ABSTRACT

WAHLEN, JORY BLAKE. Microbial Risk Assessment of Graywater Reuse using Quantitative Molecular Approaches for Estimating Pathogen Concentrations. (Under the direction of Dr. Francis L. de los Reyes III).

Graywater is a potential resource that could be reused for non-potable end uses such as irrigation. The presence of pathogens in graywater could potentially present human health risks. However, very few reports have been written to quantify pathogens present in graywater. Previous risk assessments have used traditional culture based methods to quantify fecal indicators and to assess the human health risks associated with graywater reuse. Using fecal indicators to estimate the concentration of pathogens in graywater introduces high levels of uncertainty into the risk assessment. The limitations of using indicator methods are that not all organisms are targeted and the fecal indicators do not always correspond with the actual pathogens. Sensitive molecular methods have been developed to detect and quantify potential pathogens in environmental samples. Using molecular techniques to quantify pathogens in graywater samples will reduce the uncertainty in assessing the risks of reusing graywater because the models used to estimate the pathogen concentrations can be eliminated.

The purpose of this study was to determine the microbial quality of graywater and to assess the human health risks from exposure to pathogens in untreated graywater. The microbial quality of graywater was determined by using molecular techniques to quantify pathogens and identify microbial communities present in untreated graywater. The human health risks associated with reusing graywater were evaluated using a risk assessment model.

A total of 80 samples from 28 households were collected and analyzed using molecular techniques. Samples were analyzed using qPCR to quantify the concentrations of *Bacteroides, Aeromonas hydrophila, Campylobacter jejuni,* and *Legionella pneumophila.* Five out of eighty samples tested positive for *Bacteroides*, two out of eighty samples tested positive for *Aeromonas hydrophila*, one out of eighty samples tested positive for *Legionella pneumophila*, and no samples tested positive for *Campylobacter jejuni*.

A preliminary risk assessment was performed using the concentration of fecal indicators and chemical biomarkers to estimate the concentration of *C. jejuni* in untreated graywater. It was shown that using the different indicators to estimate the concentration of pathogens in graywater resulted in widely varying results.

The results from the qPCR analysis were then used to estimate the human health risks. The estimates of infection risk from this method varied from the estimates in the preliminary risk assessment. Because there were no samples that tested positive for the *C. jejuni*, the risk of infection from exposure to *C. jejuni* in the untreated graywater samples was zero for a large portion of the population. However, it was estimated that for a small portion of the population, *C. jejuni* contamination was possible, leading to unacceptably high risks for that small portion of the population.

qPCR results indicated that *L. pneumophila* contamination of graywater is unlikely. However, the concentration of the one sample that tested positive resulted in an estimate of the risk of infection that exceeded acceptable limits.

A genomic survey was also conducted to evaluate the microbial communities present in untreated graywater. This was the first metagenomic analysis of graywater using next

generation sequencing techniques. In this analysis the microbial communities were identified at the taxonomic class level. Additionally, the different sources of graywater were determined to be significantly different based on the operating taxonomic units present in the collected samples.

A storage experiment was performed to evaluate the fate of pathogens in stored graywater. The results indicated that graywater provides an environment that is suitable for microbial growth, which suggests that graywater should not be stored for more than 24 hours.

If the risks of reusing graywater are properly managed, graywater can be a valuable resource that will help reduce the demand for potable water.

Microbial Risk Assessment of Graywater Reuse using Quantitative Molecular Approaches for Estimating Pathogen Concentrations

by
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DEDICATION

This thesis is dedicated to my beautiful daughter and my amazing wife. My daughter was born while I was conducting research for this thesis requiring an enormous amount of patience and sacrifice from her and my wife. They are the two most important people in my life and they inspire me to be the man I want to be.

BIOGRAPHY

Jory Blake Wahlen was born in Vernal, Utah and raised in Layton, Utah. He is the son of George Blake and Jana Lee Wahlen. Jory is married to Heather Marie Wahlen and is the proud father of Havyn Marie Wahlen. It is Jory's desire to use his education to pursue a career that will benefit the lives of others. After graduation Jory will begin working for Hazen and Sawyer as an Assistant Engineer.

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TABLE OF CONTENTS

LIST OF TABLES ..... **vii**

LIST OF FIGURES ..... **viii**

CHAPTER 1 OVERVIEW **1**

Background **1**

Water Reuse **4**

Graywater Reuse **5**

Graywater Treatment **8**

Graywater Regulations **11**

Quantitative Microbial Risk Assessment (QMRA) **13**

Hazard Identification 14

Exposure Assessment 14

Dose Response 15

Risk Characterization 15

Quantitative Microbial Risk Assessment (QMRA) of Graywater **15**

Molecular Techniques **20**

Polymerase Chain Reaction (PCR) 20

Quantitative Polymerase Chain Reaction (qPCR) 23

Sequencing 25

Application of Molecular Techniques on Graywater 28

Project Overview **29**

CHAPTER 2: MICROBIAL RISK ASSESSMENT oF GRAYWATER REUSE USING QUANTITATIVE MOLECULAR APPROACHES **30**

Introduction **30**

Materials and Methods **34**

Collection of Samples 34

DNA Extraction 36

Quantitative Polymerase Chain Reaction (qPCR) 36

Preliminary Risk Assessment Model 40

Updated Risk Assessment 50

Results and Discussion **52**

Collection of Samples 52

DNA Extraction 53

Quantitative Polymerase Chain Reaction (qPCR) 54

Preliminary Risk Assessment Model 61

Updated Risk Assessment 69

Conclusions **76**

CHAPTER 3: MICROBIAL COMMUNITY ANAlYIS OF UNTREATED GRAYWATER **78**
Introduction **78**
Materials and Methods **80**
Collection of Samples for Genomic Survey 80
DNA Extraction 81
Metagenomic Analysis 81
Description of Graywater Collected for Storage Experiment 82
Sampling Procedures 82
Total Coliform Assay 83
Fecal Coliform Assay 84
DNA Extraction 84
Quantitative Polymerase Chain Reaction (qPCR) 85
Results and Discussion **89**
Metagenomic Analysis 89
Total Coliform Assay 94
Fecal Coliform Assay 95
DNA Extraction 97
Quantitative Polymerase Chain Reaction (qPCR) 98
Conclusions **102**
CHAPTER 4: SUMMARY AND CONCLUSIONS **104**
CHAPTER 5: FUTURE WORK **106**
REFERENCES **107**
APPENDICES **113**
Appendix A Instruction packet given to households providing graywater samples **114**
Appendix B Optimization of Sampling Procedures **119**
Appendix C Laboratory DNA Extraction Method (Aluminum Sulfate Method) **124**
Appendix D Summary of Households Providing Graywater Samples **126**
Appendix E Summary of extracted DNA **127**
Appendix F Summary of extracted DNA from graywater storage experiment **130**

LIST OF TABLES

Table 1 Fecal Indicators in Graywater and Wastewater 6
Table 2 Primers and cycling parameters for targeted organisms 37
Table 3 Genome characteristics of targeted organisms 39
Table 4 Mass of targeted organisms 40
Table 5 Fecal Indicator Characteristics 43
Table 6 Chemical Biomarker Characteristics (Ottoson and Stenstrom, 2003) 44
Table 7 *C. jejuni* Characteristics 45
Table 8 Summary of inputs for modified pathogen concentration model 46
Table 9 Exposure Scenarios 48
Table 10 Summary of inputs for modified pathogen dose model and dose response model . 49
Table 11 Summary of distributions analyzed for importance 67
Table 12 Primers and cycling parameters for targeted organisms 86
Table 13 Genome characteristics of targeted organisms 88
Table 14 Mass of targeted organisms 88
Table 15 Results of one-way ANOSIM test based on Bray-Curtis similarity matrix derived from the distribution of microbial communities 93
Table 16 Comparison of 0.22μm and 0.45μm Sterivex Filters 122

LIST OF FIGURES

Figure 1 Reservoir Capacity in the Continental United States (Graf, 1999) 1
Figure 2 Changes in U.S. water use. Population and total U.S. water use are shown on the left axis. Per capita water use is shown on the right axis. Per capita water use includes all water uses except thermoelectric power (NRC, 2012). 3
Figure 3 Reclaimed Water by State data obtained from EPA (CEPA, 2009; TWDB, 2010; EPA, 2004; FDEP, 2012) 5
Figure 4 Water use in North America (Mayer and DeOreo, 1999) 7
Figure 5 Aqua2use® Greywater Diversion Device (GWDD) (Aqua2use®, 2013). 9
Figure 6 Aqua2use® Greywater Treatment System (GWTS) 10
Figure 7 Graywater Definitions by State (Yu et al., 2012) 12
Figure 8 States with Graywater Definitions (Yu et al., 2012) 13
Figure 9 Pathogen Concentration Model as explained by Ottoson and Stenstrom (2003) 17
Figure 10 Exposure Assessment Model as explained by Ottoson and Stenstrom (2003) 18
Figure 11 PCR Diagram (Rice, 2013) 22
Figure 12 Amplification plot. Baseline-subtracted fluorescence versus number of PCR cycles (BIO-RAD, 2013) 24
Figure 13 Pyrosequencing (Metzker, 2009) 27
Figure 14 Illumina Imaging 28
Figure 15 (A) Number of Children per Household (B) Pets per Household 35
Figure 16 Pathogen Concentration Model as explained by Ottoson and Stenstrom (2003) ... 41
Figure 17 Modified Pathogen Concentration Model 42
Figure 18 Modified Pathogen Concentration Model using Chemical Biomarkers 44
Figure 19 Exposure Assessment Model 47
Figure 20 Filtered Volumes for Collected Samples 52
Figure 21 Extracted DNA from Collected Graywater Samples 53
Figure 22 Standard Curve for *A. hydrophila* (PCR efficiency=0.87%) 55
Figure 23 Standard Curve *C. jejuni* (PCR efficiency=96%) 55

Figure 24 Standard Curve *L. pneumophila* (PCR efficiency=91%) 56
Figure 25 Standard Curve *Bacteroides* (PCR efficiency=80%) 56
Figure 26 Standard Curve for Bacterial Primers using *Bacteroides* (PCR efficiency=92%) . 57
Figure 27 Samples Testing Positive for Targeted Pathogens 58
Figure 28 Mean Pathogen Concentration 59
Figure 29 Concentration of General Bacteria 61
Figure 30 Estimates of *C. jejuni* in untreated graywater 62
Figure 31 Risk of Infection from Exposure to *C. jejuni.* Exposure Scenario 1 (Accidental ingestion of graywater) 63
Figure 32 Risk of Infection from Exposure to *C. jejuni*. Exposure Scenario 2 (Indirect ingestion from touching plants and lawns) 64
Figure 33 Risk of Infection from Exposure to *C. jejuni*. Exposure Scenario 3 (Ingestion of graywater from sprays used for irrigation and washing) 65
Figure 34 Risk of Infection from Exposure to *C. jejuni*. Exposure Scenario 4 (Ingestion of crops irrigated by graywater) 66
Figure 35 Risk of Infection Importance Analysis 68
Figure 36 Dose Model Importance Analysis 69
Figure 37 Risk of infection from exposure to *C. jejuni* (Concentration represented using a Poisson distribution fitted to qPCR data) 73
Figure 38 Distribution of the Bacteria domain as determined by taxonomic identification of partial 16S rRNA gene sequencing (at class level) 90
Figure 39 Non-metric multidimensional scaling (nMDS) ordination plot representing the potential relationship between samples from three graywater sources 92
Figure 40 (A) Positive Total Coliform Result (B) Negative Total Coliform Result 94
Figure 41 Total Coliforms in stored graywater 95
Figure 42 (A) Positive Fecal Coliform Result (B) Negative Fecal Coliform Result 96
Figure 43 Fecal Coliforms in stored graywater 97
Figure 44 Mean concentration of extracted DNA 98
Figure 45 Standard Curve General Bacteria (PCR Efficiency=92%) 99

Figure 46 Concentration of bacteria in bathroom sink water stored over time 100
Figure 47 Concentration of bacteria in laundry water stored over time 100
Figure 48 Concentration of bacteria in shower/bath water stored over time 101
Figure 49 Concentration of bacteria in combined graywater stored over time..... 101
Figure 50 Thermo Scientific Nalgene® Polysulfone Filter Holder..... 119
Figure 51 Sterivex Inline Cartridge Filter..... 120
Figure 52 Mean Filtering Times for Each Filtering Method 121
Figure 53 Mean Filtered Volume for Each Filtering Method..... 121
Figure 54 PCR Results Using a Universal Bacterial Primer..... 123

CHAPTER 1 OVERVIEW

Background

Population growth, growing water demands, and increasing concerns over water quality are all critical issues that require innovative water resource management solutions. There is an obvious need to find sustainable solutions that are both reliable and equitable to meet the water needs of current and future generations (Asano et al., 2007).

During the 20th century there was a drastic increase in the construction of water collection and storage capacity in the U.S. Figure 1 illustrates the growth in reservoir capacity from 1900 to 1996. This increase in water collection and storage in U.S. was partially the result of a population increase from 76 million people in 1900 to over 280 million in 2000 (Census Bureau, 1999, U. S. Census Bureau, 2002).

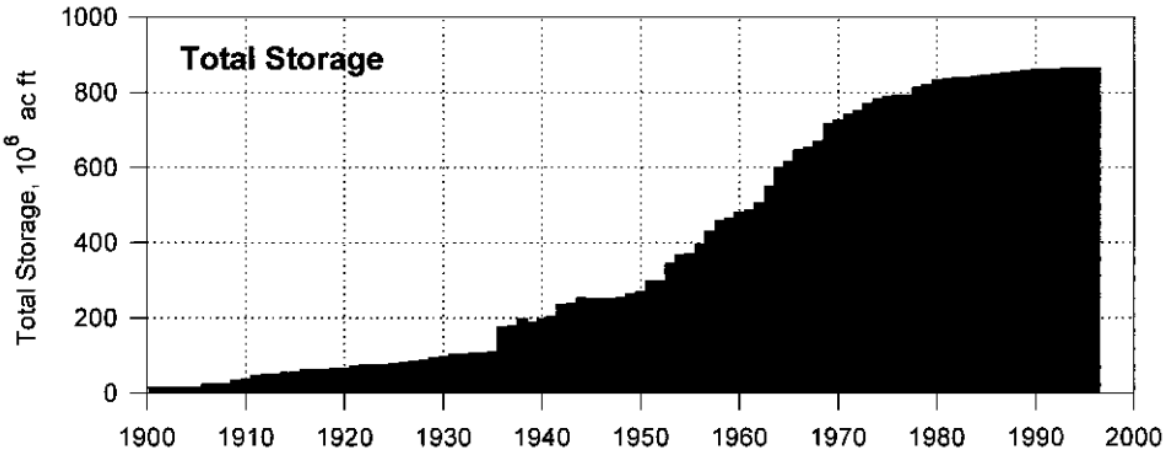


Figure 1 Reservoir Capacity in the Continental United States (Graf, 1999)

As shown in Figure 1 the rate of increase in the reservoir capacity began to decrease around 1970 (Graf, 1999). The population continued to grow but the construction of reservoirs had slowed, suggesting that the availability of water was not increasing as fast as the population's demand for water. The growing demand and decreasing rate of construction of water storage and collection facilities have led to increasing pressure to manage water resources throughout the U.S better. This pressure was and will continue to be addressed through a combination of water conservation measures and finding new sources of water (NRC, 2012).

Pressures on the U.S. water supply have caused water conservation measures to improve. Changes in the behaviors and habits throughout the nation have resulted in a lower per capita water use as shown in Figure 2.

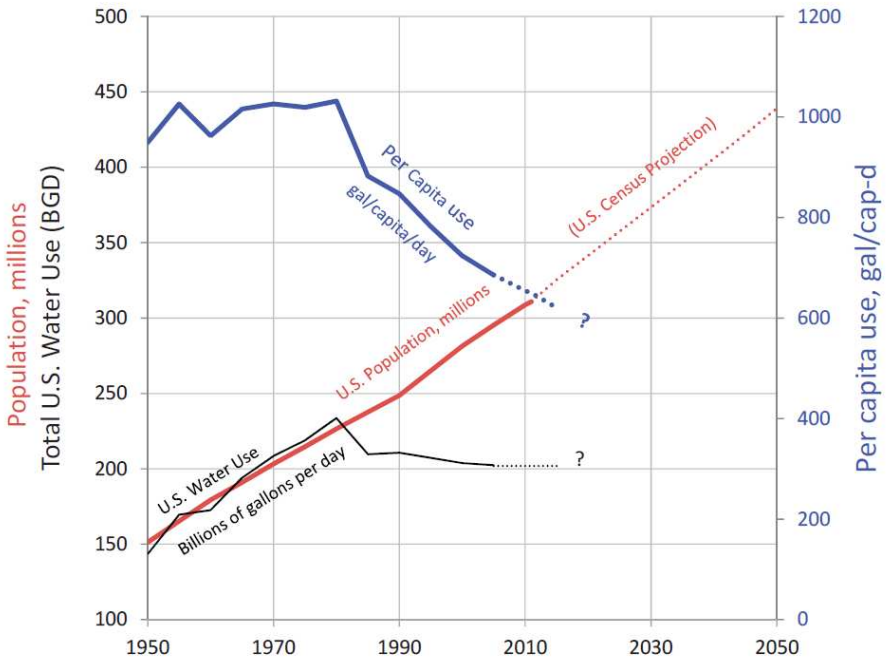


Figure 2 Changes in U.S. water use. Population and total U.S. water use are shown on the left axis. Per capita water use is shown on the right axis. Per capita water use includes all water uses except thermoelectric power (NRC, 2012).

The total water used in the U.S. corresponded with the population until about 1980 when the total water use began to decline. This decrease corresponds to the decreasing rate of reservoir capacity and indicates more efficient use of water. The per capita water use in the U.S. remained fairly steady until about 1980 when the per capita use began to decline (NRC, 2012). While the data suggests that water is being used more efficiently, there remains a need to find additional water sources.

The pressure to provide clean water is not only a challenge in the U.S., but is a concern for many countries throughout the world. Many countries have much higher population growth rates, limited technology, and lower availability of fresh water, resulting

in water scarcity issues (Asano et al., 2007). As the world population continues to grow there will be an increasing need to implement more sustainable water resource solutions.

