathogen die-off was considered (for exposures 4 and 5), as described in section 5.5. Distributions of microbial concentrations are summarized in Table 5-1. The potable reuse distribution was created by combining 0.8 parts of the surface water and 0.2 parts of the reclaimed water pathogen concentrations to create a potable reuse water that would be further 'treated' by 5 day storage and conventional drinking water treatment. The concentrations of Norovirus GII were excluded from this analysis because no gene copies were detected in reclaimed or surface water samples.

Model Parameter and Sample	Symbol	Unit	Distribution and Fit Parameter	Reference
Organism Concentration in NCT2RW			Normal fitted to log data	
Adenovirus A-F	Ν	Log ₁₀ Concentration	normal(μ:3.72,σ:1.56)	Calculated from 22 reclaimed water
Salmonella spp.		per L	$normal(\mu: 0.13, \sigma: 0.45)$	samples
Cryptosporidium spp.			normal(µ:0.22,σ:0.36)	
Giardia spp.			normal(µ:-0.22,σ:0.38)	
Organism Concentration in Surface Water			Normal fitted to log data	
Adenovirus A-F	Ν	Log ₁₀ Concentration	normal(μ:2.51,σ:1.86)	Calculated from 22
Salmonella spp.		per L	normal(µ:0.76, σ:1.08)	surface water samples
Cryptosporidium spp.			normal(μ:0.73,σ:0.53)	
Giardia spp.			$normal(\mu: 0.13, \sigma: 0.50)$	
Organism Concentration in 80/20 Mix	N	Log ₁₀ per L	Normal fitted to log data	Calculated from 80% Surface Water + 20%
Adenovirus A-F Salmonella spp.			normal(μ:2.97,σ:1.86) normal(μ:0.848,σ:0.997)	Reclaimed Water
Cryptosporidium spp.			normal(μ:0.825,σ:0.507)	
Giardia spp.			normal(µ:0.242, σ :0.507)	
Viability Adenovirus A-F			38 5	Data collected in
Salmonella spp.	Ι	%	58.5 65	Kapperud et al., 1998
Cryptosporidium spp.			25	LeChevallier et al,
Giardia spp.			13	Gennaccaro et al, 2003
Recovery Efficiency Reclaimed Water	D	% Recovered	normal(u:1.00 - 0.137)	Data Collected in Chapter 2
Auchovirus A-I	к		ποτημα(μ. τ.07,0.0.137)	

Table 5-1: Full description of model parameters

Model Parameter and Sample	Symbol	Unit	Distribution and Fit Parameter	Reference	
Salmonella spp.			normal(μ:1.0,σ:0.5)		
Cryptosporidium spp.			normal(µ:0.825, σ:0.065)		
Giardia spp.			normal(µ:0.635, σ:0.122)		
				Data Collected in	
Surface Water			$pormal(u:1,00,\sigma:0,001)$	Chapter 2	
Salmonella spp.			normal(μ :1.0. σ :0.5)		
<i>Cryptosporidium</i> spp.	R	% Recovered	normal(μ:0.958,σ:0.081)		
<i>Giardia</i> spp.			normal(u:0.783.σ:0.142)		
Charlene SPF1			10111w1(p.0.7,02,0.011 1-2)		
80/20 Blend				Data Collected in Chapter 2	
Adenovirus A-F			normal(μ:1.09,σ:0.114)	Chapter 2	
Salmonella spp.			normal(µ:1.0,σ:0.5)		
Cryptosporidium spp.	R	% Recovered	normal(μ:0.893,σ:0.073)		
Giardia spp.			normal(µ:0.709, σ:0.132)		
Volume of Water Consumed	V	L	 S1: 10mL NCT2RW S2: 100mL Surface Water S3: 2L NCT2RW S4: 2L Surface Water S5: 2L 80/20 Blended Water 5 Day Storage+ Treatment by DWTP 	US EPA, 2011	
5 Day Storage				Data Collected in	
4C				Chapter 4	
Adenovirus A-F			triangular(0.52,0.55,0.93)		
Salmonella spp.		% log ₁₀ Reduction	triangular(0.834,0.93,0.98)		
Cryptosporidium spp.	Days		triangular(0.913,0.975,0.999)		
Giardia spp.			triangular(0.913,0.975,0.999)		
20C Adenovirus A-F	Day5	% log ₁₀ Reduction	triangular(0.76,0.942,0.994)	Data Collected in Chapter 4	
Salmonella spp.			triangular(0.858,0.906,0.99)		

Model Parameter and Sample	arameter and Symbol ample		Distribution and Fit Parameter	Reference
Cryptosporidium spp.			triangular(0.982,0.987,0.996)	
Giardia spp.			triangular(0.982,0.987,0.996)	
Conventional Drinking V US EPA Scenario Adenovirus A-F Salmonella spp. Cryptosporidium spp. Giardia spp.	Water Treatr	nent % log ₁₀ Reduction	triangular(0.999,0.9999,0.99999) triangular(0.99,0.999,0.9999) triangular(0.90,0.99,0.999) triangular(0.99,0.999,0.9999)	US EPA, 2006
WHO Scenario Adenovirus A-F Salmonella spp. Cryptosporidium spp. Giardia spp.	DWTP	% log ₁₀ Reduction	triangular(0.999,0.9999,0.99999) triangular(0.99,0.999,0.9999) triangular(0.99,0.999,0.9999) triangular(0.99,0.999,0.9999)	WHO, 2011
Worst Case Scenario Adenovirus A-F Salmonella spp. Cryptosporidium spp. Giardia spp.	DWTP	% log ₁₀ Reduction	triangular(0.97488,0.999,0.99996) triangular(0.9499,0.9921,0.99874) triangular(0.9937,0.99950,0.99996) triangular(0.9937,0.99950,0.99996)	Medema and Hijnen, 2003

*DWTP is drinking water treatment plant

+80/20 is a mix of 80% surface water 20% reclaimed water

5.3.2 Recovery Efficiencies for Pathogens (R)

The recovery efficiencies for Adenovirus groups A-F, *Cryptosporidium* spp. and *Giardia* spp. in reclaimed and surface waters are summarized in Table 5-2. The recovery efficiency for Adenovirus group A-F was determined using the "adeno-like" salmonella phage PRD1 by PCR recovery methods, while the recovery efficiency for oocyts and cysts was determined using ColorSeed, a fluorescently labeled internal positive control, by US EPA Method 1623 (BTF Precise Microbiology, Inc., Pittsburgh, PA) (US EPA, 2012). The recovery efficiency for the detection of *Salmonella* spp. was not determined empirically and is assumed to be 100% by the

culture assay procedure used for the purposes of the QMRA modeling. Recovery efficiencies greater than 100% for the detection of viruses may indicate that virus particles were

disaggregated during hollow fiber ultrafiltration.

Table 5-2: Recovery efficiencies of processing control organisms in surface and reclaimed waters

	Reclaimed Water (n=26)		Surface Water (n=22)		
Microbe	Average Recovery (%)	Standard Deviation (%)	Average Recovery (%)	Standard Deviation (%)	
Adenovirus A-F (PRD1)	109	13.7	109	9.11	
Cryptosporidium spp. (ColorSeed)	82.5	6.47	95.8	8.11	
Giardia spp. (ColorSeed)	63.5	12.2	78.3	14.2	

5.3.3 Viability (I)

There are limited data available on the infectious fraction of pathogens in either reclaimed or surface waters. Data collected by ICC-qPCR on infectious adenovirus in reclaimed water samples (described in section 2.3.2) were used to determine the fraction (38.5%) of viable infectious adenovirus of those that were detected by direct qPCR. Similarly, Chapron et al. (2000) found in fresh surface waters the fraction of viable infectious adenoviruses in this water type to be 37.9%. For *Salmonella* spp., the estimated fraction of bacteria causing infection in humans from surface water exposures is 65% (Kapperud et al., 1998). Data collected on the fraction of viable (oo)cysts in surface water after chlorination was used to determine the infectious fraction of *Cryptosporidium* spp. and *Giardia* spp.; these fractions were 25% and 13% respectively (LeChevallier, 1991; Gennaccaro et al., 2003).

5.3.4 Exposure Routes (V)

To assess the potential risks of exposure to pathogens associated with the use of reclaimed water for various purposes, I considered five exposure scenarios:

- Scenario 1 (Accidental Exposure): A person is exposed to reclaimed water through a one time accidental ingestion of 10mL by consumption as drinking water. No pathogen die off is considered.
- Scenario 2 (Recreational Exposure). A person swimming in recreational water ingests 100mL of reclaimed water in a day (Haas, 1983). The person is assumed to swim for 2 hours per day on the weekends over a 5-month period, or 40 days per year. No pathogen die off is considered.
- 3. Scenario 3 (Reclaimed Water Exposure). A person ingests the 2L of water per day, as proposed by the US EPA Exposures Handbook (US EPA, 2011), in the form of tertiary treated dual disinfected reclaimed water. As reclaimed water is currently piped to households in North Carolina for non-potable reuse, there is a risk if pipes are mislabeled or water is inadvertently consumed. No pathogen die off is considered.
- 4. Scenario 4 (Surface Water Exposure). A person ingests the 2L of water per day of raw surface water. (This scenario is evaluated as a comparison of the risks of drinking reclaimed water to surface water). No pathogen die off is considered.
- 5. Scenario 5 (Potable Reuse Exposure). A person ingests 2L of blended reclaimed water after it has been combined at a 20% to 80% ratio with run-of-river intake raw source water, stored for 5 days and then treated by conventional drinking water treatment. Pathogen die-off is evaluated in the 5 day storage condition based on data presented in Chapter 4. Pathogens are assumed to be reduced by conventional drinking water treatment by 1) the US EPA required log₁₀ reductions and 2) the WHO risk based method

of reducing pathogens in water and 3) a worst-case log_{10} reduction scenario based on real world data reviewed by Medema and Hijnen (2007).

5.4 Dose-Response Modeling

For Adenovirus A-F, *Cryptosporidium* and *Giardia*, I used the exponential dose response model to determine the probability of infection from ingestion of various numbers of pathogens. The exponential model is:

$$P(inf) = 1 - e^{-k*N}$$

where P(inf) is the probability of infection resulting from daily ingestion of the number of pathogens (N) and *K* is the average dose, or the number of microorganisms that must be ingested to initiate an infection. The best fit *K* values for Adenovirus, *C. parvum*, and *G. lamblia* are 6.07E-01 (Couch et al., 1966), 0.0042 (DuPont et al., 1995), and 0.0198 (Rose et al, 1991), respectively.