Water Reuse

Traditionally water has been considered a resource that is discarded after it has been used once. However, it is clear that water can be reused. Many utilities are currently treating collected wastewater and then distributing the treated wastewater for non-potable uses such as irrigation, cleaning, cooling, dust control, industrial processes, scenic waters and fountains (EPA, 2004). This process is often termed water reclamation and the treated wastewater is often called reclaimed water. Water reuse can be beneficial because it can substitute for non-potable end uses and could reduce the discharge of the pollutants into wastewater receiving bodies (Asano et al., 2007).

Reclaimed water is already being used by many utilities throughout the U.S. It is estimated that over 2 billion gallons per (BGD) of wastewater is being reused in the U.S. (Jimenez and Asano, 2008). Most of the reclaimed water is being reused in the arid west and Florida. Figure 3 shows the states that reuse the most water.

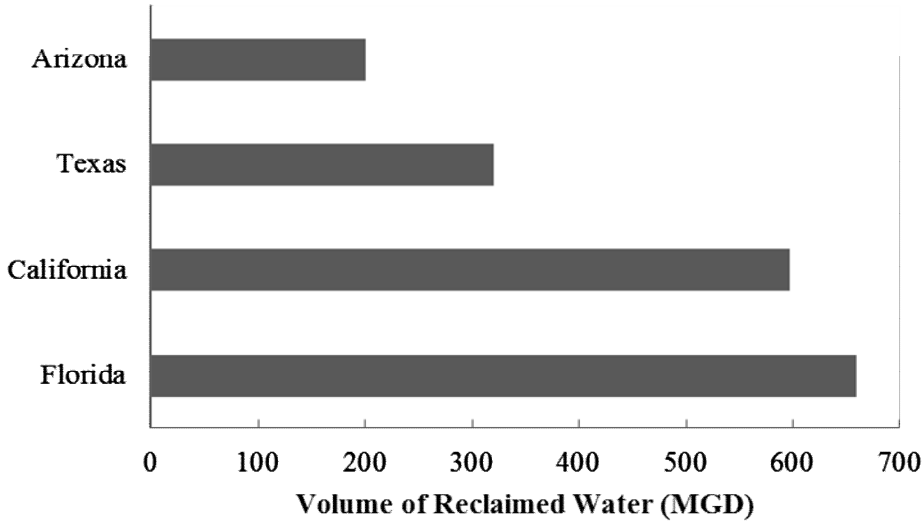


Figure 3 Reclaimed Water by State data obtained from EPA (CEPA, 2009; TWDB, 2010; EPA, 2004; FDEP, 2012)

The 2 BGD estimate represents only a small portion of the total wastewater treated in the U.S. As of 2008 there were 32 billion gallons per day of wastewater being discharged throughout the United States. Of this, 38% was being discharged into oceans and estuaries (NRC, 2012). Although it is unreasonable to assume that all of the wastewater being discharged in the oceans and estuaries could be reused, it is evident that there is abundance of wastewater that could potentially be reclaimed and reused.

Graywater Reuse

The reuse of water is not limited to centralized reclaimed water facilities. Water can be reused in residential or community settings as well. An example of decentralized water reuse is graywater reuse. Graywater is defined as wastewater from bathing and washing

facilities that does not contain concentrated human waste (i.e., flush water from toilets) or food waste (i.e., kitchen sink, food waste grinders) (Asano et al., 2007).

The absence of human and food waste implies that graywater is relatively free of pathogens and organic matter. The concentration of pathogens in graywater is typically much lower than in municipal wastewater. Fecal indicators such as total coliforms and fecal coliforms are generally lower in graywater than in wastewater (Table 1) suggesting that the pathogen load of graywater is less than combined wastewater.

Table 1 Fecal Indicators in Graywater and Wastewater

Indicator	**Concentration in raw residential Wastewater**	**Concentration in raw residential graywater**
Total Coliform	1.0×10^8 to 1.0×10^{10}	5.0×10^2 to 2.4×10^7
Fecal Coliform	1.0×10^6 to 1.0×10^8	3.2×10^1 to 7.9×10^6

(Christova-Boal et al., 1996, Otis et al., 2002, Rose et al., 1991, Winward et al., 2008)

The average indoor water use for North America is 69 gallons per capita per day (Mayer and DeOreo, 1999). Figure 4 shows that 50-75% of the indoor water used in the home contributes to graywater (Maimon et al. 2010). Based on these assumptions the average household produces between 34.5 and 51.8 gallons of graywater per day.

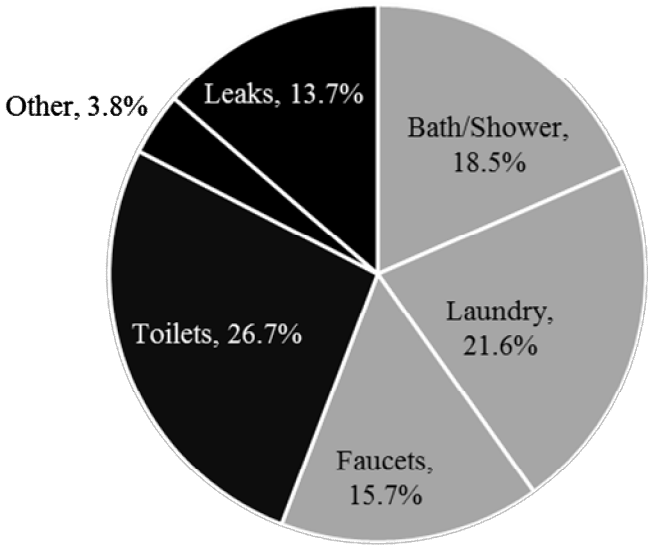


Figure 4 Water use in North America (Mayer and DeOreo, 1999)

Due to the lower pathogens load and the abundance of graywater, there is potential for implementing reuse systems that separate graywater from other wastewater sources. Separated graywater could be reused for end-uses that do not require highly treated water. These non-potable end-uses include irrigation, toilet flushing, and vehicle and home washing, among others. Reusing graywater will reduce the amount of potable water that is needed in the home and by separating graywater from wastewater, the amount of wastewater being discharged into the existing wastewater distribution system will be less, reducing the amount of wastewater that needs to be treated (Maimon et al., 2010)

According to a survey performed by the NPD Group, 7% of households in the U.S. were reusing graywater in 1999. Graywater reuse is especially popular in the arid west and

southwestern United States, with 13.9% of households in California and 11.0% of households in Texas reusing graywater. The households that are reusing graywater cited many reasons, the most popular of which are environmental considerations and water conservation (NPD, 1999).

Graywater Treatment

Graywater treatment systems range from very simple systems with limited treatment, to more complex systems that mimic traditional wastewater treatment processes (Roesner et al., 2006).

Figure 5 illustrates a simple system that is currently on the market. The Aqua2use® Greywater Diversion Device (GWDD) includes progressive density filter pads that are meant to filter out hair, lint, soap residue and other particles so graywater can be used for irrigation purposes (Aqua2use®, 2013).

Figure 5 Aqua2use® Greywater Diversion Device (GWDD) (Aqua2use®, 2013).

The Aqua2use® GWDD can also be combined with the Aqua2use® GWTS as shown in Figure 6. According to the manufacturer Aqua2use® GWDD provides pre-pretreatment before entering a chamber that is used to buffer the flow while also acting as active sludge treatment. The graywater then passes through a sedimentation basin and a biofilter before being disinfected by UV Disinfection. The effluent graywater from this system is intended for toilet flushing, laundry and irrigation (Aqua2use®, 2013).

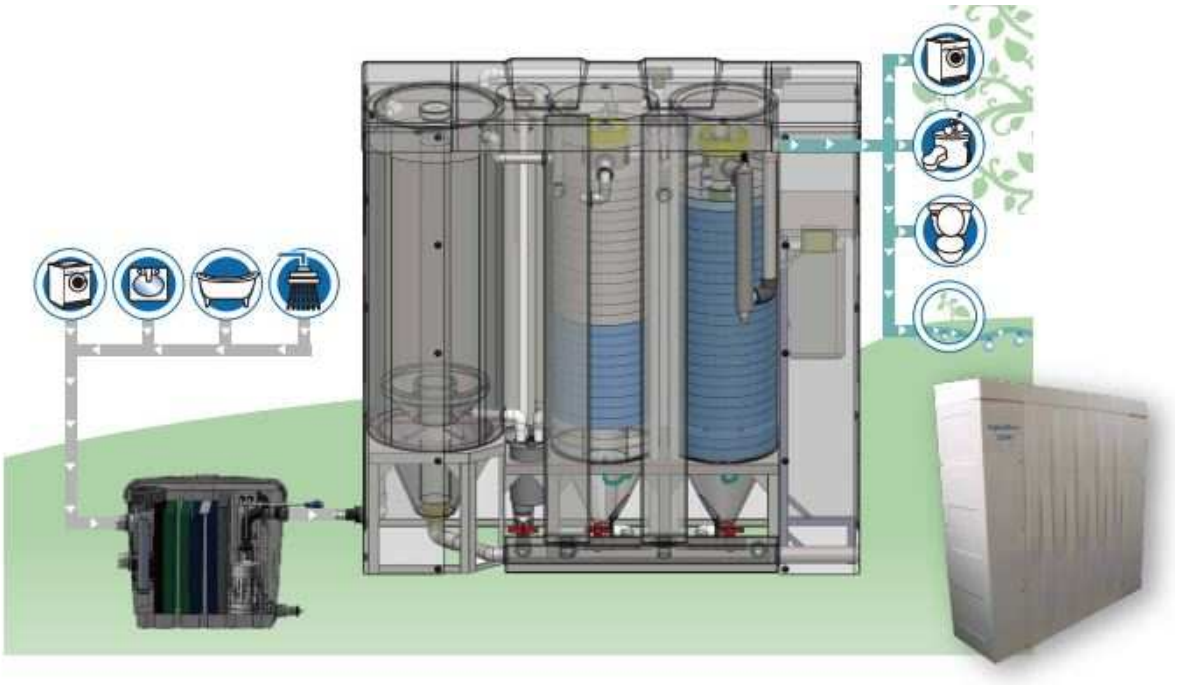


Figure 6 Aqua2use® Greywater Treatment System (GWTS)

Additional graywater systems are available. These systems include systems that utilize multiple filters (Envirolet®, 2013, FlotenderTM, 2013) and systems that include a combination of both UV and chlorine disinfection (AquacellTM, 2013). Other graywater systems that have been cited by previous studies include no treatment, sand filters, membrane filters and membrane bioreactors (Allen et al., 2010, Sharvelle et al., 2010).

The required treatment for graywater systems varies from state to state. Many states require only primary treatment of the graywater before it can be reused. Twenty states require primary treatment for subsurface irrigation, three states require primary treatment for above ground irrigation, and thirteen states require primary treatment for toilet flushing. The most commonly recommended method of primary treatment is gravity settling. A few states require more stringent treatment. Three states require secondary treatment (BOD removal) of

graywater before it can be reused. California is currently the only state that requires tertiary treatment (chlorine disinfection) of graywater before it can be reused (Yu et al., 2012). There are also states that do not specify any required treatment.

The International Plumbing Code (IPC) and the Uniform Plumbing Code (UPC), two widely accepted plumbing codes, also specify the treatment that is recommended for graywater before it can be reused. The IPC recommends that graywater should pass through a filter and a storage tank before it can be used subsurface irrigation. The IPC recommends that graywater being reused for toilet flushing should also undergo disinfection and should be colored with dye. The UPC recommends that graywater should pass through a storage tank and then be filtered before being used for subsurface irrigation (Yu et al., 2012).

Graywater Regulations

Despite the potential benefits of reusing graywater and the availability of graywater technology, many municipalities are hesitant to support the reuse of graywater at the residential level. This hesitancy is evidenced by unclear regulations regarding graywater reuse. Graywater reuse regulations and definitions vary from state to state and several states have not even acknowledged graywater reuse in their regulations. Forty one states have defined graywater in their state regulations, while nine states have no definition for graywater in their state regulations. Figure 7 shows the graywater definitions by state. Some states only define graywater in their plumbing code, some only in their other state regulations, and some states do not define graywater at all.

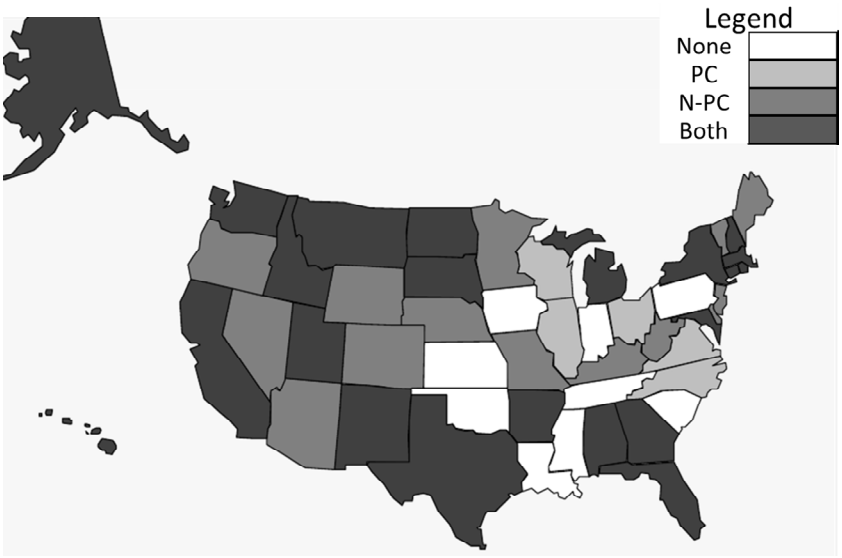


Figure 7 Graywater Definitions by State (Yu et al., 2012)

In Figure 7 the dark color identifies states that define graywater in both their plumbing code and other regulations. N-PC represents states that do not define graywater in their plumbing code but do define graywater in other regulations and PC represents states that only define graywater in their plumbing code. The actual definition of graywater also varies from state to state as shown in Figure 8.

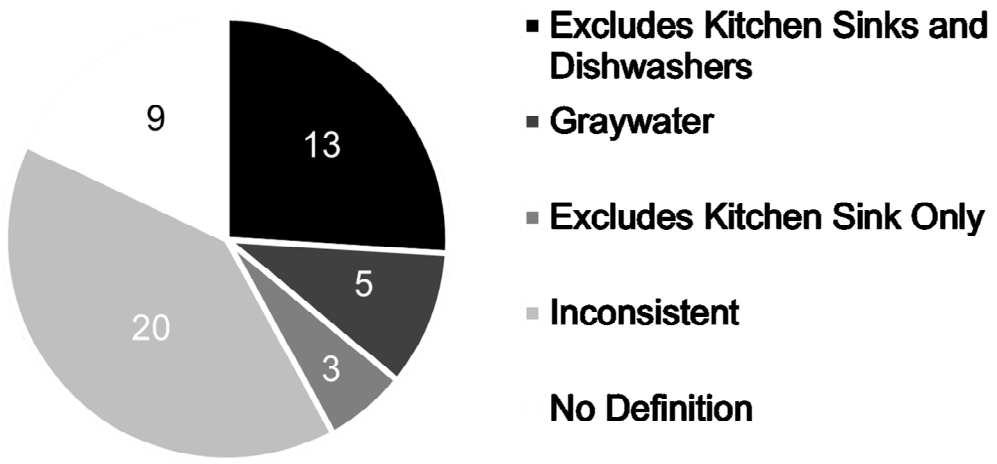


Figure 8 States with Graywater Definitions (Yu et al., 2012)

Quantitative Microbial Risk Assessment (QMRA)

One of the reasons that some regulators and municipalities have been reluctant to support graywater reuse is the unclear risks to human health from exposure to graywater. The municipal reuse industry has worked hard to change the public perception of reusing reclaimed water and they are hesitant to support decentralized graywater systems. Their reluctance is likely because they have less control over the treatment and maintenance of a decentralized graywater systems and they fear that a negative outcome from a negligent homeowner may alter the public perception of reusing reclaimed water. Currently there are no documented cases of public health impacts from exposure to graywater. That does not mean that reusing graywater is void of human health risks, just that there have been no reported incidents. There is inadequate information on human health risks from exposure to untreated graywater which suggests a need to further assess the risks of reusing graywater (Sheikh, 2010). The presence of pathogenic organisms in graywater is a major concern

because low dosages can lead to infection, and many pathogens have a high survival rate in environmental systems (Maimon et al, 2010). Thus, an assessment of the human health risks should include an evaluation of the microbial risks associated with graywater reuse.

Quantitative microbial risk assessment (QMRA) uses principles of risk assessment to estimate the consequences from exposure to infectious organisms and can provide a valuable framework for evaluating the human health risks associated with graywater reuse (Haas et al., 1999). QMRA generally includes four components, that are also common among other risk assessment paradigms, including 1) Hazard Identification, 2) Exposure Assessment, 3) Dose Response, and 4) Risk Characterization (Medema and Ashbolt, 2006).

Hazard Identification

Hazard identification refers to the identification of hazards and hazardous events affecting humans. In a microbial risk assessment this includes the identification of potentially pathogenic organisms and identifying exposure scenarios that could expose humans to the infectious organisms (Medema and Ashbolt, 2006). The identification of hazards and hazardous events requires a multidisciplinary approach involving the fields of medicine, public health, and epidemiology to identify potential sources of pathogenic organisms and scenarios that could lead to human exposure to infectious organisms (Haas et al., 1999).

Exposure Assessment

An exposure assessment describes the exposure pathway of pathogenic organisms to people and also attempts to determine the amount, or number of pathogenic organisms that an individual will be exposed to (CAMRA, 2012). An exposure assessment is performed to

determine the dose of pathogenic organisms that reaches an individual during a single exposure scenario. The dose is often evaluated by determining the concentration of the pathogenic organisms and the consumption per exposure (Haas et al., 1999).

Dose Response

The dose response describes the relationship between the pathogen dose and the probability of human infectivity (Maimon et al., 2010). Mathematical models are often generated based on human and animal exposures to pathogenic organisms to estimate the likelihood of infection for a given pathogen dose (CAMRA, 2012). The evaluation of the human response to pathogenic organism can be difficult because few incidents of infection are documented and clinical experiments (Haas et al., 1999).

Risk Characterization

The information from the hazard identification, exposure assessment, and dose response are used to characterize the risks. This process provides an estimate of the risk in terms of infection, illness, or death (CAMRA, 2012; Medema and Ashbolt, 2006).

The QMRA framework has been utilized for various applications including national security, environmental systems, and food safety (Ahmed et al., 2010, Casman and Fischhoff, 2008, Haas et al., 1999).

Quantitative Microbial Risk Assessment (QMRA) of Graywater

There have been a few studies that have utilized the QMRA framework to report the human health risks associated with graywater reuse. These reports attempted to utilize fecal indicators and traditional culturing methods to estimate the human health risks associated

with graywater reuse. The indicators used were thermal tolerant coliforms, *Enterococci* and *Escherichia coli,* to quantify pathogens such as *Salmonella* and rotaviruses (Godfrey et al., 2009; Maimon et al., 2010; Ottoson and Stenstrom, 2003). These reports defined exposure scenarios such as accidental ingestion, indirect ingestion from hand-to-mouth contact of plants and lawns irrigated with graywater, ingestion of sprays from graywater irrigation systems and ingestion of soil contaminated with graywater. Using the pathogen concentrations and exposure scenarios, the number of pathogens, or pathogen dose that an individual ingests from reusing graywater was estimated. After the dose was determined the risk of infection was calculated using exponential or beta-Poisson distributions. The reported risks were presented as risks of infection, or disability adjusted life years (DALY).

Ottoson and Stenstrom (2003) evaluated the human health risks of reusing graywater using a model to first estimate the pathogen concentration in treated and untreated graywater. The pathogen dose that an individual would consume and the risk of infection was then estimated based on the estimated pathogen concentration. The model consists of three distinct parts; 1) the pathogen concentration model as shown in Figure 9, 2) the pathogen dose model as shown in Figure 10, and 3) the dose response models.

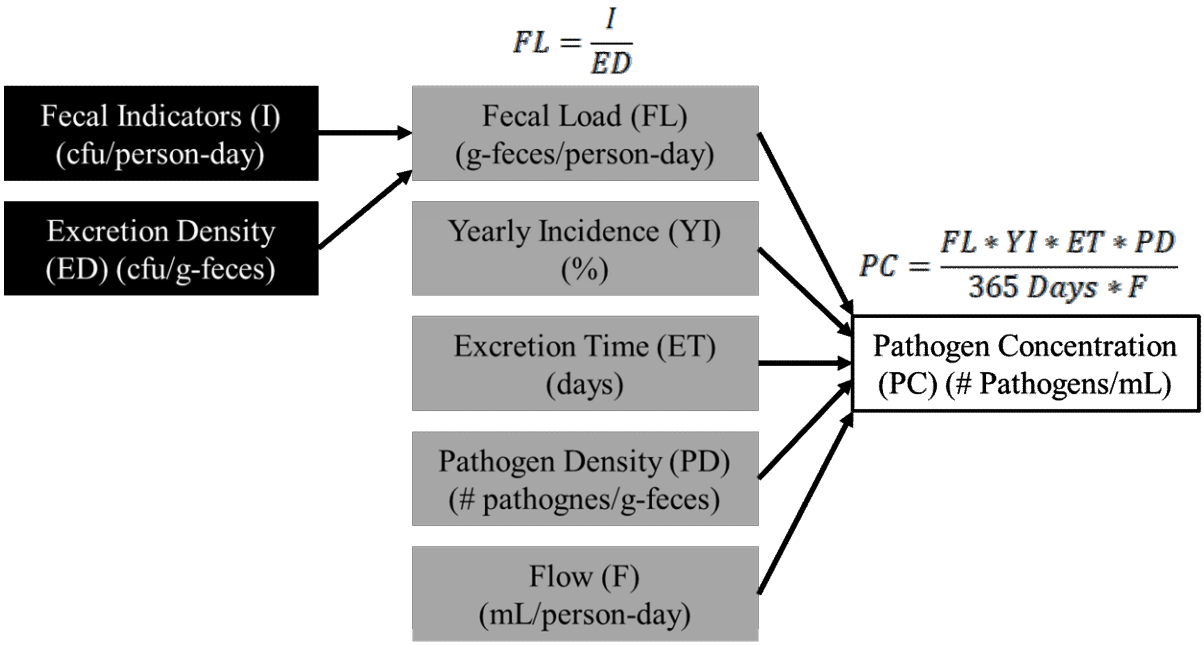


Figure 9 Pathogen Concentration Model as explained by Ottoson and Stenstrom (2003)

Figure 9 illustrates the different parameters that were used to estimate the pathogen concentration. The fecal indicators represented the indicators excreted into the collected graywater. The excretion density represented the typical number of fecal indicators in a gram of feces. The yearly incidence is the estimated number of times that an individual is likely to be infected by the specified pathogen each year. The excretion time represents the length of time an infected individual would excrete the pathogen. The pathogen density represents the number of pathogens present in the feces of an infected individual.

The pathogen concentration model was used to estimate the concentration of various pathogens including *Camplyobacter jejuni*, *Salmonella*, *Cryptosporidium*, *Giardia lamblia*, *and* rotavirus. In addition to fecal indicators, chemical biomarkers where used to estimate the concentration of the specified pathogens, following a similar model as the one shown in

Figure 9. The only differences were that the fecal indicators were replaced with the concentration of chemical biomarkers (mg/person-day) including Coprostanol and Cholesterol, and the excretion density represented the typical mass of the biomarker in feces.

The pathogen concentration was then used to estimate the pathogen dose an individual would consume during various graywater reuse scenarios as shown in Figure 10.

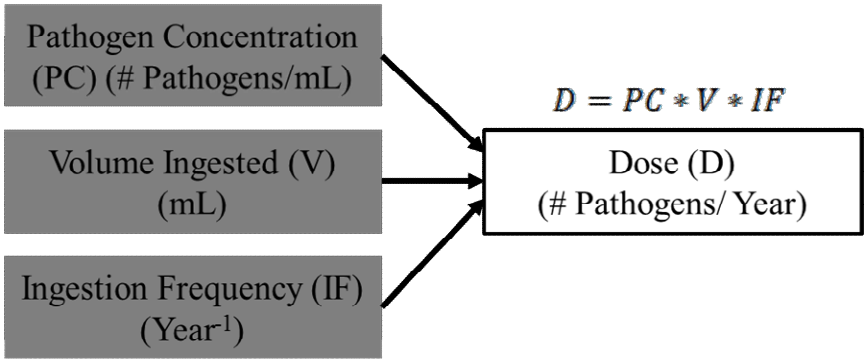


Figure 10 Exposure Assessment Model as explained by Ottoson and Stenstrom (2003)

After the dose was determined, the risk of infection was calculated using exponential or beta-Poisson distributions depending on the pathogen that was being evaluated.

Using this model Ottoson and Stenstrom (2003) determined the greatest risk of reusing graywater was due to infection by rotavirus, which represented an unacceptably high risk of infection between 0.04 and 0.60 for the defined scenarios.

Maimon et al. (2010) used a similar model correlating the concentration of fecal indicators to the concentration of pathogens in graywater. They concluded that according to the maximum tolerable risk for water borne disease set by the World Health Organization (WHO) (WHO, 2006), a safe pathogen dose would be 1.4×10^{-4} rotaviruses/mL which

according to Maimon would suggest a maximum tolerable concentration of *E. coli* between 10^2 to 10^4 per 100 mL .

Godfrey et al. (2009) evaluated the risks of reusing graywater for seven different schools in Madhya Pradesh, India that had graywater reuse systems in place at the time of the study. In this assessment the concentration of indicator organisms, thermotolerant coliforms (TTC), streptococci, and coliphages were used to estimate the risk of infection. They determined that for two of the seven schools evaluated, the risks of reusing graywater exceeded acceptable levels as outlined by WHO (2006).

There remain limitations to these estimations of the human health risks from exposure to untreated graywater. In particular, estimating the pathogen concentration using these models introduces uncertainty into the risk assessment model.

As shown in the risk assessment performed by Ottoson and Stenstrom (2003) there are many parameters that are used to estimate the risks of reusing graywater. Each of these parameters contain varying degrees of uncertainty. One of the major sources of uncertainty in these risk assessments is the models that are used to estimate pathogen concentrations. Using indicator organisms to estimate the concentration of pathogens has severe limitations because many pathogens may not correlate with indicator organisms (Harwood et al., 2005).

Sensitive molecular methods have been developed to detect and quantify potential pathogens in environmental samples (Ahmed et al., 2008). Using molecular techniques to quantify pathogens in graywater samples will reduce the uncertainty in assessing the risks of reusing graywater because the models used to estimate the pathogen concentrations can be

eliminated. Pathogen concentrations as determined by molecular techniques can be directly inputted into the pathogen dose models.

Molecular Techniques

The field of microbiology has benefitted greatly from the advancements in molecular microbiology. Molecular techniques analyze microorganisms at the molecular level, allowing organisms and cells to be identified and categorized based on their genetic characteristics. The improved ability to analyze organisms at the molecular level has led to advances in medicine, agriculture, biotechnology and microbial ecology. Sensitive techniques analyzing nucleic acid (DNA and RNA) are now widely used for pathogen detection. Many of the molecular methods are based on DNA (or RNA) synthesis or artificial replication of DNA (Madigan et al., 2012). Some of the more common methods to analyze DNA include polymerase chain reaction, quantitative polymerase chain reaction and DNA sequencing.

Polymerase Chain Reaction (PCR)

PCR is a sensitive and specific method use to amplify DNA segments. The amplified DNA can then be further analyzed using many different techniques. In this process DNA can be amplified exponentially to produce a large amount of DNA in a short time. PCR requires only a few molecules of DNA and can lead to the microbial analysis of many different environmental samples even when concentrations of the organisms in the sample are very low (Madigan et al., 2012).

The PCR amplification can be broken down into the four major steps as shown in Figure 13. The first step (1) is when the DNA template is denatured by heating the reaction to

approximately 95°C. Denaturation occurs when the hydrogen bonds between the two DNA strands melt causing the DNA strands to separate. The second step (2) is when primers that are specific to the targeted gene anneal to the DNA template. Primers are short stands of nucleotides that have a specific sequence that is unique to the gene or organisms being targeted. The annealing of the primer to the DNA template occurs at around 60°C depending on the primers and the gene being targeted. The third step (3) is the elongation of the primer, where the primer is extended by a polymerase enzyme. During elongation, nucleotides that have been added to the reaction are extended by the primer using the original DNA as a template. The fourth step (4) marks the completion of the first cycle resulting in two strands of DNA which are then used as the template for the next cycle (Madigan et al., 2012). This process is shown in Figure 11.

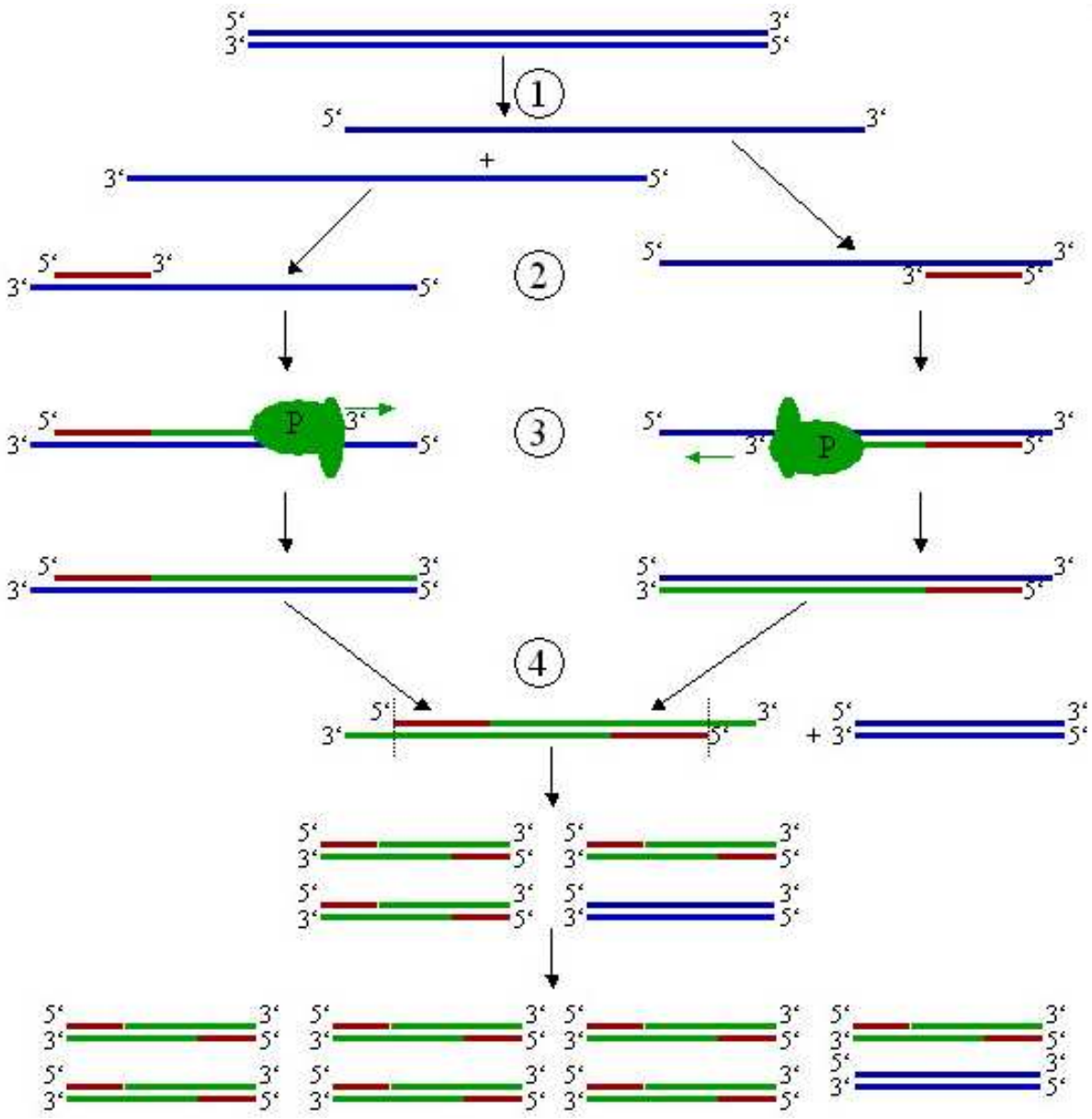


Figure 11 PCR Diagram (Rice, 2013)

The process as shown in Figure 11 is repeated twenty to forty times resulting in exponential amplification of the DNA. PCR can often result in a 10^6 to 10^9-fold increase in the targeted sequence. The amplified DNA can then be analyzed using various molecular methods (Madigan et al., 2012, Rice, 2013). During PCR, specific primers can be used to

target DNA strands that are unique to specific pathogens. PCR is a useful tool for analyzing environmental samples because samples can be collected and genes that are specific to a certain pathogen can be amplified for further analysis.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR, often referred to as Real Time PCR, utilizes the PCR amplification steps to quantify targeted DNA sequences. During qPCR a fluorescent dye that binds to double stranded DNA is added to the reaction. As the DNA is being amplified the fluorescent dye binds to the double stranded DNA resulting in the increase in intensity of the fluorescent dye. Figure 12 illustrates a typical curve that measures the fluorescent intensity for each cycle. As the number of cycles increases the fluorescence also increases. At first the rate of increase in fluorescent intensity is slow and then the fluorescence begins to increase exponentially and eventually reaches a horizontal asymptote. The point at which the fluorescence begins to increase exponentially is called the threshold level and the corresponding cycle is often labeled as C_t value or C_q value. The C_t value is recorded because it represents the relative abundance of DNA in the initial reaction. A reaction that had a large amount of the targeted DNA would reach the threshold much quicker than a reaction with very little amounts of the targeted DNA. Therefore reactions with a low C_t value contain more of the targeted DNA than reactions with high C_t values.

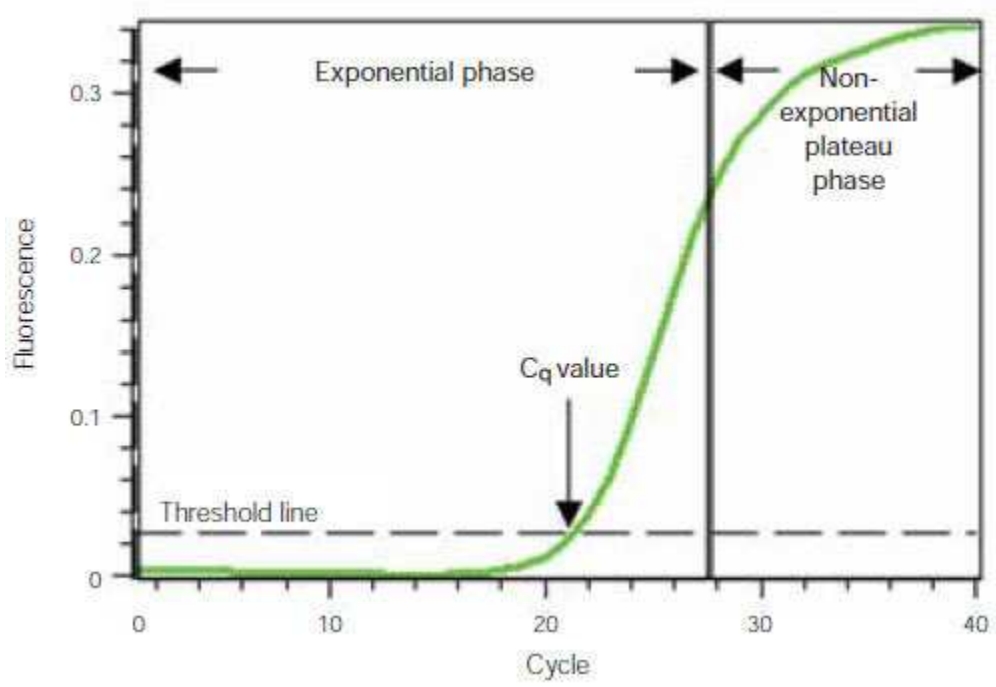


Figure 12 Amplification plot. Baseline-subtracted fluorescence versus number of PCR cycles (BIO-RAD, 2013)

qPCR can be used to quantify an organism in an environmental sample. qPCR determines the relative abundance of DNA in each PCR reaction. Therefore, a standard curve using samples with a known concentration DNA from the targeted organisms must be created to determine the absolute concentration. The standard curve is created by performing qPCR on serial dilutions of the known concentrations of the DNA of the targeted organism. The reactions with the higher concentrations will cross the threshold quicker resulting in a lower C_t value. The known concentrations of the DNA of the targeted pathogens are then plotted against the Ct value to determine the absolute concentration of the DNA of the targeted pathogens in the environmental sample.

qPCR is advantageous because the amplification is monitored in real time which means that PCR product does not need to be handled or visualized after it has been amplified. Additionally qPCR allows for organisms that are difficult to culture or present in low concentrations to be quantified relatively quickly (Madigan et al., 2012).