For Salmonella spp. I used the Beta-Poisson model, and this model is:

$$P(inf) = 1 - [1 + N \frac{(2^{\frac{1}{\alpha}} - 1)}{N_{50}}]^{-\alpha}$$

where: P(inf) is the probability of infection resulting from daily ingestion of the dose of pathogens (N); α is a pathogen infectivity constant; and N_{50} is the LD₅₀, the dose that is lethal to 50% of individuals, divided by the ID₅₀, which is the median infective dose. The optimized parameters for non-typhoid *Salmonella* are 2.1E-01 and 4.98E+01 for α and N₅₀, respectively (Meynell and Meynell, 1958).

Estimates of daily risk can be extrapolated to the risk of infection over an extended period of time using the equation below (Haas, 1983), which I used to calculate yearly risks and surface water risks at the exposure scenario of 40 days of recreational water exposure.

 $P_t = 1 - (1 - P_d)^t$

Here, P_t is the probability of infection after t days and P_d is the probability of infection after one day of exposure.

5.5 Potable Reuse Modeling

In order to evaluate the specific conditions proposed by the state of North Carolina, I developed a QMRA model, which incorporated both the 5 day storage period and conventional drinking water treatment steps. The 5 day storage condition was modeled using data described in Chapter 4, where sewage propagated indicator microorganisms were subjected to 5 day storage conditions under various mixing speeds. The \log_{10} reductions achieved on average by bacteria over a 5 day storage time were approximately -0.54 at 4°C and +2.0 at 20°C. A positive log₁₀ reduction indicates an increase in concentration over the 5 day storage period. Natural bacteria modeled over this same period had an average \log_{10} reduction of -0.61. For the purposes of this modeling, the survival of *Salmonella* bacteria over the 5 day period was modeled using the \log_{10} reduction of natural bacteria. Average viral indicator reductions were approximately -0.32 at 4°C and -1.22 at 20°C. For protozoan parasite surrogates, the average \log_{10} reduction was -1.89 at 4°C and -1.64 at 20°C. Based on the statistical analysis presented in Chapter 4, there was a statistically significant difference between each of the temperatures evaluated for all organisms except C. perfringens, but not for the three mixing speeds; accordingly, storage conditions were modeled for both temperatures. The survival of organisms in reuse water was modeled as a normal distribution of the average $\log_{10}(n_t/n_o)$ values (presented in Chapter 4) at each temperature.

In addition to modeling the state mandated 5 day storage period at two temperatures, I also modeled conventional drinking water treatment to further evaluate the full scale production of NCT2RW. For this analysis, three drinking water treatment scenarios were evaluated: 1) the

US EPA regulated log_{10} reductions for conventional drinking water treatment and disinfection (4 log_{10} for virus, 2 log_{10} *Cryptosporidium* and 3 log_{10} for *Giardia*), 2) the WHO's risk based reduction of pathogens based on Disability Adjusted Life Years (DALYs) and 3) 3) a worst-case log_{10} reduction scenario based on real world data reviewed by Medema and Hijnen, 2007. A more detailed description of these regulations is provided in Chapter 1; distributions were modeled using triangular distributions using the 95% confidence intervals (95% CIs) of the required log_{10} reductions as the lower and upper bounds.

5.6 Results, Risk Characterization and Uncertainty Analysis

In this analysis, the mean risk of infection was analyzed for reuse scenarios relevant to human health risk associated with exposure to treated and untreated reclaimed water. Table 5-3 presents a stepwise calculation of the potable reuse model for the US EPA mandated log_{10} reductions and storage at 20°C. Table 5-4 displays the mean risk of infection and upper and lower 95% CIs for the 5 risk scenarios. It is important to note that the calculation of risks for Cryptosporidium and Giardia spp. are based on total counts (not on infectivity data) and, despite accounting for infectivity in the QMRA model, risk may be overestimated. Additionally, US EPA Method 1623 does not differentiate between human infectious species and all species of *Cryptosporidium* and *Giardia*, which may also result in an overestimation of human health risks. For Salmonella spp., the risks of infection may be overestimated as a result of the lack of data on the recovery efficiency (R) and, similar to the protozoan parasite data, a lack of differentiation between human infectious species and all Salmonella species. However, for adenovirus, the fraction of infectious viruses was determined by ICC-qPCR and therefore likely more closely estimates the risk of exposure in these exposure scenarios. In order to perform risk characterizations, an annual acceptable risk level for microbial infection of 1 x 10⁻⁴ was applied

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for waterborne exposure by potable water (Regli et al., 1991; Ryu et al., 2007). For recreational exposures, I used the US EPA acceptable risk level of 30 infections per 1000 people (30×10^{-3}).

For exposure scenario 1, which involves a one time accidental exposure to 10mL of reclaimed water, the average risks for all pathogens were below the acceptable risk level with the exception of the mean risk of infection for adenovirus, which was 7.39×10^{-3} (95% CI 3.73 x 10⁻³ to 1.43 x 10⁻²). Based on this result, there is a low risk of infection from the accidental exposure to 10mL of reclaimed water from the pathogens analyzed with the exception of adenovirus, which still poses a risk at this level. For scenario 2, the risks of infection for 40 days of recreational exposure per year were compared to the US EPA acceptable risk level of 30 infections per 1000 people (3 x 10⁻⁴). Based on this level of exposure, the average annual risks were above the acceptable microbial risk level for *Giardia*, with average infection risks of 4.28 x 10^{-4} (95% CI 2.69 x 10^{-5} to 6.20 x 10^{-3}). The mean risk *Cryptosporidium* was slightly above the acceptable risk level, with a mean level of 2.58 x 10^{-3} (95% CI 8.62 x 10^{-4} to 7.63 x 10^{-3}). The mean risk of exposure for both *Salmonella* spp. and adenovirus were above the US EPA acceptable risk level with levels of 1.18×10^{-1} and 8.23×10^{-1} (respectively), with the highest levels of risk posed by adenovirus for the 40 day exposure period.

Scenario 3 involves the annual risks of infection after exposure to reclaimed water piped to a household as drinking water and consumed at a rate of 2L per day 365 days per year. In this scenario, the risks for adenovirus infection are equal to 1, indicating that there is a certainty that an individual exposed under this scenario would become infected. The average risk of *Salmonella* spp. was 8.50 x 10^{-1} (95% CI 1.29 x 10^{-1} to 1.00×10^{0}), as with adenovirus these values are above the US EPA acceptable risk level for drinking water. Additionally, the estimated risk levels for *Cryptosporidium* and *Giardia* are 1.00×10^{-1} and 2.75×10^{-1} (respectively), and are also above the acceptable risk levels set by US EPA. As all of the average risks of infection for each pathogen for this exposure level are above the US EPA acceptable risk of infection, there is a high risk of infection by this exposure route.

Scenario 4 evaluates the annual risks of infection after exposure to 2L per day for 365 days of raw surface water for comparison to the quality and risks of consuming raw reclaimed water. In this scenario, the risk of adenovirus infection was lower than that posed by reclaimed water, with an average risk of infection of 1. 56 x 10^{-1} (95% CI 5.08 x 10^{0} to 9.17 x 10^{-1}). The risk of infection from *Salmonella* spp. in surface waters was greater than the risks in reclaimed waters, with a risk of infection equal to 1, indicating that *Salmonella* infection is likely. The annual risks of infection for *Cryptosporidium* and *Giardia* were similar to the risks estimated for reclaimed water with average risks of 3.55×10^{-1} and 8.47×10^{-2} , respectively. For this scenario, all of the risks are greater than the US EPA acceptable risk level.

The potable reuse scenarios analyzed in Scenario 5 include 1) the US EPA regulated log_{10} reductions for conventional drinking water treatment and disinfection (4 log_{10} for virus, 2 log_{10} *Cryptosporidium* and 3 log_{10} for *Giardia*), 2) the WHO's risk based reduction of pathogens based on DALYs and 3) a worst-case log_{10} reduction scenario based on real world data reviewed by Medema and Hijnen, 2007. Each of these scenarios were evaluated for 5 day storage conditions at both 4 and 20°C.

Based on these log₁₀ reduction scenarios, the US EPA and WHO log₁₀ reduction targets produce reclaimed water that complies with the 10⁻⁴ annual risk of infection target set by US EPA for *Cryptosporidium* and *Giardia* at both 4 and 20°C storage temperatures. In contrast, the risks of infection from *Salmonella* spp. and adenovirus are not reduced below the acceptable level of risk for either storage temperature. The US EPA log₁₀ reduction targets are based on
reducing viruses, *Cryptosporidium* and *Giardia*, while the WHO targets are based on reducing risk of illness based on exposure to organisms in source water. For the worst-case scenario, which was based on a real world data set reviewed by Medema and Hijnen (2007), the average annual risk of infection from all microorganism was higher at all temperatures, with a similar pattern of *Cryptosporidium* and *Giardia* reduction below the acceptable risk level, and adenovirus and *Salmonella* risk continuing to be above this risk level. In general, the risks of adenovirus infection were the greatest for all exposure routes; this is partly due to the higher concentrations of adenovirus in reclaimed waters, surface water, and the combined waters. Temperature does not play a large role in reducing the average annual risk of infection based on this analysis; for all scenarios analyzed, the risks are approximately the same for both temperatures.

Uncertainty was evaluated for each microorganism and each water type by assessing the rank order correlation of uncertainty for the variables considered in this model, specifically the microbe concentration, the percent recovery, the 5-day storage time, and the expected log₁₀ reductions achieved by drinking water treatment. For reclaimed water, the microbe concentration contributed the most to uncertainty for all microorganisms; this was true for all of the drinking water treatment scenarios and the two temperatures of 5-day storage. In contrast, for surface water, microbe concentration was only the greatest contributor to uncertainty for *Salmonella* spp., *Cryptosporidium* spp., and *Giardia* spp.; for adenoviruses in surface water, percent recovery constituted the largest contributing variable to uncertainty. In waters modeled as stored for 5 days and treated by drinking water treatment, microbe concentration contributed the most to uncertainty for all microorganisms; however, for this water type surface water treatment contributed more to uncertainty than 5-day storage or percent recovery. Based on this analysis,

for most scenarios the variable that is most often associated with high-risk scenarios is microbial concentration.