Sequencing

Sequencing refers to the process of determining the sequence or order of the nucleotides of a gene or genome. The genome includes all the genetic information of an organism. Determining the genome sequence not only identifies the specific organism but can also provide useful information regarding the functions of the organism and the evolutionary history. For environmental samples, understanding the DNA sequences that are present can provide insight into the microbial communities that are present in the sample (Madigan et al., 2012).

Conventional DNA sequencing has been around since the 1970's and has been a powerful tool for analyzing biological systems. DNA sequencing has been used to sequence entire bacterial, archeal, and eukaryotic genomes. The demand for large scale sequencing projects has led to a push by many research groups to improve sequencing methods and to find alternative methods for sequencing DNA (Madigan et al., 2012, Ronaghi, 2001). This has led to the development of many new DNA sequencing methods that are often referred to as next generation sequencing technologies.

Although there are advantages to each of the next generation sequencing methods, there is no consensus that one method provides a universally better result. Two of the more

commonly used next generation sequencing technologies are Pyrosequencing and Illumina (Metzker, 2009).

Pyrosequencing involves the synthesis of DNA. During DNA synthesis pyrophosphate is released from the nucleotide being synthesized. The pyrophosphate that is released provides energy to the enzyme luciferase that is added to the reaction. The enzyme luciferase obtains the energy in the form of pyrophosphate and then emits light. When multiple nucleotides are synthesized more pyrophosphate is released resulting in an increase in the intensity of the light that is being emitted by the enzyme luciferase. The sequence of the DNA is determined by testing each of the four nucleotides at each site. The nucleotide that yields a light pulse determines the nucleotide that was inserted. A higher intensity indicates that more than duplicates of the base were inserted. The synthesis of each base is repeated for the entire strand of DNA and a flowgram is created showing the light that is being emitted and the associated base that resulted in the pulse of light. The flowgram provides the necessary information to determine the sequence of the DNA as shown in Figure 13 (Madigan et al., 2012, Metzker, 2009).

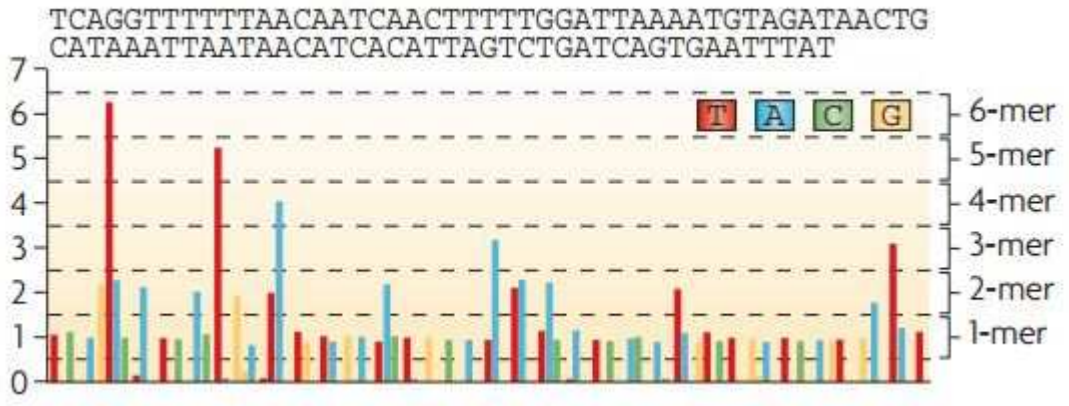


Figure 13 Pyrosequencing (Metzker, 2009)

Pyrosequencing is fast, but it can only sequence up to about 300 nucleotides, which is shorter than conventional sequencing (Madigan et al., 2012). For environmental systems pyrosequencing can be used to identify the different microbial communities present in the system. By determining short sequences of DNA (20-100 nucleotides) in the environmental samples, different organisms can be taxonomically grouped and microbial communities can be identified (Metzker, 2009, Ronaghi, 2001).

Illumina is another next generation sequencing method and is currently the method that is most commonly used. The genome analyzer used by Illumina utilizes a sequencing method known as cyclic reversible termination (CRT). CRT involves DNA synthesis where a single fluorescently modified nucleotide and a reversible terminator are synthesized for each base site. The reversible terminator momentarily terminates the synthesis allowing the fluorescent dye to be released. The termination is then reversed allowing for additionally synthesis to occur. Each of the four modified nucleotides includes a different color of fluorescent dye, thus when the dye is released the specific nucleotide can be identified. The process is repeated for each site on the DNA strand. Figure 14 illustrates the image highlights

that are the result of the different nucleotides releasing different colors during DNA synthesis (Metzker, 2009).

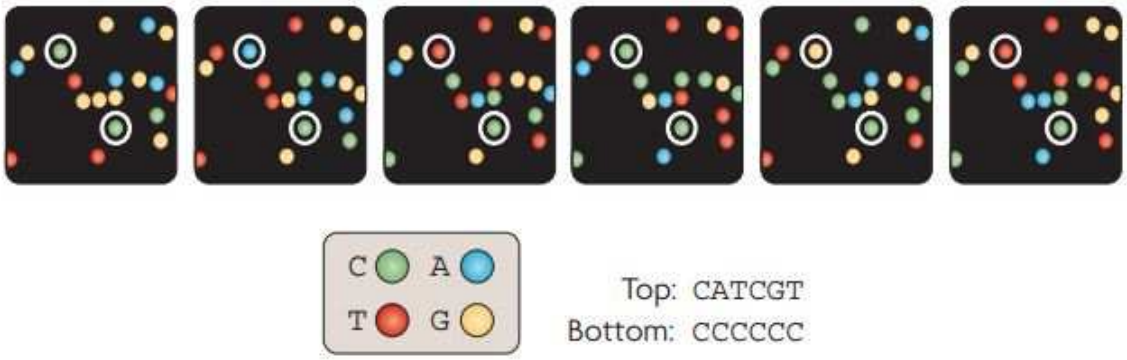


Figure 14 Illumina Imaging

Figure 14 shows six frames, and each frame represents one nucleotide being incorporated into the DNA strand and each circle represents one strand of DNA. Thus six base pairs of the sequence of each of the strands of DNA are identified (Metzker, 2009).

Illumina is able to sequence a DNA strand of about 75 to 100 bases, which is shorter than the strands that can be sequenced by pyrosequencing. Using Illumina, organisms in an environmental sample can be taxonomically grouped and microbial communities can be identified (Metzker, 2009). However, because Illumina can only sequence up to about 100 base pairs, sequencing results from Illumina will be less specific that sequencing results from pyrosequencing.

Application of Molecular Techniques on Graywater

Despite the advances in molecular microbiology, there is still very limited information on the microbial characteristics of graywater. The few reports that have sought to

quantify pathogens in graywater have used traditional culture based methods and indicator organisms to estimate the concentration of pathogens (Christova-Boal et al., 1996, Otis et al., 2002, Rose et al., 1991, Winward et al., 2008). Indicator organisms are assumed to correlate with the presence of pathogens. The limitations of using these methods are that not all organisms are targeted and indicator organisms do not always correspond with the actual pathogens (Harwood et al., 2005). No quantitative molecular microbial assays have been performed for graywater.

Project Overview

The purpose of this study was to determine the microbial quality of graywater and to assess the human health risks from exposure to pathogens in untreated graywater. The microbial quality of graywater was determined by using molecular techniques to quantify pathogens and identify microbial communities present in untreated graywater. The human health risks associated with reusing untreated graywater were assessed using a risk assessment model. This model included a preliminary assessment in which fecal indicators were used to estimate the pathogen concentration, and an updated risk assessment that used molecular approaches to determine pathogen concentrations in untreated graywater.

CHAPTER 2: MICROBIAL RISK ASSESSMENT OF GRAYWATER REUSE USING QUANTITATIVE MOLECULAR APPROACHES

Introduction

Population growth, growing water demands, and increasing concerns over water quality are all critical issues that require innovative water resource management solutions. The pressure on water resources requires sustainable solutions that are both reliable and equitable to meet the water needs of current and future generations (Asano et al., 2007). This pressure must be addressed through a combination of water conservation measures and finding new sources of water (NRC, 2012).

One potential source that has should be considered is water that has already been used. Traditionally, water has been considered a resource that should be discarded after a single use but it is clear that water can be reused. Many utilities are currently treating collected wastewater and then distributing the treated wastewater for non-potable uses such as irrigation, cleaning, cooling, dust control, industrial processes, scenic waters and fountains (EPA, 2004). This process is often termed water reclamation and the treated wastewater is often called reclaimed water. Water reuse can be beneficial because it can be substituted for non-potable end uses resulting in the conservation of freshwater supplies (Asano et al., 2007).

The reuse of water is not limited to centralized reclaimed water facilities. Water can be reused in residential or community settings as well. One example of decentralized water reuse is the reuse of graywater. Graywater is defined as wastewater from bathing and

washing facilities that does not contain concentrated human waste (i.e., flush water from toilets) or food waste (i.e., from kitchen sinks, or food waste grinders) (Asano et al., 2007).

Due to the lower pathogen load and the abundance of graywater, there is potential for implementing reuse systems that separate graywater from other wastewater sources. Separated graywater could be reused for end-uses that do not require highly treated water, such as irrigation, toilet flushing, and vehicle and home washing, among others. Reusing graywater will reduce the amount of potable water that is needed in the home and also separates graywater from wastewater, reducing the amount of wastewater that needs to be treated (Maimon et al. 2010).

According to a survey performed by the NPD Group, 7% of households in the U.S. were reusing graywater in 1999. Graywater reuse is especially popular in the arid west and southwestern United States. In California and Texas 13.9% and 11.0% of households in reuse graywater respectively (NPD, 1999).

Despite the potential benefits, municipalities are hesitant to support the reuse of graywater at the residential level. One of the reasons is the lack of understanding of the human health risks from exposure to graywater. The municipal reuse industry has worked hard to change the public perception of reusing reclaimed water and they are hesitant to support decentralized graywater systems. This is likely because they have less control over the treatment and maintenance of decentralized graywater systems and they fear that a negative outcome from a negligent homeowner may alter the public perception of reclaimed water. Despite these concerns, there is very little information regarding the public health risks associated with graywater reuse (Sheikh, 2010).

A few studies have sought to utilize fecal indicators and traditional culture methods to estimate the human health risks associated with graywater reuse. These reports have relied on indicators such as thermotolerant coliforms, *Enterococci*, and *Escherichia coli* to estimate the concentration of pathogens such as *Salmonella* and rotaviruses in graywater (Godfrey et al., 2009, Maimon et al., 2010, Ottoson and Stenstrom, 2003). These reports defined exposure scenarios such as accidental ingestion, indirect ingestion from hand-to-mouth contact of plants and lawns irrigated with graywater, ingestion of sprays from graywater irrigation systems, and ingestion of soil contaminated with graywater. Using the pathogen concentrations and defined exposure scenarios, the number of pathogens, or pathogen dose that an individual ingests from reusing graywater, was estimated. After the dose was determined, the risk of infection was calculated using exponential or beta-Poisson distributions. These studies report risk and provide a framework for an initial quantitative microbial risk assessment (QMRA) of graywater reuse. However, there are limitations to these estimations of the human health risks from exposure to untreated graywater. The main limitation is the introduction of uncertainty into the risk assessment model.

The risk assessments performed by Godfrey et al. (2009), Maimon et al. (2010), and Ottoson and Stenstrom (2003), include many parameters that are used to estimate the risks of reusing graywater. Each of these parameters contains varying degrees of uncertainty. A major source of uncertainty in these risk assessments is the models that are used to estimate pathogen concentrations. Using indicator organisms to estimate the concentration of pathogens has severe limitations because many pathogens may not correlate with indicator organisms (Harwood et al., 2005).

Sensitive molecular methods have been developed to detect and quantify potential pathogens in environmental samples (Ahmed et al., 2008). Using molecular techniques to quantify pathogens in graywater samples will reduce the uncertainty in assessing risks because the models used to estimate the pathogen concentrations can be eliminated and pathogen concentrations as determined by molecular techniques can be directly used in the pathogen dose models.

The purpose of this study was to quantify some of the pathogens in graywater and to assess the human health risks from exposure to pathogens in untreated graywater. Graywater samples from 28 households were collected, and quantitative polymerase chain reaction (qPCR) was used to quantify pathogens in the untreated graywater. The concentrations of pathogens were then used in a risk assessment model to estimate the human health risks associated with graywater reuse. The results of two approaches were compared. An assessment in which fecal indicators were used to estimate the pathogen concentrations in untreated graywater was compared with an updated risk assessment that used qPCR to quantify pathogen concentrations in untreated graywater.

Materials and Methods

Collection of Samples

Samples were collected from 28 households near Raleigh, North Carolina. When possible each household provided at least 500 mL of graywater from three sources: (1) Shower/Bath; (2) Bathroom Sink; and (3) Laundry. These samples were collected using 250mL and 500 mL sterilized bottles.

From the 28 households, 29 shower/bath samples, 28 bathroom sink samples, and 23 laundry samples were collected. An attempt was made to collect samples from a variety of households with different sizes, numbers of children and numbers of pets. Several factors including household size, pets, and information about the samples were determined using a brief survey. Each household was provided detailed instructions and the previously mentioned survey as shown in Appendix A.

The diversity of the selected households with respect to the number of pets and the number of children is shown in Figure 15.

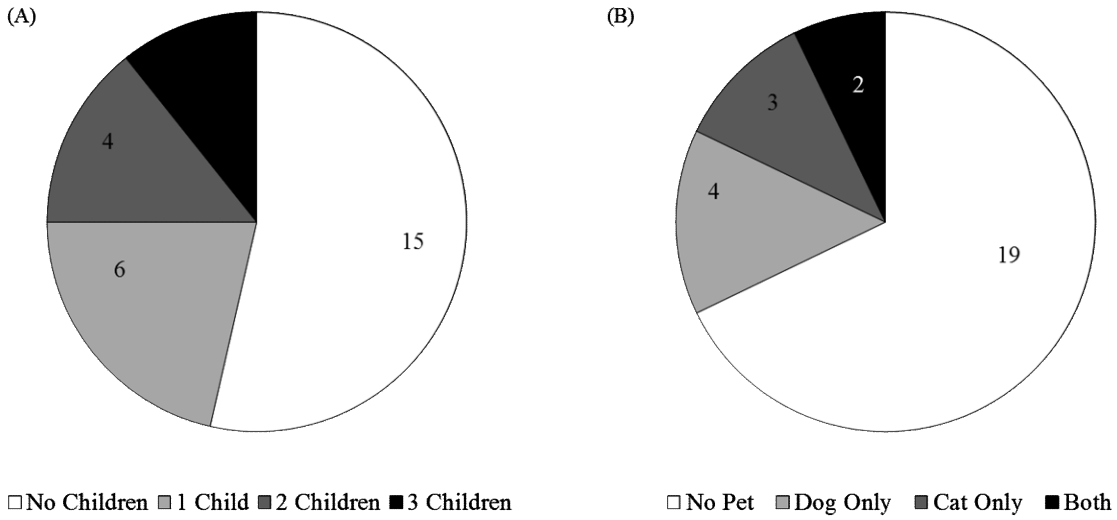


Figure 15 (A) Number of Children per Household (B) Pets per Household

Over half of the households that provided graywater samples did not have children. The average size of the households was 2.7 individuals per household, and most of the households did not have pets. However, there were nine households that had pets (dogs and cats). A summary of the households that provided graywater samples is shown in Appendix D.

After samples were collected, they were transported on ice to the NCSU Environmental Engineering Laboratory and filtered within 24 hours of collection. All samples were filtered using Millipore (Billerica, MA) 0.45µm Sterivex filter and peristaltic pump. Filtered volumes and filter times were recorded. After filtration, Sterivex filters were stored in the -20°C freezer.

DNA Extraction

Filter paper was then removed from Sterivex filters and the total DNA was extracted from filter paper using the Aluminum Sulfate method developed in the NCSU Environmental Engineering Laboratory (Hicks et. al., in preparation) as explained in Appendix C. The concentration of the extracted DNA was determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). A_{260}/A_{280} and A_{260}/A_{230} ratios were also measured using the Nano Drop Spectrophotometer ND-1000 to determine the purity of the extracted DNA. Values of A_{260}/A_{280} and A_{260}/A_{230} close to 2.00 are desirable because it indicates that DNA is free from carbohydrate and protein contamination. A summary of the extracted DNA is shown in Appendix E.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed to detect and quantify targeted pathogens in graywater samples. Three waterborne pathogens were targeted: *Legionella pneumophila, Campylobacter jejuni,* and *Aeromonas hydrophila.* Additionally *Bacteroides* was targeted as a fecal indicator. For the samples that tested positive for any of the four targeted organisms, a general bacterial primer was also used to quantify the bacteria in the samples.

Using qPCR methods requires that standard curves are created by plotting C_t values versus known concentrations of DNA. DNA from *A. hydrophila* ATCC 7966D-5, *C. jejuni* ATCC 700819D-5, *L. pneumophila* ATCC 33152D-5, and *Bacteroides vulgatas ATCC 8482D-5* were purchased form the American Type Culture Collection (ATCC). Additionally a standard curve was developed for the general bacterial primers using Bacteroides *vulgatas ATCC 8482D-5.*

Tenfold dilution series ranging from 1 ng/µL to 1 x 10^{-9} ng/µL for *A. hydrophila,* 5 ng/µL to 5 x 10^{-9} ng/µL for *C. jejuni*, 50 ng/µL to 5 x 10^{-6} ng/µL, and 50 ng/µL to 5 x 10^{-4} for *Bacteroides* were prepared. qPCR was then performed on triplicates of the dilution series using the Bio-Rad iQ5 icycler Multicolor Real-Time PCR Detection System.

The total volume of the each qPCR reaction was 25 µL. This includes 7 µL sterile water, 0.25 µL of both the forward and reverse primers (25 µM), 12.5 µL SYBR Green mix (Bio-Rad iQ SYBR Green Supermix), and 5 µL of the DNA template. Primers and cycling parameters for each of the targeted organisms are shown in Table 2.