Table 5-3: Step-wise potable reuse example calculation

			Salmonella	Cryptosporidium	Giardia
	Units	Adenovirus	spp.	spp.	spp.
	Organisms per				
Microbe Concentration, C	liter	1	1	1	1
Average Reduction by 5 day storage (20°C), 5 day	% Reduction	0.94	0.91	0.98	0.98
Reduction by EPA Mandated conventional drinking water treatment, DWTP	% Reduction	0.9999	0.999	0.99	0.99
Potable Reuse Water, RW	Organisms per liter	6.00E-06	9.00E-05	2.00E-04	2.00E-04
Recovery Efficiency, R	%	109	100	82.5	63.5
Viability, I	%	38.5	65	25	13
Volume of Water Consumed, V	Liters per day	2	2	2	2
Exposure by drinking water, N	Organisms per day	4.62E-04	1.17E-02	1.00E-02	5.20E-03
Dose-response, r	Probability of infection per organism	6.07E-01	2.10E-01	4.20E-03	1.98E-02
Risk of infection, Pinf,d	Per day	2.80E-04	2.46E-03	4.20E-05	1.03E-04
Risk of infection, Pinf,y	Per year	9.73E-02	5.93E-01	1.52E-02	3.69E-02
Formulas:	$RW = C*1-DWT$ $N = RW*C*R^{-1}$ Pinf,d=N*r	TP*1-5day 1*I*V			

Pinf,y=1-(1-Pinf,d)^365

Scenario 1 (One Time Accidental Exposure to 10mL Reclaimed Water)					
Organism	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval		
Salmonella spp.	2.63E-05	1.90E-06	3.60E-04		
Adenovirus A-F	7.39E-03	3.73E-03	1.43E-02		
Cryptosporidium spp	• 1.45E-06	2.27E-07	9.68E-06		
Giardia spp.	4.41E-06	6.81E-07	3.21E-05		

Table 5-4: Risks of infection for pathogens from five exposure scenarios to reclaimed water

Scenario 2 (Recreational Exposure to 100mL Surface Water, 40 days)

Organism	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Salmonella spp.	1.18E-01	2.25E-02	4.85E-01
Adenovirus A-F	8.23E-01	4.36E-01	9.94E-01
Cryptosporidium spp.	2.58E-03	8.62E-04	7.63E-03
Giardia spp.	4.28E-04	2.69E-05	6.20E-03

Scenario 3 (Reclaimed Water Exposure 2L/day, 365 days)

Organism	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Salmonella spp.	8.50E-01	1.29E-01	1.00E+00
Adenovirus A-F	1.00E+00	1.00E+00	1.00E+00
Cryptosporidium spp. Giardia spp.	1.00E-01 2.75E-01	1.64E-02 4.85E-02	5.07E-01 9.04E-01

Scenario 4 (Surface Water Exposure 2L/ day 365 days)

		Lower 95%	Upper 95%
Organism	Mean	Confidence	Confidence
		Interval	Interval
Salmonella spp.	1.00E+00	9.92E-01	1.00E+00
Adenovirus A-F	1.56E-01	-5.08E+00	9.17E-01
Cryptosporidium spp.	3.55E-01	1.62E-01	6.34E-01
<i>Giardia</i> spp.	8.47E-02	7.18E-03	6.23E-01

		Storage at 4°C			Storage at 20°C		
Organism	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval	
Salmonella spp.	4.62E-03	4.76E-04	3.69E-02	4.58E-03	4.51E-04	3.53E-02	
Adenovirus A-F	3.63E-02	6.41E-03	1.43E-01	1.01E-02	1.44E-03	4.86E-02	
Cryptosporidium spp.	1.57E-04	2.61E-05	6.81E-04	4.55E-04	6.32E-05	2.38E-03	
Giardia spp.	7.63E-07	2.38E-08	2.43E-05	2.22E-06	6.03E-08	7.44E-05	

Scenario 5-1 (Potable Reuse Exposure - US EPA 2L/day 365 days)

Scenario 5-2 (Potable Reuse Exposure - WHO 2L/day 365 days)

		Storage at 4°C			Storage at 20°C		
Organism	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval	
Salmonella spp.	4.69E-03	4.62E-04	3.88E-02	4.62E-03	4.49E-04	3.76E-02	
Adenovirus A-F	3.59E-02	6.23E-03	1.42E-01	9.95E-03	1.42E-03	4.84E-02	
Cryptosporidium spp.	1.60E-05	2.75E-06	7.06E-05	4.63E-05	6.56E-06	2.44E-04	
Giardia spp.	7.64E-07	2.35E-08	2.42E-05	2.22E-06	6.17E-08	7.49E-05	

Scenario 5-3 (Potable Reuse Exposure - Worst Case 2L/day 365 days)

		Storage at 4°C			Storage at 20°C		
Organism	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval	Mean	Lower 95% Confidence Interval	Upper 95% Confidence Interval	
Salmonella spp.	2.62E-02	3.01E-03	1.84E-01	2.54E-02	2.94E-03	1.78E-01	
Adenovirus A-F	5.70E-01	9.79E-02	9.74E-01	2.06E-01	2.46E-02	7.02E-01	
Cryptosporidium spp.	9.65E-06	1.50E-06	4.34E-05	2.79E-05	3.47E-06	1.55E-04	
Giardia spp.	4.71E-07	1.42E-08	1.48E-05	1.37E-06	3.63E-08	4.60E-05	

5.7 Summary and Conclusions

My evaluation of the North Carolina potable reuse scheme indicates that the proposed combination of 80% surface water with 20% reclaimed water, followed by 5 days of storage and conventional drinking water treatment, does not adequately decrease the risks of infection for the pathogens assessed if log₁₀ reduction targets set by US EPA and WHO are met by drinking water utilities. My goal in this study was to evaluate the health risk associated with exposure to

pathogens in reclaimed water after specific exposures related to potable reuse and, based on the four scenarios presented here, the risk of exposure to pathogens is not adequately reduced after complete treatment. Adenovirus and *Salmonella* were not reduced below the US EPA acceptable level of risk for drinking water exposures. For the US EPA log₁₀ reduction targets, the average annual risk of infection for adenovirus was approximately 10⁻² for storage at 4°C and 20°C, while for the WHO log₁₀ reduction targets the average annual risk was 10⁻² at 4°C and 10⁻³ at 20°C. For *Salmonella*, under the US EPA and WHO log₁₀ reduction targets, annual risks were on average 10⁻³ at both temperatures. The risks of infection from both *Cryptosporidium* and *Giardia* were reduced below the US EPA acceptable risk level by potable reuse treatment by both the US EPA and WHO log₁₀ reduction targets at both temperatures.

A secondary goal was to evaluate the risk of infection from recreational exposure to reclaimed water (Scenario 2). I found that the average annual risks for this route of exposure for adenovirus and *Salmonella* spp. were higher than the US EPA acceptable limit of 3×10^{-4} per year. As mentioned in section 5.6, my risk calculations are conservative estimates and may be higher than what can be expected from potable reuse or drinking water treatment in practice. The numbers for recreational exposure do not consider sunlight exposure and potential die-off as a result of UV exposure, a factor that is likely to play a role in microorganism survival.

Since high levels of adenovirus were detected by qPCR, additional methods were used (ICC-qPCR) to determine the infectivity of adenoviruses in reclaimed water samples. Rodríguez et al. (2013) and Polston et al. (2014) have previously evaluated the infectivity of adenovirus in wastewater and surface samples and found that approximately 50% of raw sewage and 44% of surface water samples positive for adenovirus were also infectious. I found that approximately 38% of positive reclaimed water samples were also infectious. A combination of a high

concentration of adenovirus in the reclaimed water (detected by qPCR) and a high percentage of infectious adenovirus (informed by ICC-RTqPCR) results in a high annual risk for this pathogen under scenarios considering incomplete drinking water treatment steps. However, when full drinking water treatment processes were evaluated, the risk of adenovirus infection decreased but was still not reduced below the US EPA acceptable level of risk. Additional study is needed to further evaluate the relationship between direct detection of viruses (DNA/RNA) and the infectivity of those viruses after disinfection in wastewater and surface water treatment applications.

Although conventional wastewater treatment is known to reduce the numbers of *Cryptosporidium* and *Giardia* by 3 and 4 log₁₀ respectively, numbers of (oo)cysts are often detected in tertiary treated effluents (Gennaccaro et al., 2003). The risk of infection related to exposure to *Cryptosporidium* and *Giardia* in reclaimed waters has been previously evaluated for non-potable uses (Ryu et al., 2007) as well as from surface water exposures (Jacob et al., 2015), in which studies there were found low but detectable levels of protozoan parasites in these waters used for either agricultural use or drinking water purposes, but with health risks below (<10⁻⁴) the US EPA acceptable risk level. I detected both *Cryptosporidium* and *Giardia* in nearly all reclaimed water samples at low concentrations; despite this (similar to the previously cited studies), the average annual risks of infection were below the US EPA average annual risk of infection. Since I did not assess infectivity, further study is needed to evaluate the infectivity of human infectious protozoan parasites after UV treatment.

Based on the analysis presented here, potable reuse under conditions that provide for advanced drinking water treatment or storage options is possible and potentially a viable option for communities with the capacity to first treat wastewater by a dual disinfection process. As the

risks of adenovirus and *Salmonella* infections for drinking water and recreational exposures are still above the US EPA acceptable risk limit, it may be advisable for wastewater treatment plants to increase or re-evaluate virus treatment methods. As a 5 day storage time is required for potable reuse in NC, open air tanks with some form of mixing may be an option for decreasing the microbial concentrations in reclaimed waters blended with raw surface waters for further treatment to reduce pathogens, preferably with additional sunlight exposure, such as by aeration that sprays the water into the air.

Reclaimed water has become a more attractive option given increases in the population (particularly around urban centers) and, with the appropriate use of available technology, it is possible to use waters currently produced by wastewater utilities together with the treatment capabilities of drinking water treatment plants to provide high quality reclaimed water for potable reuse.

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CHAPTER 6: QUANTITATIVE MICROBIAL RISK ASSESSMENT OF NORTH CAROLINA TYPE 2 RECLAIMED WATER FOR AGRICULTURAL REUSE

6.1 Introduction

Treated wastewater used for agricultural purposes has been proposed as a means of enhancing food security while addressing water scarcity issues related to growing water demands (Asano, 2002). Most reclaimed water usage in the world comes from agricultural uses, with Mexico and Egypt reported to have the highest usage of treated wastewater for irrigation (Jiméz, 2006). In North Carolina, high quality reclaimed water, designated Type 2, has been proposed for the irrigation of food crops (NC DENR, 2011; 2014). Despite this level of wastewater treatment, the actual microbial quality and potential health risks from pathogens in this type of reclaimed water are not known and therefore are still of concern.

In order to evaluate the microbial risks of reclaimed water for agricultural use, the WHO has recommended the use of QMRA to assess the additional disease burden (WHO, 2006). QMRA is an analytical tool used to estimate the health effects resulting from exposure to microorganisms (Peterson et al., 2006; Medema and Peterson/WHO, 2016). There are four key steps to the QMRA process: 1) hazard identification, 2) exposure assessment, 3) dose-response assessment, and 4) risk characterization. Previous researchers conducting QMRAs on the agricultural reuse of reclaimed water have focused on the use of reclaimed water on either specific crops (Hamilton et al., 2006) or by specific irrigation methods (Oron et al., 2000). These studies have also examined the use of secondary effluent, final effluent or reclaimed water treated by chlorine disinfection only. As the concentrations of pathogens and the associated risks from these pathogens are potentially different in waters that have been previously evaluated, it is

necessary and important to evaluate the risks of using NC Type 2 reclaimed water for agricultural proposes.

I selected five representative pathogens from the three groups of microorganisms (bacteria, virus, protozoan parasites) included in the NC legislation on reclaimed water. Although the NC reclaimed water legislation is focused on fecal indicator microorganisms to specify water quality and treatment system performance requirements, my goal in this risk assessment was to select representative pathogens from each group to model the risk of exposure posed by reclaimed water via raw fruit and vegetables ingested by consumers under various exposure scenarios. The selected pathogens from each group include *Salmonella* spp. bacteria, Adenovirus groups A-F, Norovirus GII (as representative enteric viruses) and *Cryptosporidium* spp. and *Giardia* spp.as protozoan parasites. The exposure scenarios evaluated in this analysis are focused on three irrigation water delivery types, specifically, spray, drip, and subsurface drip irrigation.

The presence of enteric pathogens, including bacteria, viruses, and protozoan parasites in water, has been previously evaluated by risk assessment tools (Regli et al., 1991; Asano et al., 1992; Ryu et al., 2007; Jacob et al., 2015); however, the NC system consisting of tertiary treatment by conventional physical and biological processes followed by dual disinfection to produce Type 2 waters for agricultural irrigation has not been examined using QMRA. My objective is to perform quantitative risk assessments for adenovirus groups A-F, Norovirus GII, *Salmonella* spp., and *Cryptosporidium* spp. and *Giardia* spp., in various exposure scenarios relevant to the agricultural reuse of the North Carolina approved Type 2 reclaimed water.

6.2 Methods

6.2.1 Study Design and Sampling

Reclaimed water samples were collected bi-monthly for 1 year, during and after storm events, as grab samples using approved techniques (SMEWW) from 4 different water reclamation facilities located in central North Carolina, resulting in 22 reclaimed water samples. North Carolina Type 2 reclaimed water is characterized by tertiary physical and biological treatment (typically, primary sedimentation, secondary biological treatment and direct granular media filtration) followed by dual disinfection (typically by UV radiation and chlorine disinfection). Reclaimed water samples were collected in 10L volumes, transported on ice to the laboratory and stored at 4°C until analyzed.

6.2.2 Pathogen Recovery

A total of 22 samples of reclaimed water were assayed for *Salmonella* spp., *Cryptosporidium* spp., *Giardia* spp., Norovirus GII, and Adenovirus A-F using the methods described in section 2.2. *Salmonella* spp. were analyzed in 1000 mL volumes of reclaimed and water samples by an established MPN method (Krometis et al., 2010) using triplicate sample volumes of 300 mL, 30 and 3 mL that were pre-enriched, initially in peptone water, followed by selective enrichment in RV broth and streak plating for colony isolation on SS agar to detect presumptive Salmonella spp. colonies. Presumptive positive colonies were confirmed by biochemical testing using Triple Sugar Iron Agar medium slants. Concentrations were reported as MPN per 100mL. Enteric viruses and protozoan parasites were analyzed in 10L volumes of reclaimed water and 12L volumes of surface water by hollow fiber ultrafiltration and elution following the procedure described by Hill et al. (2007) and Polaczyk et al. (2008) using Fresenius Optiflux F250NR hollow fiber ultrafilters (dialyzers). Enteric viruses were further concentrated by polyethelyeneglycol (PEG-8000) precipitation by the method described in Yamamoto et al. (1970), while secondary concentration and purification for protozoan parasites was performed by immunomagnetic separation (IMS) and immunoflorucescent (oo)cyst microscopic examination. Concentrations of (oo)cysts were reported as (oo)cysts per 100mL and concentrations of enteric viruses were reported as genome equivalent copies (GEC) per 100mL. An integrated cell culture-polymerase chain reaction assay (ICC-PCR) was used to detect infectious Adenovirus as described by Rodríguez et al. (2013) and Polston et al. (2014), and as further described in section 2.2.8. Norovirus GII data was excluded from this analysis because noroviruses were not detected in the reclaimed water samples.

6.2.3 Statistical Analysis

Data were entered into a Microsoft Excel (Microsoft Cooperation, Redmond, WA) spreadsheet and calculations for risk and Monte Carlo simulations were performed using Analytica 4.6 (Lumina Decision Systems, Los Gatos, CA), with random variables sampled 10,000 times for each analysis. Details on the components of the risk assessment model, assumptions, recovery efficiencies, etc. will be presented in detail in the sections below. A diagram of the QMRA model designed in Analytica is presented in Figure 6-1. Uncertainty analyses were conducted for microorganism in each water type by evaluating the rank order correlation of uncertainty with variables used in the model. The results of this analysis are presented in section 6.5.



Figure 6-1: Analytica diagram of agricultural reuse model

6.3 Exposure Assessment

My focus in this exposure assessment is the estimation of the likelihood of exposure by an individual to the identified hazard. The key elements and variables included in this exposure assessment are the concentrations and survival of key pathogens, specifically *Salmonella* bacteria, adenoviruses, *Cryptosporidium* and *Giardia* spp., on raw vegetable crops watered by drip, spray or subsurface drip irrigation over a period of 30 days.

In order to more fully evaluate the exposure of individuals due to the ingestion of contaminated foods, the survival (and decay) of those pathogens on food products was considered. Natural processes, such as temperature, dissolved solids, UV/sunlight radiation, exposure, relative humidity and moisture content (water activity), may impact the survival of pathogens on food products in the supply chain. As a component of this model, a decay constant was incorporated into the pathogen concentration term (C_C). The decay constants used for this

analysis for each class of microorganism are listed in Table 6-1. Microbial concentrations were calculated using the following equation:

$$C_c = C_{RW}[exp(-kt_d)]$$

in which C_c is the concentration of pathogens in organisms/L at elapsed time t_d after irrigation or at consumption, C_{RW} (organisms/L) is the initial pathogen concentration in reclaimed water samples, *k* is the kinetic decay constant (d⁻¹), and t_d is the elapsed time between final irrigation and consumption in days.

The exposure due to the ingestion of contaminated food can be estimated as the product of contaminant concentration in the consumed food and the amount of food consumed per day (Hammad and Manocha, 1995), as represented in the equation developed by Hamilton et al. (2006) below:

$$D_i = f_{raw} M_{body} M_i C_c V_{eq} exp(-kt_d)$$

where D_i is the daily dose of contaminant (organism per capita per day), f_{raw} is the fraction of fruits and vegetables consumed raw, M_{body} is human body weight (kg), M_i is daily consumption per capita per kg of body weight (g/(kg ca d)), C_c is the pathogen concentration (organisms/L) of irrigation water , V_{eq} is the volume of reclaimed water in g⁻¹ retained on raw vegetables after irrigation, k is the kinetic decay constant (d⁻¹), and t_d is the elapsed time between final irrigation and consumption in days. This equation evaluates the combined effects of human consumption habits as related to the applied volume of wastewater at a specific quality and application method. The variables used in this equation are described in Table 6-1.

6.3.1 Exposure Scenarios

For this analysis, I used the data collected on the microbial quality of North Carolina Type 2 reclaimed water to model the health risk from pathogens of consuming raw fruits and vegetables irrigated by specific techniques. The irrigation techniques evaluated include spray irrigation (SI), drip irrigation (DI), and subsurface drip irrigation (SDI). Elapsed times of 0, 15, and 30 days between irrigation and harvest were evaluated. The variables used in this model are summarized in Table 6-1.

6.3.2 Irrigation Method and Reclaimed Water Quality

Agricultural crops are typically contaminated in one of two ways: 1) by direct external plant contact with wastewater; and, 2) penetration of microorganisms through the root system or another pathway into a plants' internal parts (Oron et al., 1991). Three different types of irrigation methods were considered to capture the risks of both types of agricultural contamination by microbial pathogens. As contact contamination typically depends on the type of irrigation method, it is important to evaluate the three irrigation methods: SI, DI, and SDI. With SI, relatively large amounts of reclaimed water and aerosols are in contact with the crop surface, causing high amounts of contamination. With DI, reclaimed water is provided through on-surface laterals, which only contaminate plants if the laterals are directly attached to the emitters. Oron et al. (1991) estimated that the contamination levels when using DI are at least 2 orders of magnitude lower than when using SI. With SDI, estimated contamination levels are even lower as reclaimed water will only come into contact with the root of the plant. The distributions and mean values for equivalent volumes of reclaimed water on fruit and vegetable crops are summarized in Table 6-1.