Table 2 Primers and cycling parameters for targeted organisms

Target	**Primer Sequence (5'-3')**	**Cycling Parameters**
A. hydrophila lip gene[a]	AAC CTG GTT CCG CTC AAG CCG TTG (F), TTG CTC GCC TCG GCC CAG CAG CT (R)	15 min at 95°C, 35 cycles of 60 s at 94°C, 60 s at 62°C, and 90 s at 72°C
C. jejuni mapA gene[a]	GCT AGA GGA ATA GTT GTG CTT GAC AA (F), TTA CTC ACA TAA GGT GAA TTT TGA TCG (R)	10 min at 95°C, 50 cycles of 15 s at 95°C and 30 s at 59°C
L. pneumophila mip gene[a]	GCA ATG TCA ACA GCAA (F), CAT AGC GTC TTG CATG (R)	15 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 54°C, and 60 s at 72°C
Bacteroides 16S rRNA gene[a]	AAC GCT AGC TAC AGG CTT (F), CAA TCG GAG TTC TTC GTG (R)	15 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 59°C, and 60 s at 72°C
Bacteroides 16S rRNA gene[b]	ATG GCTGTCGTCAGCT (F), ACGGGCGGTGTGTAC (R)	3 min at 50°C, 10 min at 95°C 45 cycles of 30 s at 95°C, 60 s at 50°C, and 20 s at 72°C

a-(Ahmed et al., 2008)
b-(Harms et al., 2003)

C_t values for the serial dilutions were determined and a standard curve plotting the C_t value versus the log concentration (ng/µL) of the template DNA was created for each pair of primers. A linear trendline was fit to the data to represent the relationship between the DNA

concentration and the C_t values. The R^2 and the PCR efficiencies were measured for each standard curve. The PCR efficiency represents how efficiently the DNA replicates. Ideally each cycle of qPCR will result in the DNA doubling. As a result, a 10-fold increase should occur after 3.32 cycles. Thus for a standard curve featuring 10-fold dilutions, the Ct value should increase by 3.32 for each dilution, resulting in a slope of -3.32. The PCR efficiency can be calculated using equation 1.

Equation 1

$$PCR\ efficieny = 10^{-1/slope} - 1$$

Thus for perfect doubling the PCR efficiency is 100%.

qPCR was first used to screen the extracted DNA from graywater samples to determine which of the targeted organism were in each of the graywater samples. This was accomplished by analyzing a single qPCR reaction for each of the graywater samples. The concentration of each of targeted organism was then quantified in triplicate reactions for the samples that tested positive in the initial screening process. The C_t values were then converted to a concentration (ng/µL), which was subsequently converted to cells/mL. Equation 2 was used to determine the mass of each organism.

Equation 2

$$DNA\ (fg) = \left(genome\ size\ (bp)\right) * \left(\frac{MW\ (Da)}{bp}\right) * \left(\frac{1.66 * 10^{-27}\ kg}{Da}\right) * \left(\frac{10^{18}\ fg}{kg}\right)$$

MW represents the molecular weight in Daltons. The relative weight of a base pair is 615.3830 for AT pairs and 616.3711 for GC pairs. The average molecular weight can be calculated using Equation 3.

Equation 3

$$MW(Da/bp) = (GC\ content(\%)) * 616.3711 + (AT\ content\ (\%))(\frac{MW\ (Da)}{bp})*615.3830$$

The genome size, GC content, and the AT content are shown in Table 3.

Table 3 Genome characteristics of targeted organisms

Organism	**Genome Size (bp)**	**GC Content**	**AT Content**	**Reference**
A. hydrophila ATCC 7966	4,744,448	61.5%	38.5%	Seshadri et al. (2006)
C. jejuni ATCC 700819	1,641,481	30.6%	69.4%	Parkhill et al. (2000)
L. pneumophila ATCC 33152	3,353,636	38.4%	61.6%	NCBI (2013)
Bacteroides Vulgatus ATCC 8482	4,781,702	42.0%	58.0%	Cuiv et al. (2011)

Note: Values shown for *L. pneumophila* represent the average value from several studies that have analyzed the genome sequences of various strands of *L. pneumophila*

Using Equations 2 and 3 the relative mass of each organism was found as shown in Table 4.

Table 4 Mass of targeted organisms

Organism	Mass per Cell (fg/cell)
A. hydrophila ATCC 7966	4.85
C. jejuni ATCC 700819	1.68
L. pneumophila ATCC 33152	3.43
Bacteroides vulgatus ATCC 8482	4.89

The number of cells per mL in each reaction was then calculated by taking the concentration (ng/µL) as determined by qPCR reactions and dividing that value by the mass per cell as shown in Table 4. The number of cells per mL in the collected graywater samples was then determined using equation 4 .

Equation 4

$$\frac{cells}{mL} = \left(DNA\ concentration\ \left(\frac{ng}{\mu L}\right)\right) * \left(\frac{100\ \mu L}{Extract}\right) * \left(\frac{Volume\ Filtered\ (mL)}{Extract}\right) * \left(\frac{10^6\ fg}{ng}\right) * \left(\frac{Cell)}{Mass\ of\ Cell\ (fg)}\right)$$

Preliminary Risk Assessment Model

A preliminary risk assessment model was used to evaluate the effect of using different fecal indicators to estimate pathogen concentrations and the risk of infection. The initial risk assessment model that was performed evaluated the risk of infection from exposure to a single pathogen (*Camplyobacter jejuni*) in untreated graywater. The concentration of this pathogen in graywater was estimated using several different fecal indicators.

The risk assessment was based on the model used by Ottoson and Stenstrom (2003) . The model consisted of three distinct parts: 1) the pathogen concentration model as shown in Figure 17; 2) the pathogen dose model as shown in Figure 18; and 3) the dose response models.

Figure 16 shows the pathogen concentration model used by Ottoson and Stenstrom (2003). This model uses fecal indicators to estimate the concentration of pathogens in graywater.

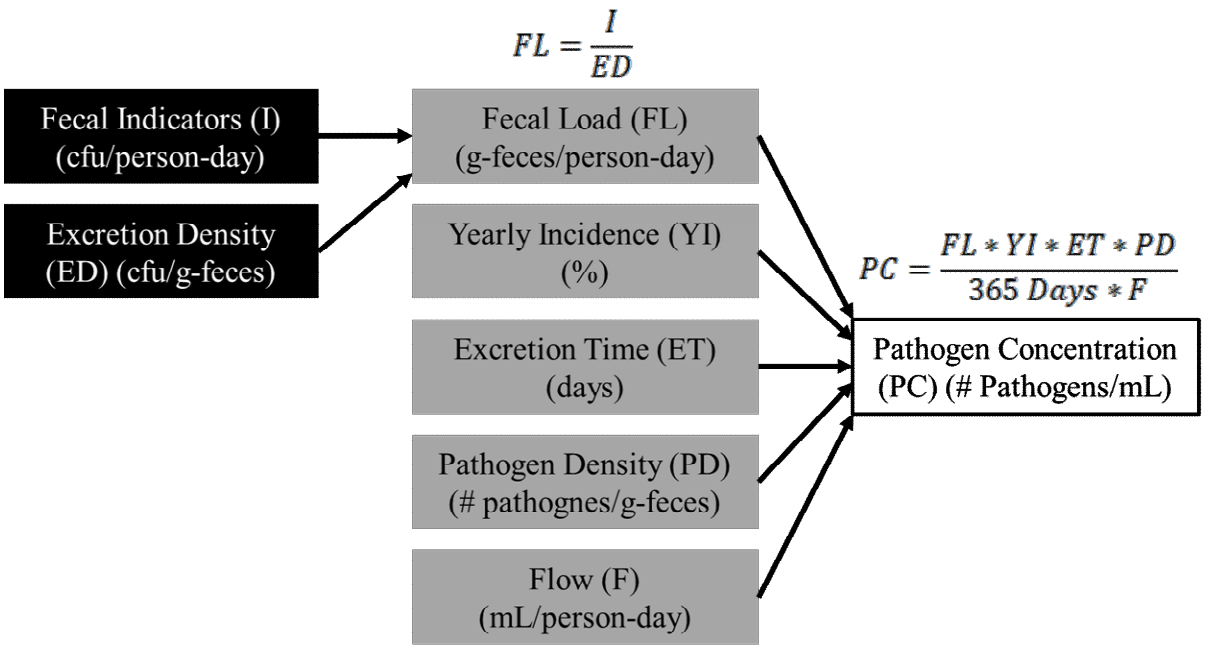


Figure 16 Pathogen Concentration Model as explained by Ottoson and Stenstrom (2003)

The fecal indicators used in the model by Ottoson and Stenstrom (2003) were in units of cfu per person per day. However, the values for the fecal indicators used in this study were obtained from other published reports. These studies quantified fecal indicators in units of

cfu per mL of graywater. This resulted in a slight modification of the pathogen concentration model. Figure 17 represents the model that was used in the preliminary risk assessment.

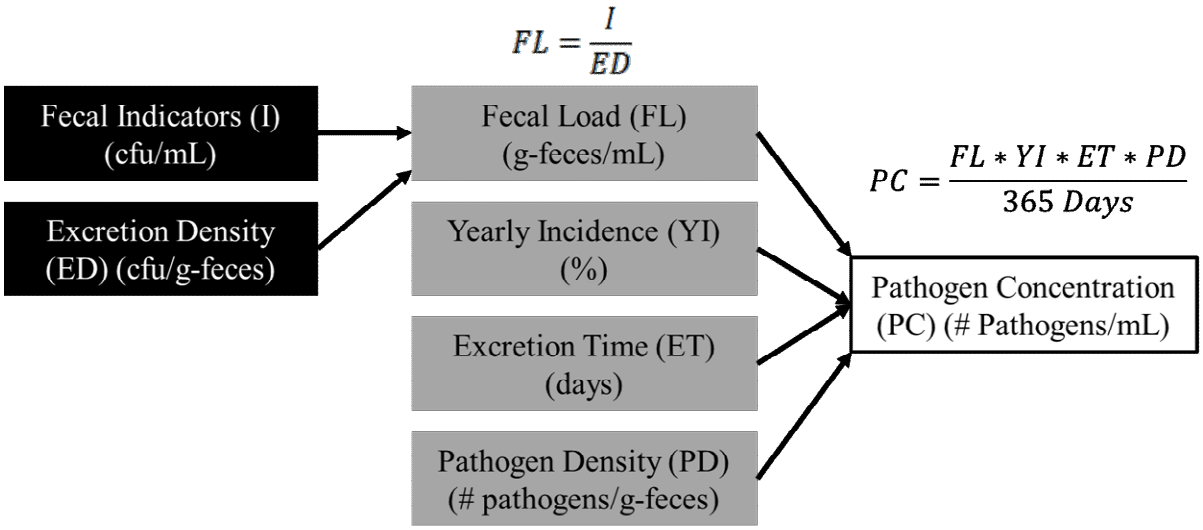


Figure 17 Modified Pathogen Concentration Model

The parameters shown in black represent the characteristics of the fecal indicators. The parameters shown in gray represent the characteristics of the pathogens. As shown in the model, fecal indicators represent the concentration of the indicators of fecal contamination in graywater. The excretion density represents the estimated concentration of each fecal indicator in human feces. Yearly incidence is the percentage of individuals who become infected each year. Excretion time is the duration that an infected individual will excrete the specified pathogen. Pathogen density is the estimated number of pathogens excreted.

Four different fecal indicators, *E. coli*, fecal coliforms, total coliforms, and *Enterococci*, were used to estimate the concentration of the pathogen *C. jejuni* in untreated graywater. A wide range of values for the concentration of fecal indicator concentration has

been reported. However, there is no obvious distribution for the concentrations of the four fecal indicators that were used in the model. Therefore the concentrations of the four fecal indicators were represented by uniform distributions based on maximum and minimum values from several previously published studies as shown in Table 5. Data for the excretion density was obtained from previously published studies and is also shown in Table 5. When a range of values was reported for the excretion density, a uniform distribution was used because there was no obvious distribution.

Table 5 Fecal Indicator Characteristics

Fecal Indicator	**Fecal Indicators in Graywater (cfu/mL)[a b]**	**Excretion Density (cfu/g feces)[c d]**
E. coli	$0 - 2.4 \times 10^4$	1.0×10^7
Total Coliforms	$2.4 \times 10^2 - 2.4 \times 10^5$	$1.0 \times 10^7 - 1.0 \times 10^9$
Fecal Coliforms	$1.7 - 3.3 \times 10^3$	$1.0 \times 10^5 - 1.0 \times 10^6$
Enteroccoci	$0.0 - 2.0 \times 10^2$	3.2×10^6

a (Birks and Hills, 2007)
b (Christova-Boal et al., 1996)
c (Geldreich, 1978)
d (Ottoson and Stenstrom, 2003)

Additionally two chemical biomarkers, coprostanol and cholesterol, were used as indicators to estimate the concentration of the pathogen *C. jejuni* in untreated graywater using the model shown in Figure 18.

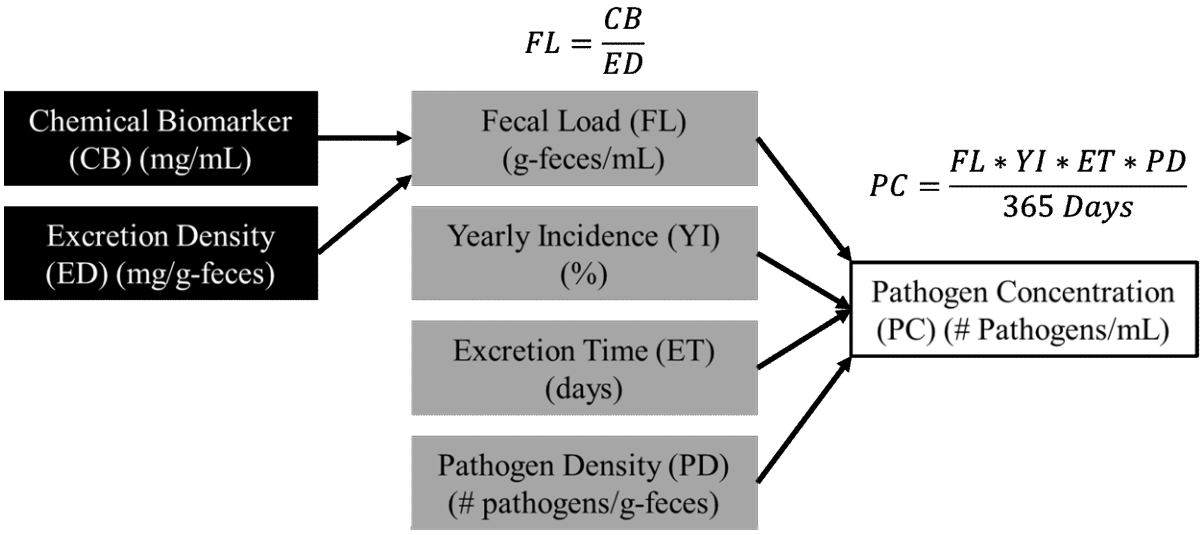


Figure 18 Modified Pathogen Concentration Model using Chemical Biomarkers

Figures 18 represents the same model as shown in Figure 17 except that chemical biomarkers were used to estimate the fecal load instead of fecal indicators.

The concentrations of the two biomarkers were represented by lognormal distributions as determined by Ottoson and Stenstrom (2003) (Table 6).

Table 6 Chemical Biomarker Characteristics (Ottoson and Stenstrom, 2003)

Chemical Biomarker	**Mean Biomarker Concentration (µg/L)**	**Standard Deviation (µg/L)**	**Excretion Density (mg/g feces)**
Coprostanol	8.6	4.4	12.74
Cholesterol	17.3	8.4	5.08

(Ottoson and Stenstrom, 2003)

Table 7 shows the other parameters that were used to estimate the concentration of *C. jejuni* in graywater.

Table 7 *C. jejuni* Characteristics

Parameter	**Value**
Yearly Incidence (YI) (%)	15.6%
Excretion Time (ET) (Days)	$\mu = 16.14$, $\sigma = 2.11$
Pathogen Density (PD) (#/g feces)	$\mu = 10^8$, $\sigma = 10$

(Ottoson and Stenstrom, 2003)

A summary of the values that were input into the modified pathogen concentration model is shown in Table 8.

Table 8 Summary of inputs for modified pathogen concentration model

Input	Distribution	Source
Fecal Indicators		
E. coli (CFU/mL)	Uniform (0, 2.4×10^4)	Birks and Hills (2007); Christova-Boal et al (1996)
Total Coliforms (CFU/mL)	Uniform (2.4×10^2, 2.4×10^5)	Birks and Hills (2007); Christova-Boal et al (1996)
Fecal Coliforms (CFU/mL)	Uniform (1.7, 3.3×10^3)	Birks and Hills (2007); Christova-Boal et al (1996)
Enterococci (CFU/mL)	Uniform (0, 2.2×10^2)	Birks and Hills (2007); Christova-Boal et al (1996)
Coprostanol (μg/L)	Lognormal(8.6, 4.4)	Ottoson and Stenstrom (2003)
Cholesterol (μg/L)	Lognormal(17.3, 8.4)	Ottoson and Stenstrom (2003)
Excretion Density		
E. coli (CFU/g feces)	1.0×10^7	Geldreich (1978)
Total Coliforms (CFU/g feces)	Uniform (1.0×10^7, 1.0×10^9)	Geldreich (1978)
Fecal Coliforms (CFU/g feces)	Uniform (1.0×10^5, 1.0×10^6)	Geldreich (1978)
Enterococci (CFU/g feces)	Uniform (1.7, 3.3×10^3)	Geldreich (1978)
Coprostanol (mg/g feces)	12.74	Ottoson and Stenstrom (2003)
Cholesterol (mg/g feces)	5.08	Ottoson and Stenstrom (2003)
Yearly Incidence (%)	15.6%	Ottoson and Stenstrom (2003)
Excretion Time (Days)	Lognormal(16.14, 2.11)	Ottoson and Stenstrom (2003)
Pathogen Density (pathogens/g feces)	Lognormal(10^8, 10)	Ottoson and Stenstrom (2003)

Once the pathogen concentration was determined, an exposure assessment was performed to estimate the dose, or the number of pathogens an individual would consume. This exposure assessment is shown in Figure 19.