Model Parameter and Sample	Symbol	Unit	Distribution and Fit Parameter	Reference
Organism Concentration in NCT2RW			Normal, fitted to log data	Calculated from 22 reclaimed water samples
Adenovirus A-F	Cnw	Log ₁₀	normal(µ:3.72,σ:1.56)	water sumpres
Salmonella spp.	CRW	per L	$normal(\mu:0.13,\sigma:0.45)$	
Cryptosporidium spp.			normal(µ:0.22,σ:0.36)	
Giardia spp.			normal(µ:0.22,σ:0.38)	
Daily Fruit and Vegetable Consumption	M_{i}	g (kg ca da) ⁻¹	Point Estimate*, 313	US EPA, 2011
Percentage of Fruit and Vegetables Consumed Raw	fraw	-	Triangular(0.25, 0.5, 0.75)	Van Ginneken and Oron, 2000
Kinetic Decay Constant				
Viruses	lr.	Dourl	PE, 0.69	Asano et al., 1992
Bacteria	ĸ	Day	PE, 0.147	
Protozoan Parasites			PE, 0.0365	Reinoso et al., 2008
Body Mass	M_{body}	kg	Lognormal(μ:61.429, σ:13.362)	US EPA, 2011
Equivalent Volume Spray irrigation			PE, 1.6E-04	
Drip irrigation	V_{eq}	g ⁻¹	Triangular(1.6E-07, 1.6E-06, 1.6E-05)	Van Ginneken
Subsurface drip irrigation			Triangular(1.6E-08, 1.6E-07, 1.6E-06)	and Oron, 2000
Period Between Irrigation and Consumption *PE is a point estimate	t _d	Days	0, 15, 30	-

Table 6-1: Summary of distributions and fit parameters used in models

6.4 Dose-Response Modeling

For Adenovirus A-F, *Cryptosporidium* and *Giardia*, the exponential dose response model was used to determine the probability of infection from ingestion of various numbers of pathogens. The exponential model is:

$$P(inf) = 1 - e^{-k*N}$$

where P(inf) is the probability of infection resulting form daily ingestion of the number of pathogens (N) and *K* is the average dose, or number of organisms, that must be ingested to initiate an infection. The best fit *K* values for Adenovirus, *C. parvum*, and *G. lamblia* are 6.07E-01 (Couch et al., 1966), 0.0042 (DuPont et al., 1995), and 0.0198 (Rose et al, 1991), respectively.

For Salmonella spp., the Beta-Poisson model was used; this model is:

$$P(inf) = 1 - [1 + N \frac{(2^{\frac{1}{\alpha}} - 1)}{N_{50}}]^{-\alpha}$$

where P(inf) is the probability of infection resulting from daily ingestion of the dose of pathogens (N), α is pathogen infectivity constant, and N_{50} is the LD₅₀, the dose that is lethal to 50% of individuals, divided by the ID₅₀, which is the median infective dose. The optimized parameters for non-typhoid *Salmonella* are 2.1E-01 and 4.98E+01 for α and N₅₀, respectively (Meynell and Meynell, 1958).

Estimates of daily risk can be extrapolated to the risk of infection over an extended period of time using the equation below (Haas, 1983). I used this equation to calculate yearly risks of reclaimed water under the exposure scenario of 365 days of exposure to raw vegetables irrigated with Type 2 reclaimed water.

 $P_t = 1 - (1 - P_d)^t$

Here, P_t is the probability of infection after t days and P_d is the probability of infection after one day of exposure.

In order to further evaluate the risk of illness from exposure to pathogens in reclaimed waters used for agricultural purposes, it is necessary to calculate the DALYs associated with the illness from the diseases associated with the pathogens examined. The first step in this calculation is to estimate the risk of diarrheal illness per year (P_{ill}) using the formula:

$$P_{ill} = P_t x P_{ill/inf}$$

where P_t is the probability of infection after t days (in this case 356 days or 1 year) and P_{illµnf} is the probability of illness given infection. This parameter is organism specific; the value for *Salmonella is* 0.3, *Cryptosporidium* and *Giardia* are 0.7, and the value for adenovirus is 0.5 (WHO, 2011). DALYs per case is also organism specific and the relevant values are 9.6 x 10^{-4} for *Salmonella*, 1.5 x 10^{-3} for *Cryptosporidium* and *Giardia*, and 2 x 10^{-3} for adenovirus, respectively (Peterson et al., 2006). The health outcome target (HT), or DALYs per year, is calculated using the equation below:

$$HT = P_{ill} x \, db \, x \, f_s \div 100$$

where f_s is the fraction of the population susceptible to a given pathogen; for this analysis 100% of the population is assumed to be susceptible for each pathogen.

6.5 Results and Risk Characterization

Table 6-2 displays the DALYs per person per year as well as upper and lower 95% confidence intervals (CIs) for the risk scenarios based on irrigation type and period between irrigation and consumption using North Carolina Type 2 reclaimed water. It is important to note that the calculation of risks for *Cryptosporidium* and *Giardia* spp. are based on total counts (not on infectivity data) and, despite accounting for infectivity in the QMRA model, the risk may be

overestimated. Additionally, US EPA Method 1623 does not differentiate between human infectious species and all species of *Cryptosporidium* and *Giardia*, which may also result in an overestimation of human health risks. However, for adenovirus, the fraction of infectious viruses was determined by ICC-qPCR and likely more closely estimates the risk of exposure in these exposure scenarios. In order to perform risk characterizations, a health based target aimed at evaluating a DALY loss of $\leq 10^{-6}$ per person per year through waterborne exposure by potable reuse water (WHO, 2006).

SI, which involves the use of sprinklers to distribute reclaimed water onto land surface, which then either evaporates into the air, or soaks into the soil, causes a large amount of aerosolized particles to come into contact with crop surfaces and the ground, resulting in a large amount of contamination if microorganisms remaining in irrigation water and coming in contact with the produce. Based on my analysis, the DALYs associated with this irrigation method are relatively high compared to DI and SDI. The protozoan parasites had DALYs higher than the acceptable level of 10^{-6} per person per year, with average levels of 8.74×10^{-5} for *Cryptosporidium* and 3.44×10^{-4} for *Giardia*. The DALYs for *Salmonella* were also above the acceptable level with an average of 7.49×10^{-6} (95% CI 5.07 x 10^{-7} to 8.93×10^{-5}). In contrast, the average DALY for adenovirus was 1.15×10^{-9} (95% CI 4.98x 10^{-10} to 2.64×10^{-9}), indicating that there is little DALY risk due to adenovirus at this acceptable risk level.

In DI, water is delivered directly to the root zone of a plant, where it seeps into the soil. It is expected that less direct contact with plant surfaces will result in a lower annual risk of infection from microbial contaminants. Based on my results, the annual microbial risks of infection are lower than those estimated for SI. For this type of irrigation, the DALY for adenovirus (3.66×10^{-11} , 95% CI 7.25 x 10^{-12} to 1.28×10^{-10}), and *Salmonella* spp (DALY of

2.25 x 10^{-7}) were below the acceptable level, while the DALYs for *Cryptosporidium* and *Giardia* were above the acceptable DALY risk level of 1 x 10^{-6} , at 2.76 x 10^{-6} and 1.26 x 10^{-5} , respectively.

SDI involves the use of embedded pipes or tubing to irrigate crops, typically in rows or fields. As this method involves even less contact with the surface of the plant, Oron et al. (1991) proposed that there may be even less risk associated with this type of irrigation than with DI or SI; and for all pathogens I examined, with the exception of *Giardia*, the DALYs were below the acceptable level. The DALY for adenovirus was 3.63×10^{-12} (95% CI 6.92×10^{-13} to 1.28×10^{-11}), the DALY for *Salmonella* spp. was 2.28×10^{-8} (95% CI 1.12×10^{-9} to 4.05×10^{-7}), and the DALY for *Cryptosporidium* was 2.73×10^{-7} (95% CI 2.57×10^{-8} to 2.66×10^{-6}). For *Giardia* spp., the mean annual risk was 1.27×10^{-6} (the 95% CI 1.10×10^{-7} to 1.25×10^{-5}).

It is important to note that the assessments performed here only consider fruits and vegetables consumed in the raw state. Additionally, my results indicate that the irrigation type and exposure of the various crops to reclaimed water (and the resulting microorganisms in reclaimed water) have an impact on the annual risks of infection. I performed sensitivity analyses for all irrigation scenarios by assessing the rank order correlation of uncertainty for the variables considered in this model, specifically the microbe concentration, time between irrigation and harvest, human body weight, the equivalent volume of water irrigated onto crops, and the fraction of fruits and vegetables consumed raw. I found that microbe concentration contributed the most to uncertainty for all irrigation types; however, the equivalent volume also had an important impact on the magnitude of the health outcome for the DI and SDI models.

	Scenario		DALY per year					
Irrigation Type	Organism	Average	Lower Confidence Limit	Upper Confidence Limit				
Spray	Salmonella spp.	7.49E-06	5.07E-07	8.93E-05				
	Adenovirus A-F	1.15E-09	4.98E-10	2.64E-09				
	Cryptosporidium spp.	8.74E-05	1.27E-05	4.81E-04				
	Giardia spp.	3.44E-04	5.38E-05	9.98E-04				
Drip	Salmonella spp.	2.25E-07	1.16E-08	4.00E-06				
	Adenovirus A-F	3.66E-11	7.25E-12	1.28E-10				
	Cryptosporidium spp.	2.76E-06	2.61E-07	2.54E-05				
	Giardia spp.	1.26E-05	1.14E-06	1.16E-04				
Subsurface	Salmonella spp							
Drip	Saimoneita spp.	2.28E-08	1.12E-09	4.05E-07				
	Adenovirus A-F	3.63E-12	6.92E-13	1.28E-11				
	Cryptosporidium spp.	2.73E-07	2.57E-08	2.66E-06				
	Giardia spp.	1.27E-06	1.10E-07	1.25E-05				

Table 6-2: Annual risk of infection for irrigation scenarios based on 10,000 Monte Carlo simulations

Time between irrigation and consumption is assumed to be 15 days.

6.6 Summary and Conclusions

My goal in this study was to evaluate the health risk associated with pathogens in reclaimed water used for agricultural purposes, specifically as water for the irrigation of fruit and vegetable crops. Based on the types of irrigation I examined, using North Carolina Type 2 reclaimed water, there is evidence it is not clear that the annual risk of infection is not always reduced below the acceptable risk level of $\leq 1 \times 10^{-6}$ for some exposure conditions. Based on my analysis of North Carolina Type 2 reclaimed water, for all irrigation types, the risks of viral infection by adenoviruses groups A-F were below the acceptable risk level; however, for *Salmonella* spp., *Cryptosporidium*, and *Giardia*, the annual risk of infection was higher than is considered acceptable. Potential reasons for the higher level of risks for bacteria and protozoan parasites include increased levels of survival on plant surfaces after irrigation as well as a

variation in the volume of water retained on the produce after irrigation. My sensitivity analyses indicated that the microbe concentration in reclaimed water played the largest role in the variations between the pathogen risks.