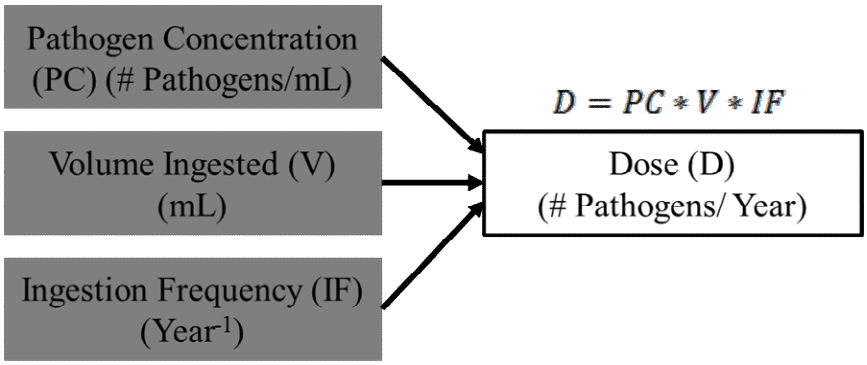


Figure 19 Exposure Assessment Model

The ingested volume indicates the volume that a person would ingest during a given exposure scenario and the exposure frequency represents the number of exposures per year. Both the ingested volume and the exposure frequency are the result of four different exposure scenarios that were evaluated for this report. These exposure scenarios include accidental ingestion of graywater, hand-to-mouth contact from plants and lawns irrigated by graywater, ingestion of sprays used for irrigation and washing, and the consumption of crops irrigated with graywater. The ingested volumes and exposure frequencies from previous reports (Maimon et al., 2010, Tanaka et al., 1998) are shown in Table 9 for each exposure scenario.

Table 9 Exposure Scenarios

Exposure Scenario	Description [a,b]	Ingested Volume (mL)[a,b]	Ingested Volume Range	Exposure Frequency[a,b]	Ingestion Frequency Range
1	Accidental Ingestion of Graywater	100	50 - 200	1 per year	0.5 to 2 per year
2	Indirect Ingestion From Touching Plants and Lawns	1	0.5 – 2.0	90 per year	45 to 180 per year
3	Ingestion of Graywater From Sprays used for irrigation and washing	0.1	0.05 – 0.2	90 per year	45 to 180 per year
4	Ingestion of Crops Irrigated by Graywater	10	5 - 20	365 per year	183 to 730 per year

a (Maimon et al., 2010)
b (Tanaka et al., 1998)

To account for variability due to the ingested volume and the exposure frequency, an estimate of the range of each parameter was made. This information was used in the model as a triangular distribution making the assumption that the average also represented the most likely value.

After the pathogen dose was estimated, the risk of infection was estimated using a dose response model. A Beta-Poisson dose response model (Equation 5) was used to estimate the risk of infection from exposure to *C. jejuni* ($N_{50} = 896$, $\alpha = 0.145$) (Ottoson and Stenstrom, 2003).

Equation 5

$$P(response) = 1 - \left[1 + dose\frac{(2^{\frac{1}{\alpha}} - 1)}{N_{50}}\right]^{-\alpha}$$

The dose response model estimates the probability that an individual will be infected for a given dose. All quantitative evaluations were modeled using probabilistic modeling software, Analytica of Lumina Decision Systems, Inc. Analytica utilized the Monte Carlo method with 1000 iterations.

The inputs for the pathogen dose model and the dose response model are shown in Table 10.

Table 10 Summary of inputs for modified pathogen dose model and dose response model

Input	Distribution	Source
Ingested Volume (mL)		
Scenario 1 (Accidental Ingestion)	Triangular (50, 100, 200)	Maimon (2010)
Scenario 2 (Indirect Ingestion)	Triangular (0.5, 1, 2)	Maimon (2010)
Scenario 3 (Ingestion of Sprays)	Triangular (0.05, 0.1, 0.2)	Maimon (2010)
Scenario 4 (Ingestion of Crops)	Triangular (5, 10, 20)	Tanaka (1998)
Exposure Frequency (Year^{-1})		
Scenario 1 (Accidental Ingestion)	Triangular (50, 100, 200)	Maimon (2010)
Scenario 2 (Indirect Ingestion)	Triangular (0.5, 1, 2)	Maimon (2010)
Scenario 3 (Ingestion of Sprays)	Triangular (0.05, 0.1, 0.2)	Maimon (2010)
Scenario 4 (Ingestion of Crops)	Triangular (5, 10, 20)	Tanaka (1998)
Dose Response *C. jejuni*		
N_{50}	896	Ottoson and Stenstrom (2003)
α	0.145	Ottoson and Stenstrom (2003)

The preliminary risk assessment provided a rough estimate of the risks of reusing graywater. One major source of uncertainty is the model that used fecal indicators to estimate the concentration of *C. jejuni* in untreated graywater. There is uncertainty in this model because many pathogens may not correlate with indicator organisms (Harwood et al., 2005).

To evaluate which parameters contributed most to the uncertainty in the estimates of risk, an importance analysis was performed. Importance analysis is an analytical tool available in the Analytica package that estimates the most important inputs of a given model. This method estimates how much each uncertain input contributes to the uncertainty in the output. Importance analysis was used to evaluate which parameters in the risk assessment model contributed the most to the uncertainty of the model. From the four exposure scenarios and the six indicators that were evaluated, there were 24 distributions that were generated. Four of these distributions were evaluated for importance. The four distributions that were evaluated represented each of the exposure scenarios and four of the six fecal indicators.

Updated Risk Assessment

Using the pathogen concentrations from the qPCR results, the human health risks were evaluated again. Quantifying pathogens in graywater using qPCR reduces the uncertainty in the risk assessment model because it eliminates the need to use fecal indicators to estimate the concentration of pathogens in graywater.

The risk assessment model used in the preliminary risk analysis was used to estimate the risk of infection from exposure to the targeted pathogens in graywater. However, the model could be simplified because the pathogen concentrations were determined using qPCR

analysis, allowing for the pathogen concentration model shown in Figure 17 to be eliminated from the risk assessment model. The same exposure scenarios that were used in the preliminary risk assessment were used to estimate the pathogen dose (Figure 19).

The risks of infection from exposure to *C. jejuni* and *L. pneumophila* in graywater were both estimated. The risk of infection from exposure to *A. hydrophila* was not calculated because there is a lack of correlation between *A. hydrophila* and the pathogenicity for humans (Morgan et al., 1985). A Beta-Poisson dose response model (Equation 6) was used to estimate the risk of infection from exposure to *C. jejuni* ($N_{50} = 896$, $\alpha = 0.145$) (Ottoson and Stenstrom, 2003).

Equation 6

$$P(response) = 1 - \left[1 + dose\frac{(2^{\frac{1}{\alpha}} - 1)}{N_{50}}\right]^{-\alpha}$$

An exponential dose response curve (Equation 7) was used to estimate the risk of infection from *L. pneumophila* ($k = 5.99 \times 10^{-2}$) (Muller et al., 1983).

Equation 7

$$P(response) = 1 - e^{-k \times dose}$$

The dose response models estimate the probability that an individual will be infected for a given dose. A P(response) equal to one suggests that an individual will be infected and a P(response) equal to zero indicates that there is no risk of infection.

Results and Discussion

Collection of Samples

During the filtering process the filter pores would often clog resulting in variability in the volume of sample that was filtered. A summary of the filtered volumes is shown in Figure 20.

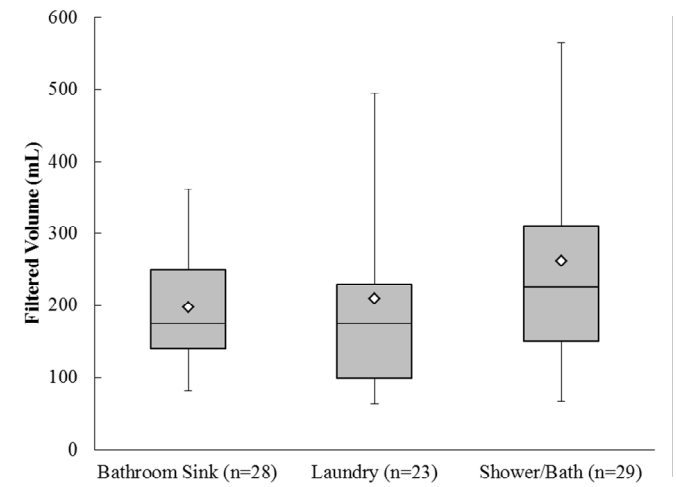


Figure 20 Filtered Volumes for Collected Samples

The boxplots shown in Figure 20 illustrate the median, the upper quartile and the lower quartile of the filtered volumes. The mean volume filtered is shown by the white diamond. Errors-bars represent the 5^{th} and 95^{th} percentiles of the filtered volumes. Boxplots represent 28 samples filtered from the bathroom sink, 23 samples from laundry, and 29 samples from shower or bath.

The mean volume that was filtered was 197 mL, 209 mL, and 262 mL for samples collected from the bathroom sink, laundry, and shower/bath respectively. The median volume

that was filtered was 175 mL, 175 mL, and 225 mL for samples collected from the bathroom sink, laundry, and shower/bath respectively.

DNA Extraction

The concentration of the extracted DNA varied from sample to sample as shown in Figure 21.

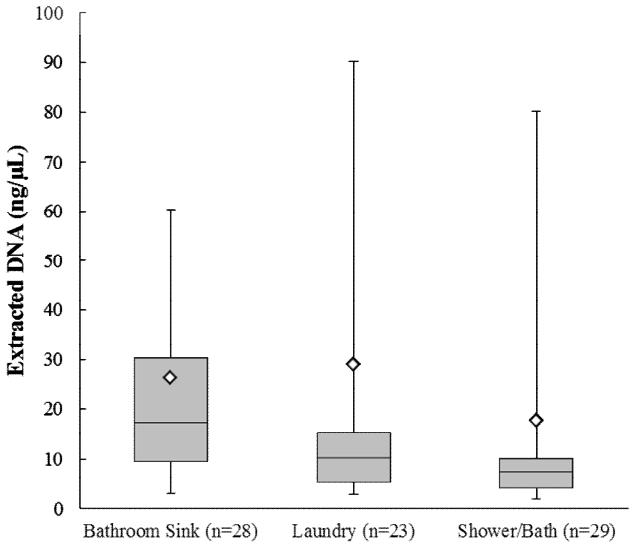


Figure 21 Extracted DNA from Collected Graywater Samples

The boxplots shown in Figure 21 illustrate the median, the upper quartile and the lower quartile of the extracted DNA. The mean volume filtered is shown by the white diamond. Errors-bars represent the 5^{th} and 95^{th} percentiles of the Extracted DNA. Boxplots represent 28 DNA extracted from 28 bathroom sink samples, 23 laundry samples, and 29 shower or bath samples.

The mean DNA concentration that was extracted was 26.4 ng/µL, 29.0 ng/µL, and 17.8 ng/µL for samples collected from the bathroom sink, laundry, and shower/bath

respectively. The median DNA concentration that was extracted was 17.4 ng/µL, 10.3ng/µL, and 7.6 ng/µL for samples collected from the bathroom sink, laundry, and shower/bath respectively.

A_{260}/A_{280} and A_{260}/A_{230} ratios were not always close to the desirable value of 2.0. However, PCR amplification using a general bacterial primer was used to verify that DNA could still be amplified. PCR amplification yielded positive results indicating that DNA could be used for further analysis (Appendix B). A summary of the extracted DNA is shown in Appendix E.

Quantitative Polymerase Chain Reaction (qPCR)

The concentration of the targeted pathogens was quantified for each sample that was collected. A positive control containing a tenfold serial dilution of the targeted organism and a no template control was included on every qPCR plate.

The standard curves that were developed to quantify the targeted organisms are shown in Figures 22 through 26.

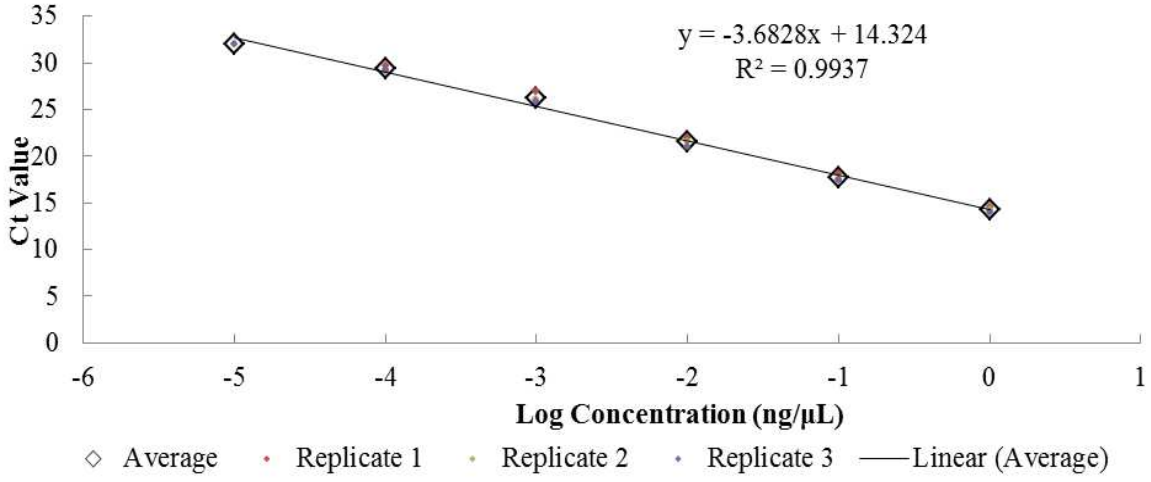


Figure 22 Standard Curve for *A. hydrophila* (PCR efficiency=0.87%)

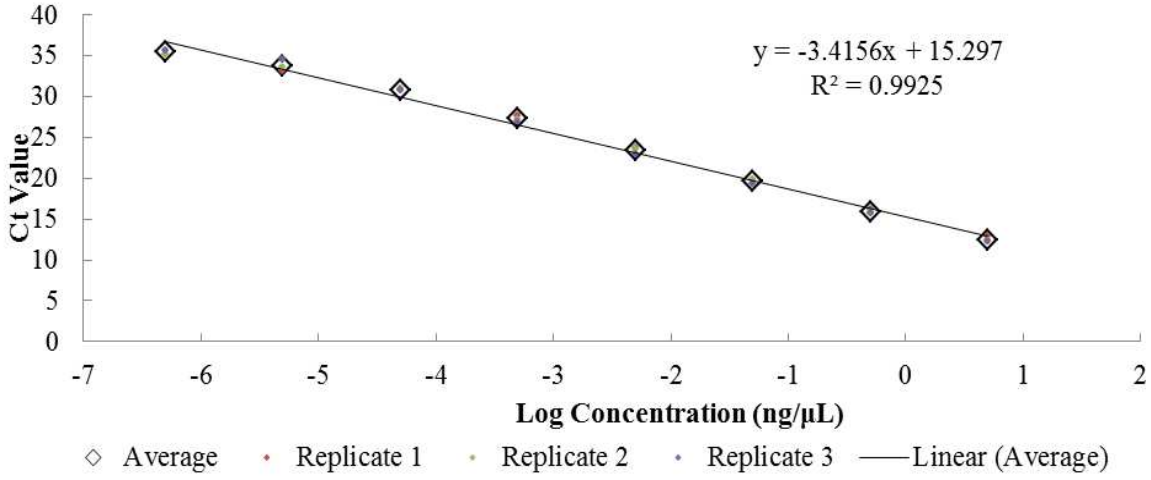


Figure 23 Standard Curve *C. jejuni* (PCR efficiency=96%)

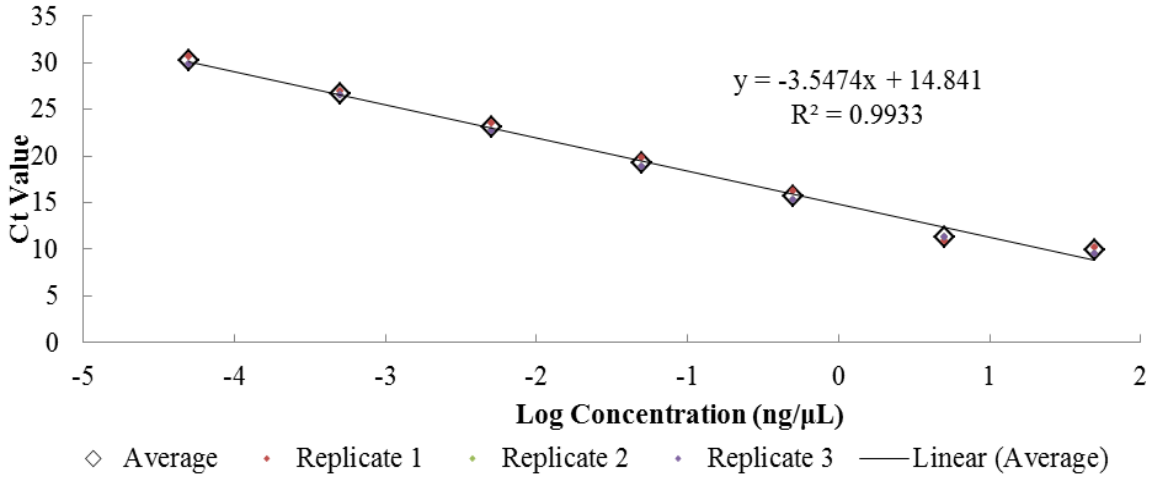


Figure 24 Standard Curve *L. pneumophila* (PCR efficiency=91%)

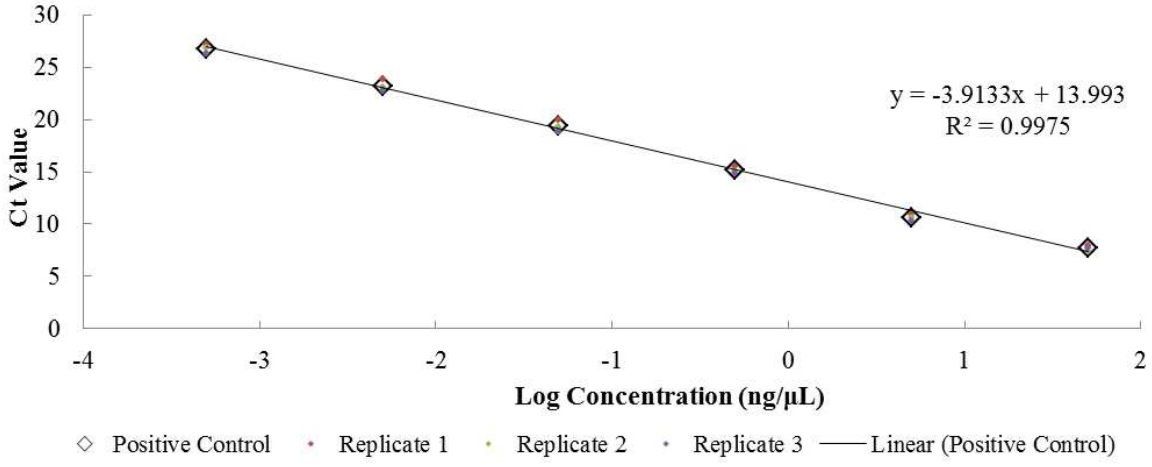


Figure 25 Standard Curve *Bacteroides* (PCR efficiency=80%)

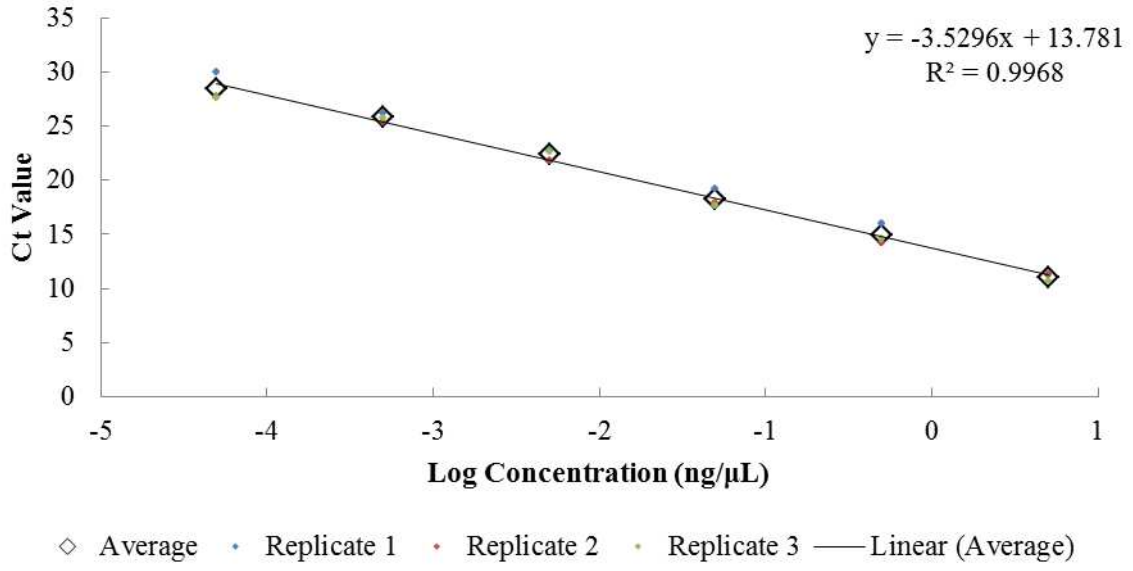


Figure 26 Standard Curve for Bacterial Primers using *Bacteroides* (PCR efficiency=92%)

Each of the standard curves resulted in a linear trendline with an R^2 value of 0.99 or greater, indicating a high correlation between C_t values and the DNA concentration. The PCR efficiency of the qPCR standard curves ranged from 80% to 96%. For each of the standard curves, only the data points that represented a linear relationship were included.

Very few samples tested positive for the targeted organisms. A summary of the samples that tested positive for each of the targeted organisms is shown in Figure 27.

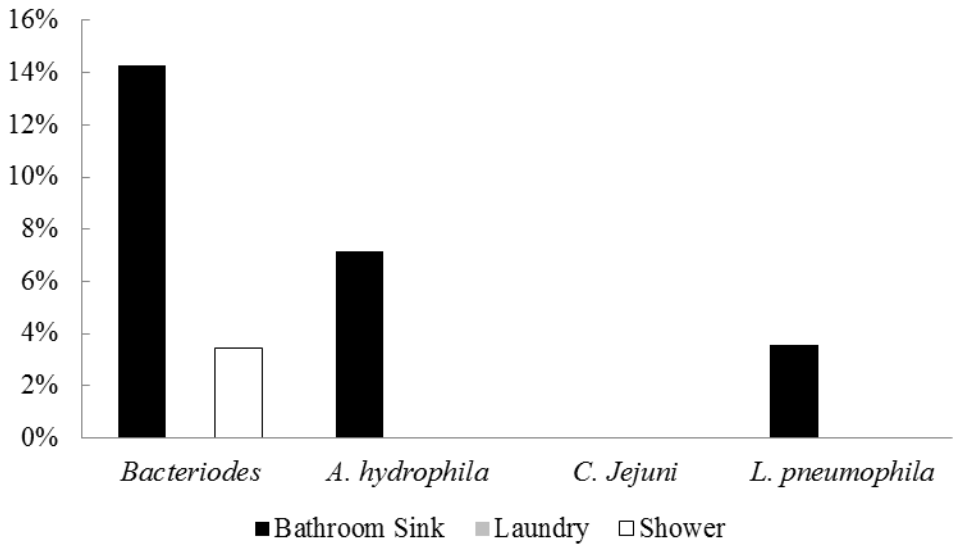


Figure 27 Samples Testing Positive for Targeted Pathogens

As shown in Figure 27 five out of eighty samples tested positive for *Bacteroides,* two out of eighty samples tested positive for *A. hydrophila*, and one out of eighty samples tested positive for *L. pneumophila*. No samples tested positive for *C. jejuni*. Each of the two samples that tested positive for *A. hydrophila,* and the one sample that tested positive for *L. pneumophila* also tested positive for *Bacteroides.* One household tested positive for *Bacteroides* in both their bathroom sink sample and their shower sample. Of the households that tested positive for *Bacteroides,* two households had children and two did not have any children.

The bar graph shown in Figure 28 shows the concentration of each of the samples that tested positive for the targeted organism.

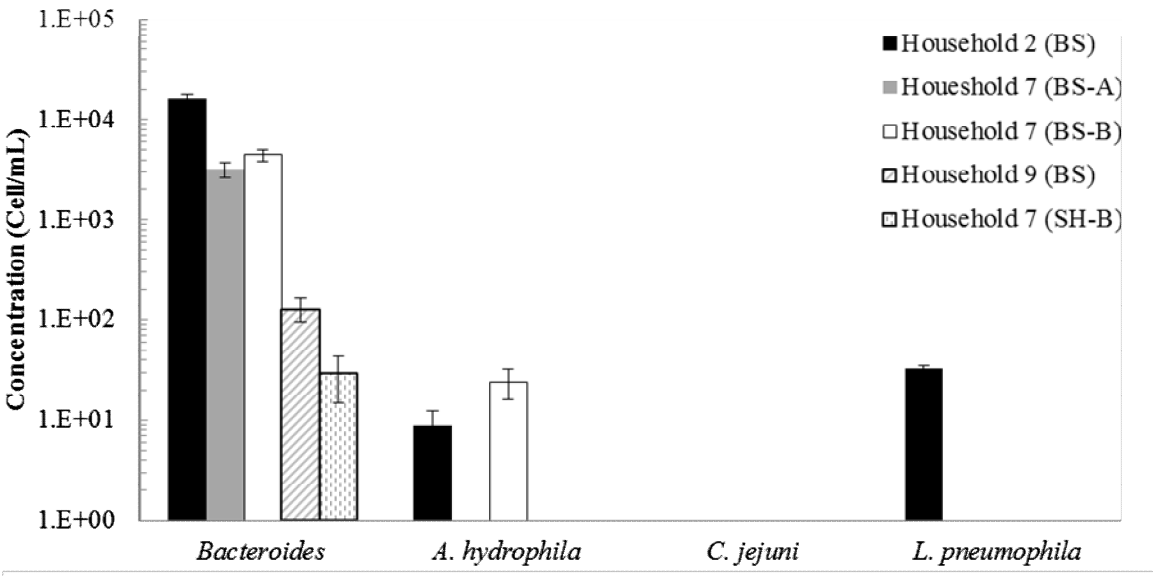


Figure 28 Mean Pathogen Concentration

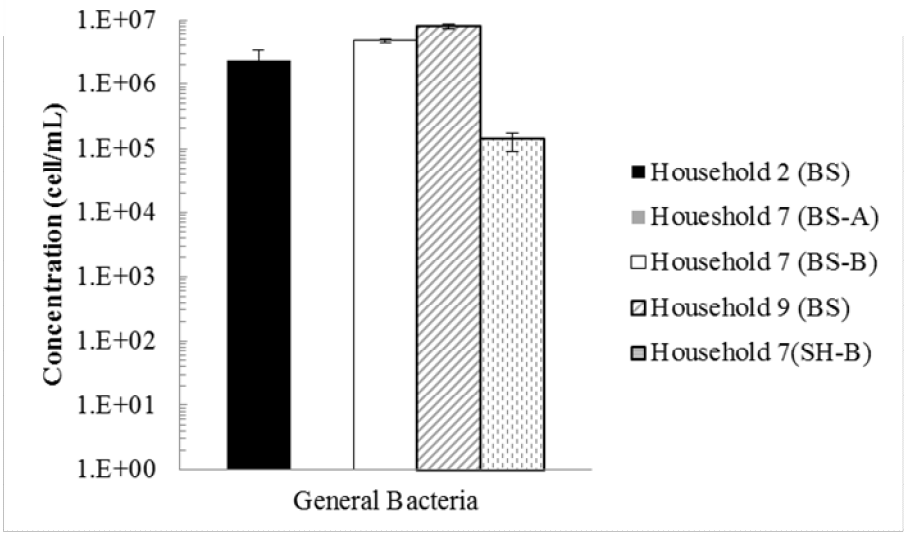
As shown in Figure 28, the average concentrations range from 29.3 to 1.63×10^4 cells per mL for *Bacteroides,* 8.7 to 24.3 cells per mL for *A. hydrophila* and 33.3 cells per mL for *L. pneumophila.* Concentrations are based on the presence of targeted DNA and do not account for the viability of the organisms. This suggests that these concentrations are conservative estimates of the actual viable organisms present in the graywater samples.

The houses that tested positive for the targeted organisms include households 2, 7 and 9. Household 2 included two adults (1 male, 1 female) and no children or pets. Only the bathroom sink graywater from household 2 tested positive for *A. hydrophila*, *L. pneumophila* and *Bacteroides*. The collected bathroom sink graywater from household 2 included graywater generated from hand washing, face washing, and brushing teeth.

There were five people in household 7, two adults and three children. Household 7 also had both a dog and a cat present in the home. Two graywater samples were collected from both the bathroom sink graywater and the shower/bath. One of the samples collected from the bathroom sink graywater tested positive for *A. hydrophila*. Both the bathroom sink samples and one of the shower/bath samples tested positive for *Bacteroides*. The collected bathroom sink graywater was generated from shaving and tooth brushing. The collected shower/bath graywater was generated from baths and showers of all five members of the household.

Household 9 included one adult male and no children or pets. Only the bathroom sink graywater from household 9 tested positive for *Bacteroides*. The collected bathroom sink graywater from household 9 was generated from hand washing, face washing, and brushing teeth.

The concentration of bacteria present in each of the samples is shown in Figure 29.



Note: (BS) indicates the bathroom sink samples and (SH) indicates the shower/bath samples
Two (2) bathroom sink samples and two (2) shower/bath samples were collected from household 7
Error bars represent the range

Figure 29 Concentration of General Bacteria

As shown in Figure 29 the mean concentration of bacteria in the samples that tested positive for the other organisms ranged from 1.41×10^5 to 7.97×10^6 cells per mL. This indicates that the organisms targeted by qPCR represent only a small portion of the bacteria present in graywater samples. qPCR was not performed for Household 7 (BS-A) because there was no DNA remaining after previous qPCR assays were performed.

Preliminary Risk Assessment Model

The pathogen concentration model resulted in six distributions representing the concentration of *C. jejuni* in untreated graywater as estimated using the various indicators. The results of the pathogen concentration model are shown in Figure 30.

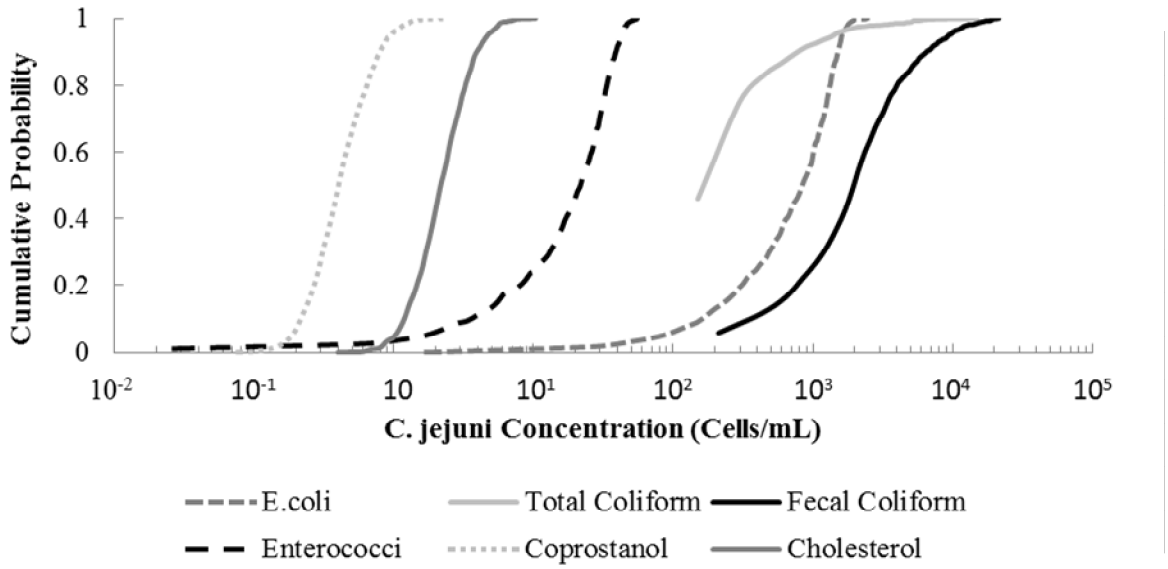


Figure 30 Estimates of *C. jejuni* in untreated graywater

The distributions in Figure 30 show that using different indicators to predict the concentration of *C. jejuni* resulted in many different cumulative probability distributions. It is unclear which indicator most accurately estimates the pathogen concentration. This is primarily due to the limitations of using fecal indicators to predict pathogen concentrations. The breaks in the distributions shown in Figure 30 are the result of using a lognormal scale to plot the estimated concentration of *C. jejuni*.

The model estimates the median concentration of *C. jejuni* to range from 0.41 to 2100 cells/mL and the estimated mean ranged from 0.47 to 2900 cells/mL. Using coprostanol to estimate the concentration of *C. jejuni* resulted in the lowest estimates while using fecal coliforms resulted in the highest estimates. Using fecal coliforms to estimate pathogen concentration resulted in an estimate of the mean that was approximately 6000 times larger than the estimated mean using coprostanol. It is unclear which indicator would most

accurately estimate the pathogen concentration, but it is clear that using different fecal indicators to estimate the concentration of *C. jejuni* can result in very different results. This further proves that using fecal indicators to predict pathogen concentration has severe limitations.

Using the *C. jejuni* concentration distributions as estimated by the pathogen concentration model, the dose and then the risk of infection were then calculated. The dose was based on four exposure scenarios. The risk of infection for the four exposure scenarios is shown in Figures 31 through 34.

The distributions shown in Figure 31 represent the risk of infection from exposure to *C. jejuni* during graywater reuse Scenario 1, which represents accidental ingestion of graywater.

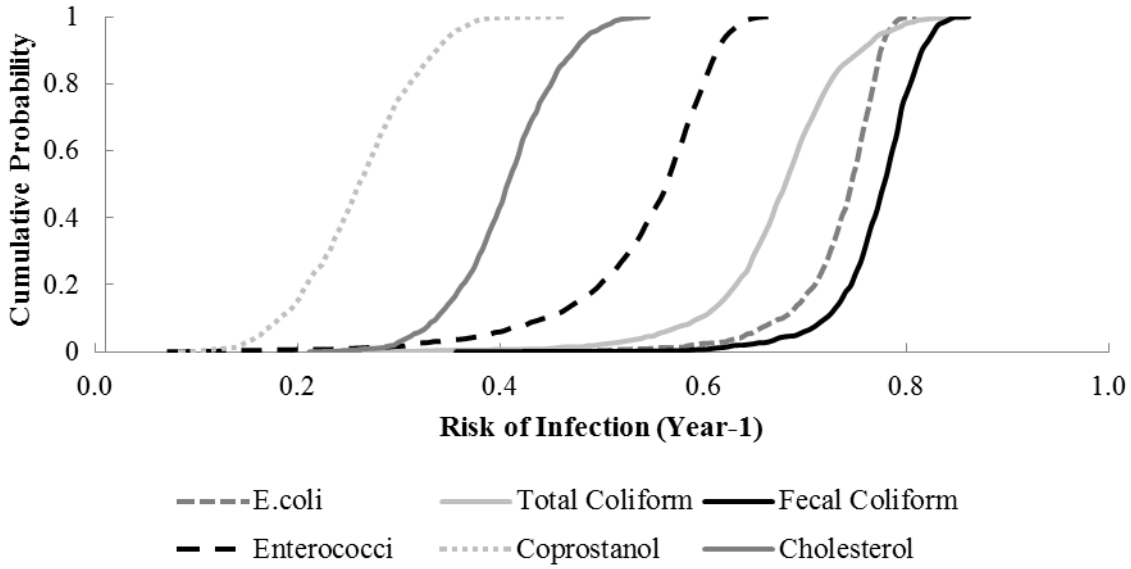


Figure 31 Risk of Infection from Exposure to *C. jejuni.* Exposure Scenario 1 (Accidental ingestion of graywater)

Each of the distributions shown in Figure 31 represent the estimation of the risk of infection from the accidental ingestion of graywater. The median risk of infection ranges from 0.26 to 0.77 infections per year and the mean ranges from 0.26 to 0.78 infections per year as estimated by the model. An acceptable level of microbiological risk in water systems is often cited as less than 1 infection per 10,000 people per year (10^{-4} infections per year) (Fewtrell and Bartram, 2001). Both the median and the mean estimates of risks for Scenario 1 exceed acceptable levels of risk.

The distributions shown in Figure 32 represent the risk of infection from exposure to *C. jejuni* during graywater reuse Scenario 2. Scenario 2 represents indirect ingestion from touching plants and lawns irrigated with untreated graywater.