Previous researchers (Van Ginneken and Oron, 2000; Hamilton et al., 2006, Amha et al., 2015) have evaluated reclaimed water for agricultural purposes and have compared the risks from these exposures to benchmarks set for drinking water risks (10⁻⁴ infections per year); whereas I evaluated the reclaimed water proposed for agricultural reuse in NC and compared it to recent WHO targets for reuse applications. Van Ginneken and Oron (2000) found that the estimated average annual risk of infection from SI was 10⁻⁶, and the risks from DI and SDI were 10⁻⁸ and 10⁻⁹, respectively. Hamilton et al. (2005), using secondary effluent water, also found a similar risk of virus infections modeled using coliphage virus data on different types of vegetables when a 14 day period was considered between irrigation and consumption, with average annual risks ranging from 10⁻⁵ for lettuce, to 10⁻⁷ for cucumber and broccoli. Amha et al. (2015) evaluated the average annual risks of infection for *Salmonella* and found that these risks (on average between 10⁻² to 10⁻³ per year) were higher than those of viral infection. These values for *Salmonella* spp. are within my estimated ranges of average annual risk.

The risk of *Cryptosporidium* in irrigation waters has been evaluated by Mota et al. (2009) and Agulló-Barceló et al. (2012). Mota et al. (2009) found that, assuming 120 days of exposure per year, the annual risks of *Cryptosporidium* infection for tomatoes, bell peppers and cucumbers were all approximately 10^{-5} . The discrepancies between this value and the risks reported by this present study could be a result of the lower period of exposure to fresh produce. Agulló-Barceló et al. (2012) found average annual risk levels of 4.37 x 10^{-2} in tertiary treated effluent for total *Cryptosporidium* (not infectious oocysts). I found that the DALYs related to illness from

exposure to agricultural reuse waters were reduced below the WHO proposed levels for all irrigation methods, with the exception of SI. As it is not clear if the oocysts I evaluated were infectious, further study is needed to evaluate the infectivity of human infectious protozoan parasites after UV treatment. If UV treatment reduces the infectivity of the oocysts, the health risks would be lower than those calculated here. Although infectivity data is possible for *Cryptosporidium* using a cell culture infectivity assay system, little information is available on *Giardia* infectivity due to the lack of a cell culture infectivity system and therefore the resulting annual risks are uncertain due to the lack of cyst infectivity data.

My results indicate that irrigation method plays an important role in the characterization of annual risk from microbes in reclaimed water used for agricultural purposes. Based on my analysis, SDI reduces the risks of infection to the lowest level. However, in the evaluation of the North Carolina potable reuse and food irrigation scheme for agricultural use, it appears that for some microorganisms there remains a significant risk of infection after application to fruits and vegetables. For viruses, however, the annual risk of infection is lower than the acceptable risk level, despite high levels of infectious viruses in the reclaimed water. Because all of the pathogens studied are present in the reclaimed water, this type of water type must be compared to other irrigation water sources to assess their suitability for direct application in agriculture.

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CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 Summary of Significant Findings

The goal of this study was to address the need for real world data on the microbiological quality of the North Carolina approved reclaimed water, designated as type 2 (NCT2RW). This goal was accomplished by conducting field studies on the microbiological quality of reclaimed water currently produced by four wastewater reclamation facilities producing North Carolina type 2-like water as well as on ambient surface water sources used or potentially useable for drinking at 4 drinking water treatment facilities. These water samples were evaluated for both fecal indicator microorganisms of interest to or regulated by the state of North Carolina, and enteric pathogens of public health interest. In addition, microorganisms in these waters were evaluated for their survival characteristics under different conditions of temperature, mixing and sunlight exposure in a blended water consisting of 20% NCT2-like RW and 80% surface water over the state mandated 5-day storage period required for potable reuse in NC. Finally, quantitative microbial risk assessments (QMRAs) were conducted for various water exposure scenarios for both potable reuse and agricultural reuse applications.

This research provides further health-related microbiological and associated health risk assessment information on the type of reclaimed water proposed by North Carolina for potable reuse purposes. As potable reuse is becoming a topic of increased interest to areas experiencing either drought or population growth or both, the microbial quality of source waters proposed for the expansion of water resources for potable use is an important component of the design water reuse and water resource management systems (NAS, 2012). Although chemical contaminants

are also of concern when evaluating reclaimed water schemes, microbial hazards can also pose health risks of an immediate nature if treatment is not designed to remove or inactivate pathogens prior to consumption of reclaimed waters. In the North Carolina potable reuse scheme, potable reuse is designed as wastewater treatment involving full tertiary treatment by physical and biological processes, typically primary and secondary treatment followed by granular media filtration as tertiary treatment to produce well oxidized reclaimed water that is then subjected to dual disinfection, (typically by UV irradiation and then chlorine disinfection. The resulting reclaimed water is then blended at up to 20% of the flow with at least 80% ambient surface water and stored for 5 days, followed by conventional drinking water treatment to produce potable water. This treatment scheme is designed to remove and inactivate microorganisms (by wastewater treatment and disinfection) and to further reduce microbial hazards by storage and combining with surface waters currently used as drinking water sources.

Conducted at a pilot scale, previous research done on the NC proposed tertiary treated, dual disinfected reclaimed water by Sobsey et al., indicated that the dual UV and chlorine disinfection system applied to tertiary treated effluent was effective in reducing numbers of fecal indicator bacteria, viruses and protozoan parasite surrogates for the production of high quality reclaimed water (Sobsey et al., 2005). When using a single disinfection step (either UV or chlorine), other studies have demonstrated that reclaimed waters may still have detectable levels of pathogens, particularly by molecular detection of nucleic acid genome targets for viruses and by microscopic detection of protozoan parasites in contrast to the detection of infectious pathogens (Harwood et al., 2005). Based on the limited amount of available literature on the microbial quality type 2 reclaimed water, there was a need and motivation for additional data on both the microbial quality of this water and its associated health risks from potable reuse

exposures as drinking water and produce irrigation water. This study sought to evaluate not only the microbial quality of the reclaimed water proposed for potable reuse, but also the microbial quality of surface waters currently used as sources for drinking water in North Carolina, and the microbial risks posed by these potable reuse-derived waters as determined by QMRA. Key research questions included:

- Can NCT2-like RW-producing treatment plants that meet the indicator performance requirements, based on their fecal indicator microbe concentrations and log₁₀ microbial reductions, also reduce enteric pathogens to the same or similar extent?
- 2. If pathogens are reduced to low levels in NCT2RW as documented by required log₁₀ reductions, are they also reduced to sufficiently low levels based on their concentrations to achieve acceptably low human health risks, if used as drinking source water?
- 3. What is the microbial quality of run of river surface waters based on concentrations of microbial indicators and pathogens as potential source waters proposed for blending as at least 80% of combined flow with up to 20% NCT2RW as a combined source water for drinking water supply?
- 4. Does 5-day storage of this blended water have any effect on the concentrations of fecal indicator microorganisms?
- 5. Is the NCT2RW used to make drinking water by blending at a ratio of no more than 20:80 by volume with surface source water, then stored for 5 days and subjected to conventional water treatment likely to achieve the US EPA acceptable microbial drinking water risk level of 10⁻⁴ infections/person/year, based on the allowable

parameters and conditions for treatment, microbial quality, log₁₀ microbial reductions, blending and storage?

- 6. Is the NCT2RW used for agricultural purposes likely achieve the WHO acceptable agricultural reuse health risk level of $\leq 10^{-6}$ DALYs per person per year, based on the allowable parameters and conditions for treatment, microbial quality, and microbial reductions when used for produce irrigation?
- 7.1.1 Pathogens Detected in Reclaimed and Surface Waters

In this research, both indicators relevant to the NC reclaimed water regulations (NC DENR 2011; 2014) and pathogens of public health concern were detected in 22 North Carolina type 2-like reclaimed water samples and 22 sewage impacted surface water samples. The indicators examined in this research included total coliforms, *E. coli*, and *Enterococcus* spp. as the bacteria, F+/male-specific, somatic, and total coliphage as indicator viruses, and vegetative and spore forming *Clostridium perfringens* as the protozoan parasite surrogate. The pathogenic microorganisms detected by this research included, *Salmonella* spp. as the pathogenic bacteria, Norovirus GII and Adenovirus groups A-F as target human enteric viruses, and *Cryptosporidium* spp. and *Giardia* spp. as the protozoan parasites.

In the evaluation of pathogen concentrations in NC type 2-like reclaimed waters, nearly all samples of reclaimed water had low but detectable levels of pathogens after tertiary wastewater treatment and dual disinfection. This result is particularly important for the concentrations of adenoviruses, as the levels detected by real time qPCR (genome copies, not infectious units) were on average quite high at 5.24×10^2 GEC per 100mL. In addition, levels of infectious adenoviruses, detected by ICC-RTqPCR were also high, at average concentrations of 6.79 x 10^1 MPNIU per 100mL. Protozoan parasites were also detected by immunofluorescent microscopy in nearly all samples of reclaimed water, but these cysts and oocysts were not

assayed for infectivity so their health risks are uncertain. In surface waters pathogens were also detected in many samples, with adenoviruses above the detection limits in 41% of samples, with an average concentration of 1.44×10^4 GEC per 100mL. For the protozoan parasites,

Cryptosporidium oocysts were detected in 86% and *Giardia* cysts were detected in 81% of all surface water samples analyzed by immunofluorescent microscopy assay. Because the North Carolina potable reuse scheme proposes to combine type 2 reclaimed waters up to 20% of flow with ambient surface waters at 80% of flow or more, the quality of such surface waters becomes an important contributor to the potential microbial risks associated with potable reuse. In many of the samples evaluated, the concentrations of pathogens were equal to or higher in the surface waters than in the NC type 2 reclaimed water. Although infectivity was not evaluated for the protozoan parasites, there are similar concentrations of both cysts and oocysts in surface waters at 5.37 and 1.35 per 100 mL, respectively. For *Salmonella* spp., there were also more bacteria in the surface water with concentrations of 5.75 per 100mL compared to 1.35 per 100mL in the reclaimed water. For adenovirus, concentrations were higher in the tertiary treated reclaimed waters with concentrations of 5.25 x 10^4 compared to concentrations in surface waters of 3.24×10^3 per 100mL.

Based on the results of the samples analyzed for microbial quality in this study, the reclaimed water samples produced by type 2-line water reclamation facilities are of higher or comparable microbial quality than the samples run of river or sewage impacted surface waters currently used by drinking water treatment facilities. Therefore, the addition of 20% of the volume of higher quality NCT2RW to the lower quality ambient surface water to be further treated as source water by these drinking water treatment facilities may reduce the microbial

risks associated with their source waters. However, the high concentrations of adenoviruses in both of these water types along with the occurrence of protozoan parasites in both water types is of health concern for potable reuse applications.

7.1.2 Log₁₀ Microbial Reductions Relative to NCT2RW Performance Targets for Potable Reuse

The State of North Carolina specifies that source water for further drinking water treatment to produce potable water when there is potable reuse must be a combination of up to 20% NCT2RW with at least 80% surface source water, followed by a 5-day storage time under unspecified conditions. Performance targets for reclaimed water are defined as reductions in log₁₀ concentrations of indicator bacteria, viruses, and a protozoan parasite surrogate as well as monitored monthly geometric mean and single sample maximum daily concentrations that must not be exceeded for each microorganism. Log₁₀ reduction targets are 6-log₁₀ for bacteria, a 5log₁₀ for viruses, and a 4-log₁₀ for protozoan parasite surrogates. The monthly geometric mean and daily maximum targets for bacteria are a monthly geometric mean of less than or equal to 3 per 100mL (CFU) with a daily maximum of less than or equal to 25 per 100mL. For coliphage and *C. perfringens*, the monthly geometric mean concentration can be no more than 5 per 100mL (PFU and CFU respectively), with a daily maximum concentration of less than or equal to 25 PFU or CFU per 100mL, respectively.

Based on these reduction targets, and the results presented in Chapter 2, it is not clear that the log_{10} reductions achieved by the wastewater reclamation systems studied meet the NC log_{10} reduction standards for indicator microorganisms. The log_{10} reduction targets for bacteria (*E. coli*) and the protozoan parasite surrogate (both pasteurized and unpasteurized *C. perfringens*) were met, with reductions of 6.36, 4.26, and 4.43 respectively. However, the quantifiable reductions for indicator viruses were below the 5-log₁₀ reduction performance target for somatic, F+, and total coliphages. As several of these log_{10} reduction values, specifically the *E. coli*, and

the coliphage values, are based on upper and lower limit detection values, it is unclear whether these log_{10} reductions are indicative of those actually achieved because they are censored values and could be greater than the log_{10} reductions reported. Even though the log_{10} reduction performance targets were not met for the viral indicators, the reclaimed water samples examined were below the state mandated monthly geometric mean and daily maximum concentration values for all indicator organisms (including viruses).

In contrast to the low concentrations of fecal indicator microorganisms in the NCT2RW, there were high occurrences and concentrations of pathogenic bacteria (Salmonella spp.), enteric viruses (enteric adenoviruses) and protozoan parasites (Cryptosporidium and Giardia) in these reclaimed water samples. Of particular interest were the high (~10²-10³ GEC/100 mL) concentrations of adenovirus groups A-F detected by real time qPCR. Although these viruses were not detected by infectivity methods, this high concentration of adenovirus in tertiary treated, dual disinfected reclaimed waters is of potential health concern for potable reuse applications. Additionally, the concentrations of human enteric viruses detected by the Single Agar Layer (SAL) method, which were often not detectable at all the 100 mL sample volumes analyzed. The relationship between infectious and genome copies of adenoviruses detected in the NCT2RW samples analyzed is discussed in section 7.1.3 that follows.

7.1.3 Detection of Adenovirus Infectivity Based on ICC-RTqPCR

To address the North Carolina T2RW regulation performance for indicator viruses, somatic, F+, and total coliphage viruses were detected by US EPA method 1602, the SAL method in 100mL reclaimed water samples. The NCT2RW samples analyzed met the concentration limits for these coliphages, with very low or non-detectable levels in 100 mL sample volumes. The average concentrations of somatic, F+ and total coliphages were 1.17, 1.28,

and 1.17 per 100mL respectively, below the allowable limit of 5 per 100 mL. However, as stated previously, high levels of enteric adenoviruses were detected in reclaimed water samples. In order to evaluate the number of infectious adenoviruses in reclaimed water samples, adenovirus infectivity was evaluated based on a semi-quantitative integrated cell culture real time RT-qPCR assay. Out of the 22 reclaimed water samples examined, 19 were positive for adenoviruses by direct qPCR, with the average viral genome copies of the positive samples at 1.36×10^5 per 100mL. Out of the 19 positive samples, only 7 were positive for infectious adenoviruses, with an average mRNA-IU concentration of 6.79×10^1 per 100mL.

Rodríguez et al., 2014 and Polston et al., 2014, have previously evaluated the infectivity of adenovirus in wastewater and surface samples and in those studies approximately 50% of raw sewage and 44% of surface water samples positive for adenovirus were also infectious. This discrepancy between infectious adenovirus and genome copies detected by real time qPCR has also been examined by Rodríguez et al., 2013 and Polston et al., 2014. They found that approximately 50% of raw sewage and 44% of surface water samples respectively testing positive for adenovirus DNA were also positive for infectious adenovirus. The results presented here indicate that approximately 32% of type 2 reclaimed water samples positive for adenovirus DNA were also positive for infectious adenovirus. As the virus indicator specified by the State of NC, coliphages, were very low in the reclaimed water samples (~1 PFU/100mL), these results suggest that this indicator may not accurately reflect the concentrations of pathogenic viruses present in these reclaimed water samples after treatment. The log_{10} reduction targets as well as the daily and monthly maxima are designed as the monitoring approach for wastewater utilities to examine the quality of type 2 reclaimed water before it is used for potable reuse. However, these results indicate that the tertiary treatment plus dual disinfection scheme for type 2 like

reclaimed water may not be effective in reducing pathogenic viruses, and that the NC standards for reclaimed water (which include a coliphage reduction target) may not be adequate for monitoring the virological quality of this water.

7.1.4 NCT2RW Quality and US EPA Acceptable Risk Level for Potable Reuse

In order to assess the risks associated with potable reuse exposures, a quantitative microbial risk assessment (QMRA) model was built to evaluate multiple exposure scenarios. These scenarios included 1) accidental exposure, 2) recreational exposure, 3) reclaimed water exposure by drinking as piped water, 4) ingestion of surface water by drinking water exposure, 5) ingestion of drinking water as 80/20 blend + 5 day storage + conventional drinking water treatment, and 6) agricultural exposure through irrigation with reclaimed water. Based on this analysis, there were no potable reuse scenario for which the acceptable risk level was not exceeded by one or more classes of pathogen; however, agriculture reuse using the DALY target of 10^{-6} was met using subsurface drip irrigation.

For the analysis of drinking water exposures, the risks of adenovirus infection were the greatest for all exposure routes, this is partly due to the higher concentrations of adenovirus in reclaimed waters, surface water, and as a result in the combined waters. In the 4th scenario, in which pathogen die-off and further water treatment effect were considered, the risk of adenovirus infection still does not meet the acceptable annual risk level of 1×10^{-4} set by US EPA. However, in the analysis of bacteria and protozoan parasites in this analysis, the risks are lower than the US EPA acceptable risk level.

In the analysis of agricultural risks for three types of irrigation using North Carolina type 2 reclaimed water, it was clear that the type of irrigation (spray, drip and subsurface drip) played an important role in assessment of risk from pathogens in irrigation water, as the type of irrigation affects the amount of water remaining on the crop at harvest. For agricultural reuse, the
risks from bacterial and protozoan parasites was greater than those for viruses, but these risks were reduced below the WHO acceptable DALY risk level of 1 X 10⁻⁶ for all classes of pathogens if subsurface drip irrigation was evaluated.

Based on these analyses, it is not clear that the waterborne health risks associated with consumption of potable reuse water, are reduced below the annual risk level of 1×10^{-4} set by US EPA. The results have implications for the practical use of this type of reclaimed water, which is currently only used for landscape irrigation. However, if subsurface drip irrigation is an appropriate method of irrigation for crops that could potentially be irrigated with NCT2RW, then this water could be used for such agricultural purposes without unacceptable health risk. 7.2 Implications of Significant Findings

This research was conducted with funding from the University of North Carolina System Water Resources Research Institute (WRRI) with collaboration from 5 local wastewater treatment plants and 4 local drinking water plants in the Research Triangle area of Raleigh, NC. The participating wastewater treatment plants included, the Orange Water and Sewer Authority (OWASA), the North Durham Water Reclamation Facility, the Neuse River Resource Recovery Facility, the Utley Creek Water Reclamation Facility, and the Cary Water Reclamation Facility. The participating drinking water treatment plants included, the Hillsborough Drinking Water Treatment Plant, the Cary/Apex Drinking Water Treatment Plant, the E.M. Johnson Water Treatment Plant, and the Smithfield Drinking Water Treatment Plant. This collaborative effort between academic, research, and public water and wastewater utilities provides a unique opportunity for scientific data to inform policy and practice. This research is important to stakeholders and members of the scientific community because it provides much needed information on the quality of NC type 2 like reclaimed water as well as the risks associated with potable reuse applications. This is especially important with increasing population growth in the triangle area of NC (where this type of potable reuse expansion has been proposed), and with changes in climate and drought conditions worldwide.

Previous lab experiments based on the evaluation of NC type 2 like waters examined one system (OWASA) at a pilot scale level (Sobsey et al., 2005). In this previous study, secondary effluent and final reclaimed water samples were examined for indicator organisms as well as protozoan parasites. The research presented here is the first to examine the full scale production of NC type 2 reclaimed water by 4 water reclamation facilities practicing the full tertiary treatment and dual disinfection processes. Additionally, this work presents the first full scale quantitative evaluation of the concentrations of both fecal indicators and target pathogens in exposure waters, followed by the use of quantitative microbial risk assessment in the examination of microbial health risks of potable reuse as proposed by the state of North Carolina. This evaluation provides valuable information that can be applied to the design and use of reclaimed water systems, specifically related to performance characteristics of wastewater treatment processes and systems for NCT2RW, storage of the NCT2RW after blending with ambient surface water sources, and consideration of possible sunlight exposure during storage of the blended source water. This evaluation also provides information that informs possible future legislation on wastewater reuse, water reclamation and the management of the microbial quality of drinking and agricultural waters and consideration of the associated microbial health risks.

The use of reclaimed waters to expand the quantity of freshwater resource available for drinking has clear advantages; however, based on this analysis there are potential microbial health risks associated with the potable reuse of NC Type 2 like reclaimed water. Although tertiary treatment, dual disinfection treatment is relatively simple and cost effective when compared to membrane treatments, such as micro-, ultra- and nano-filtration or reverse osmosis

techniques, the reduction of viruses, particularly adenoviruses, is not sufficient to reduce the risks of infection to an acceptable level. For agricultural reuse a similar issue is seen with the risks of infection from bacterial and protozoan parasite pathogens, based on their survival on raw fruits and vegetables after irrigation. After treatment there are still significant numbers of pathogenic bacteria, virus, and protozoan parasites contributing to the risk associated with either potable reuse, or with agricultural exposures associated with irrigation.

The goal of this research was to address the need for real world data on the quality of NC type 2 like reclaimed water and to assess the risks from potable reuse exposures. The data presented here addresses this need for field studies, and provides additional analysis on the risks associated with a number of alternative potable reuse scenarios. As the levels of pathogenic microorganisms detected in the reclaimed water were sufficiently high as to pose a risk greater than the US EPA acceptable level, it is not clear that potable reuse in this context is an appropriate approach for communities looking to expand water resources, unless the risks can be further reduced to acceptable levels.

Another important implication of this work is this discrepancy between the detection of fecal indictor microorganisms and key target pathogens in the type 2 like reclaimed water. In many cases, indicators were not detected when high levels of pathogens were detected. This result suggests that the monitoring scheme and allowable concentrations of the fecal indicators for this type of reclaimed water may need to be reevaluated to produce higher quality reclaimed water for potable reuse applications and improve the lower limit detection of fecal indicator microorganisms, achieve and document greater log₁₀ microbial reductions and further reduction of levels of health risk.

7.3 Research Limitations

Although this research was designed to address many of the research gaps associated with type 2 like reclaimed water, there are several important limitations of both the study design and its technical details. In terms of study design there are important questions related to pathogen infectivity that are not addressed adequately by this research, specifically for the protozoan parasites. This study does not quantify the infectivity of the protozoan parasites detected, but rather total (oo)cysts are counted by US EPA method 1623 using immunomagnetic separation for recovery and purification and fluorescence microscopy for visualization and enumeration. Although this method is widely accepted, it does not determine the number of viable or infectious (oo)cysts, and therefore provides an overestimation of the number of protozoan parasites that may cause infection in a given sample. In this analysis, particularly in the QMRA, literature values were used to estimate the infectivity of oocysts and cysts in order to better determine the risk of infection from potable reuse exposures; however, this lack of infectivity data is a limitation and should be addressed in future studies.

In addition to the limited information and data on the infectivity of protozoan parasites in these waters, there was also limited data on the infectivity of adenoviruses both in raw sewage samples and surface waters. As there is limited information on these values in the literature and these are important factors influencing microbial risk, particularly the infectivity of adenovirus in surface waters, for inclusion in the risk assessment models, this limitation should be addressed in future studies.

Another limitation in the data provided in this study is the lack of pathogen survival data; the survival of pathogens is estimated by the survival of fecal indicator microorganisms in the 80/20 blend of water. Although indicators are assumed to provide an approximation of the dieoff of pathogens, the data in this study have shown that the relationship between pathogens and

indicators is variable, uncertain and therefore unclear in wastewater and surface water. Therefore, additional information on the survival of pathogens over the 5-day storage period under different conditions, including with and without sunlight exposure is important for the design of reclaimed water systems.

A limitation in the method for the detection of indicator microorganisms included the sample volume, which was only 100mL per indicator organism, which limited the range of detectable concentrations and at times resulted in the need to estimate microbial concentrations using lower detection limit values. The use of such censored lower detection limit values also causes issues in the calculation of log₁₀ reductions, which for some microorganisms, also reflect the limited upper detection limit values, with too few microorganisms present, which, when coupled with no microorganisms detected in the final reclaimed water after treatment at the volume analyzed, resulted in censored log₁₀ reduction values that were lower than could be fully quantified.

Additional limitations include the number of samples analyzed, specifically across and within treatment plants. At the beginning of this study, there were a limited number of treatment plants producing NC type 2 like reclaimed water, resulting in only 4 plants that were willing and able to provide tertiary treated, dual disinfected reclaimed water samples. The limited number of samples (22 total) across the 4 treatment plants resulted in a low number of samples within and between treatment plants over the course of the study. As with the wastewater treatment plants, there were also fewer than desirable samples of run of river or sewage impacted surface water treatment facilities, considered candidates for potable reuse, also resulting in a small number of total samples.

7.4 Recommendations

While this research has addressed some of the knowledge gaps on the quality of NC type 2 like reclaimed water, there are still many questions that remain about the risks associated with both potable and agricultural reuse of NCT2RW. As much of this analysis relies on an assumption of microbial infectivity, one of the main areas of additional research is the incorporation of infectivity analysis into the protozoan parasite detection, specifically for *C. parvum*. In this study, *Cryptosporidium* and *Giardia* were detected by US EPA method 1623, which results in a total count of (oo)cysts rather than a determination of infectivity. Future research on the number of infectious oocysts in both treated reclaimed water and in surface waters used for drinking will expand and further inform the current risk assessment on potable reuse applications. Additionally, more research should be done to expand the information available on the infectivity of adenovirus, particularly as many GECs were detected in reclaimed water and surface water samples. Future research should include integrated cell culture RT-qPCR assays for mRNA for all samples analyzed.

Another research area in need further of study is the microbiological quality of the surface water, particularly on sewage impacted, or run of river waters that may be candidates for this type of combination of NCT2RW and surface water for potable reuse. There is little to no available data on the enteric pathogen content of such surface waters and such data could be used to inform future risk assessments, as this water is used for many purposes (drinking, irritation, recreation, etc.).

This research also addressed knowledge gaps in the area of the survival of indicator organisms in the NC approved 80% surface water 20% reclaimed water blend; however, little is known about the survival of pathogens in this matrix. Future research could determine the survival of enteric pathogens over the 5 day storage period in this matrix to determine if the die-

off or survival relationship between fecal indicators and pathogens are similar, as wells as to determine the survival kinetics of pathogens in this type of water under different conditions. Additionally, the survival of relevant pathogens has also not been examined after sunlight exposure of these waters. Because this was an important exposure factor for fecal indicators, it is possible that exposure to sunlight would also have a large impact on pathogen survival as well. Quantifying and charactering the survival and persistence of both fecal indicator and pathogenic microorganisms may help to inform design and operation characteristics of reclaimed water systems, particularly if a specific characteristic (sunlight or temperature) has a significant impact on survival.

Finally, much of the data presented in this report could be further reexamined and reanalyzed for various trends in estimating microbial concentrations, log₁₀ reductions and human health risks. In particular the relationship between indicators and pathogens in raw sewage and reclaimed water could be further characterized by using additional assumptions and alternative analytical methods such as Bayesian analysis. Although these aspects were not central questions in the current study, it may be relevant and of interest for future work.

7.5 Conclusions

A key step in assessing infectious disease risk is collecting and evaluating microbial data on exposure and human health effects to inform quantitative microbial risk assessments that conservatively estimate the health hazard associated with microbial exposure. From the results of this study, it is concluded that the risks of potable reuse, based on exposures to drinking water produced from raw sewage by tertiary treatment, dual disinfection, followed by 5 day storage, and then drinking water treatment, are higher than the US EPA allowable annual 1 x 10^{-4} risk of infection. This result is largely based on high concentrations of infectious adenovirus in the treated reclaimed water and the detection of all classes of pathogens (bacteria, viruses, and

protozoan parasites) in reclaimed and surface waters. Irrigation of raw food crops with type 2 like reclaimed water (expected to be eaten raw) also poses a higher risk of infection from the levels of bacteria and protozoan parasite pathogens and also may be higher than a tolerable level of risk, depending the level of risk considered tolerable.

This research is consistent with the work of Harwood et al., 2005 in that fecal indicator microorganisms do not always, if at all, correlate with pathogens in wastewater or treated wastewater samples. This present research shows that in many cases, fecal indicator microorganisms were not detected in reclaimed waters samples of standard volume (100 mL), but that pathogens were detected in this water with detectable levels also in 100mL volumes. In the case of the coliphage viruses, very low levels of coliphages below the specified allowable level, were detected in almost all samples, but high levels of adenovirus DNA was detected by direct qPCR and infectious adenoviruses were detected as mRNA of adenovirus-infected mammalian host cells. Additionally, in the analysis of the correlation between indicators and pathogens in surface waters, very few organisms were highly positively correlated. However, log₁₀ concentrations of *Salmonella* spp. were correlated with log₁₀ concentrations total coliforms, and log₁₀ concentrations of adenovirus were correlated with log₁₀ concentrations of F+ coliphage presence.

In conclusion, at the present treatment level and disinfection efficacy, NC type 2 like reclaimed water may not be of high enough quality to be used for potable reuse applications, based on the data and data analysis of this study. Further study is needed to evaluate the infectivity of the protozoan parasites detected in the reclaimed water, as well as the surface water currently used for drinking water sources. Such studies are recommended to better assess human health risks because the risk of virus infection based on the proposed potable reuse scheme and

the concentrations of viruses detected in NCT2RW and ambient sources water is higher than the acceptable risk level of US EPA for drinking water exposure. Additional treatment steps would be necessary to further decrease the risk of virus infection from the levels of risk determined in this study. For agricultural use, the survival of bacteria and protozoan parasites on raw fruits and vegetables is of concern and additional treatment steps are necessary to either reduce the concentrations of these organisms by disinfection processes or physically remove them before distribution to consumers.

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