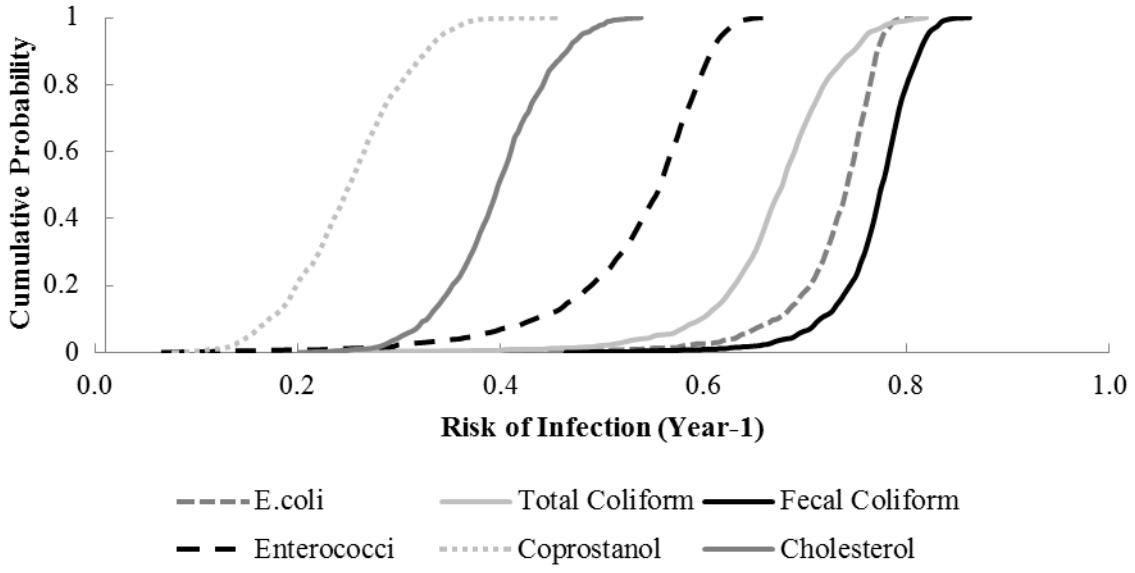


Figure 32 Risk of Infection from Exposure to *C. jejuni*. Exposure Scenario 2 (Indirect ingestion from touching plants and lawns)

As shown in Figure 32, indirect ingestion of graywater results in multiple distribution curves representing the estimated risks of infection. The median values of risk of infection range from 0.25 to 0.77 per year and the mean estimates range from 0.25 to 0.77 per year. Both the median and the mean estimates of risks for Scenario 2 exceed acceptable levels of risk.

The distributions shown in Figure 33 represent the risk of infection from exposure to *C. jejuni* during graywater reuse Scenario 3, which represents ingestion of graywater from sprays used for irrigation.

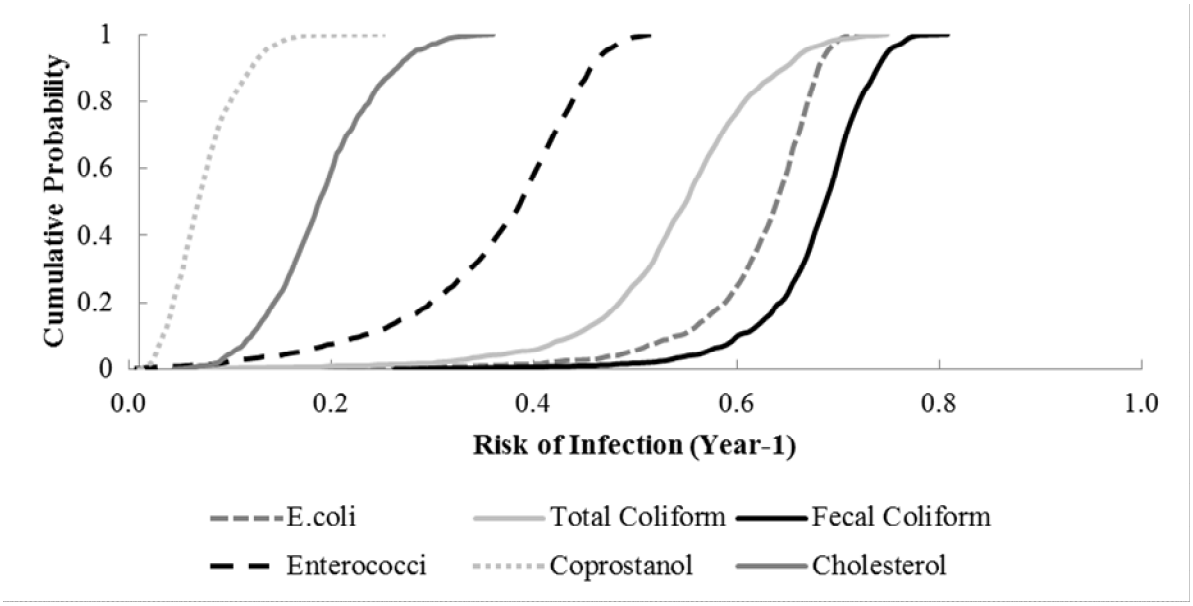


Figure 33 Risk of Infection from Exposure to *C. jejuni*. Exposure Scenario 3 (Ingestion of graywater from sprays used for irrigation and washing)

As shown in Figure 33, ingestion of graywater from sprays used for irrigation and washing results in multiple distribution curves representing the estimated risks of infection.

For exposure Scenario 3 the median values of risk of infection range from 0.19 to 0.69 per year and the mean estimates range from 0.07 to 0.68 per year. Both the median and the mean estimates of risks for Scenario 3 exceed acceptable levels of risk.

The distributions shown in Figure 34 represent the risk of infection from exposure to *C. jejuni* during graywater reuse Scenario 4, which represents ingestion of crops irrigated with untreated graywater.

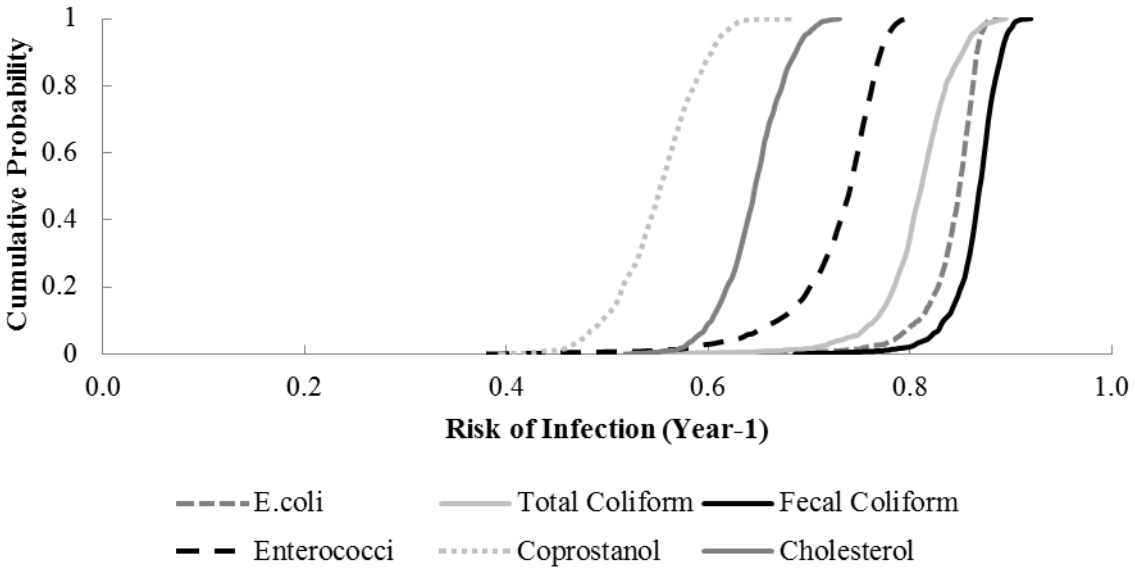


Figure 34 Risk of Infection from Exposure to *C. jejuni*. Exposure Scenario 4 (Ingestion of crops irrigated by graywater)

As shown in Figure 34, ingestion of crops irrigated with graywater resulted in multiple distribution curves representing the estimated risks of infection. For exposure Scenario 4 the median values of risk of infection range from 0.55 to 0.87 per year and the mean estimates range from 0.55 to 0.86 per year. Both the median and the mean estimates of risks for Scenario 3 exceed acceptable levels of risk.

Using coprostanol to estimate the risks of infection from exposure to *C. jejuni* in untreated graywater resulted in the lowest estimates for each exposure scenario. Additionally, using fecal coliforms to estimate the risks resulted in the highest estimates for each exposure scenario. The range of estimates is largest for Scenario 3. Scenario 3 also represents the exposure scenario with the lowest dose of graywater. This suggests that the risk model is more sensitive to the pathogen concentration for exposure scenarios where low doses of graywater are consumed.

The results of the preliminary risk assessment suggest that the human health risks from exposure to untreated graywater during different reuse scenarios are unacceptably high. This model also suggests that the exposure scenario that presents the highest human health risks is the ingestion of crops irrigated by graywater. These estimates of risk are limited due to uncertainties in the pathogen concentration model.

The inputs that contributed the most to the uncertainty of the risk assessment model were evaluated using an importance analysis. The distributions that were evaluated are summarized in Table 11 and the results from this analysis are shown in Figure 35.

Table 11 Summary of distributions analyzed for importance

Importance Analysis	Exposure Scenario	Fecal Indicator
#1	1	Total Coliform
#2	2	*E. coli*
#3	3	*Enteroccoci*
#4	4	Cholesterol

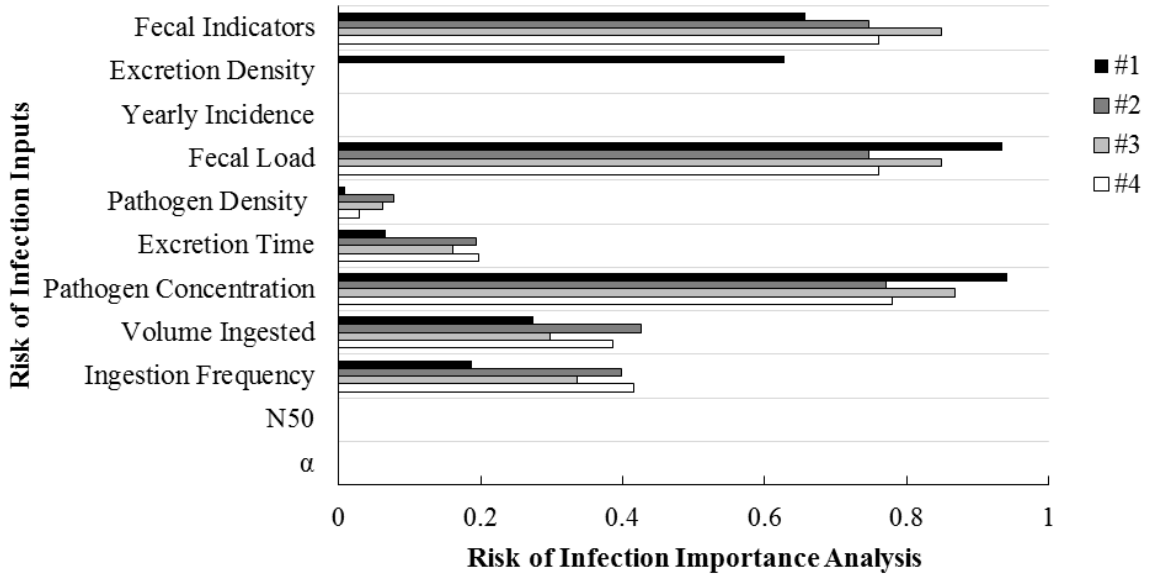


Figure 35 Risk of Infection Importance Analysis

Figure 35 shows the importance of each of the parameters that were input into the risk assessment model and includes intermediate parameters. The inputs with the highest importance are fecal indicators, fecal load, and pathogen concentration, which are all a part of the pathogen concentration model. In addition to importance analysis that was performed for all of the inputs, an importance analysis was performed for the pathogen concentration model (Figures 36).

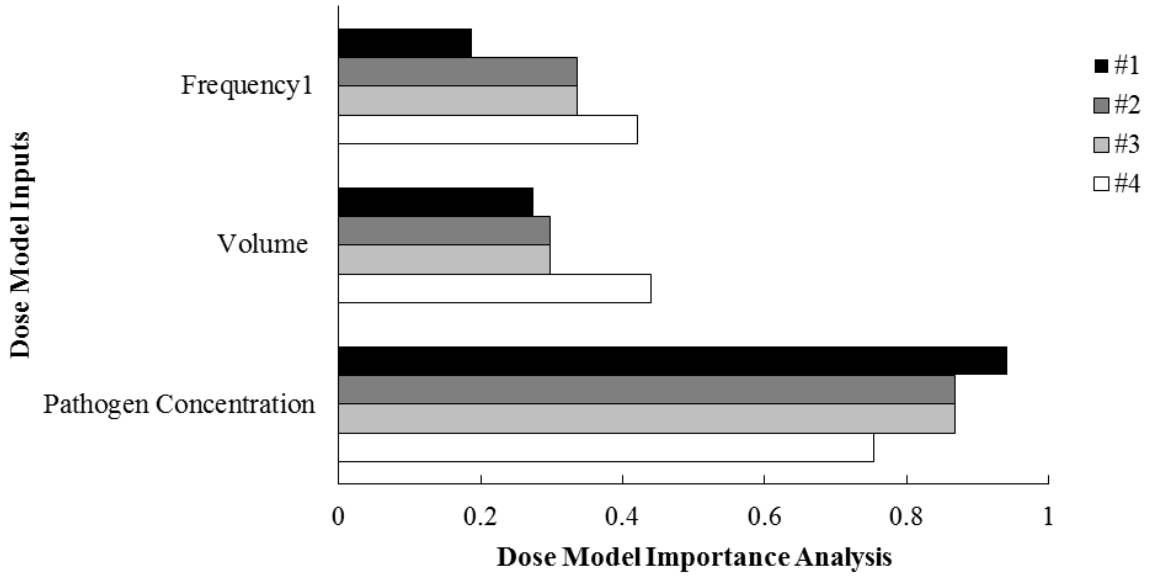


Figure 36 Dose Model Importance Analysis

As shown in Figure 36, pathogen concentration contributed the most to the uncertainty in the dose model.

The importance analysis suggests that the parameters used to estimate the pathogen concentration contribute the most to the uncertainty in the risk assessment model. This indicates that reducing uncertainty in the concentration of pathogens in graywater will greatly reduce the uncertainty in the estimates of risk.

Updated Risk Assessment

The risks of infection from exposure to *C. jejuni* and *L. pneumophila* were estimated using the exposure assessment model and the dose response curve explained in the Materials and Methods section. Although the presence of *A. hydrophila* was assessed, the risk of infection was not evaluated because Morgan et al. (1985) determined that there was no direct

correlation between the dose of *A. hydrophila* and infection. Thus the risk of infection from exposure to *A. hydrophila* in untreated graywater could not be estimated.

There were no samples that tested positive for *C. jejuni* and there was only one sample that tested positive for *L. pneumophila.* This suggests that the risks of infection from exposure to *C. jejuni* and *L. pneumophila* in untreated graywater are low. However, to accurately estimate the risks it was necessary to represent the data from the qPCR analysis using a probability distribution curve.

The presence of *C. jejuni* and *A. hydrophila* was below detectable limits for most of the collected samples making it difficult to fit a probability distribution to the qPCR data, thus it was necessary to identify a probability distribution that conceptually matched the characteristics of a microbial environment. A Poisson distribution (Equation 8) was used to represent the data for both *C. jejuni* and *A. hydrophila* because it conceptually represents the occurrence of microorganisms in the environment. A Poisson distribution is a discrete distribution in which any random integer greater than or equal to zero is possible. A discrete distribution was beneficial in this estimate because a pathogen concentration of zero is possible and could not be represented accurately by a continuous distribution. (Haas et al, 1999).

Equation 8

$$P(X) = \frac{\lambda^{X}}{X!} e^{-\lambda}$$

The Poisson distribution has only one parameter, lambda (λ), which is represented by the mean pathogen concentration of the graywater samples. To fit Poisson distributions to the concentration of *C. jejuni* and *L. pneumophila* it was necessary to determine the mean pathogen concentration of samples. Using qPCR to quantify the pathogen concentration results in a lower limit of detection there is a lower limit of detection which was determined by the standard curves and was assumed to be the lowest concentration of targeted pathogen that was detected. Many samples were below the detection limit for the targeted pathogens and it was assumed that all concentrations between zero and the detection limit were equally probable. The lower limit of detection was 1.49 cells (2.5 (1.49 cells) per qPCR reaction and 7.29 cells (2.5×10^{-5} ng) per qPCR reaction for *C. jejuni* and *L. pneumophila* respectively. These lower limits represent the mass of DNA that could be detected by qPCR analysis. The lower limits of detection for the pathogen concentration needed to be determined using the conversions shown in Equation 9.

Equation 9

$$\frac{\textbf{Limit of detection (Cells/rxn)} \times \left(\frac{\textbf{rxn}}{\textbf{5}\mu\textbf{L}}\right) \times \textbf{(100}\mu\textbf{L of Extracted DNA)}}{\textbf{Volume Filtered (mL)}}$$

The volume of graywater filtered varied, resulting in varying lower limits of detection for the pathogen concentration. The mean lower limits of detection for the pathogen concentration were 0.19 cells per mL and 0.95 cells per mL for *C. jejuni* and *L. pneumophila* respectively. To determine the mean pathogen concentration of the samples below the detection limit it was assumed that the concentration could be represented by a uniform

distribution ranging from zero to the detection limit. Thus the mean pathogen concentration of the samples below the detection limits was half of the mean lower limit of detection for the pathogen concentration. The mean pathogen concentration was then determined to be 0.097 cells per mL and 0.89 cells per mL for *C. jejuni* for *L. pneumophila* respectively. The mean concentration of *C. jejuni* was half of the lower limit of detection for *C. jejuni* because there were no samples above the detection limit while the mean concentration of *L. pneumophila* was greater than half of the detection limit for *L. pneumophila* because there was one sample that had a concentration of *L. pneumophila* above the detection limit. The pathogen concentration was represented by a Poisson distribution with lambda equal to 0.097 cells per mL and 0.89 cells per mL for *C. jejuni* and *L. pneumophila* respectively.

For the assessment of the risk of infection from exposure to *C. jejuni* the mean was very small because the lower level of detection was small and all of the samples were below the detectable limit. The resulting risks of infection from exposure to *C. jejuni* are shown by the distributions in Figure 37. Each of the distributions represents the risk of infection from exposure to *C. jejuni* in untreated graywater from four different exposure scenarios.

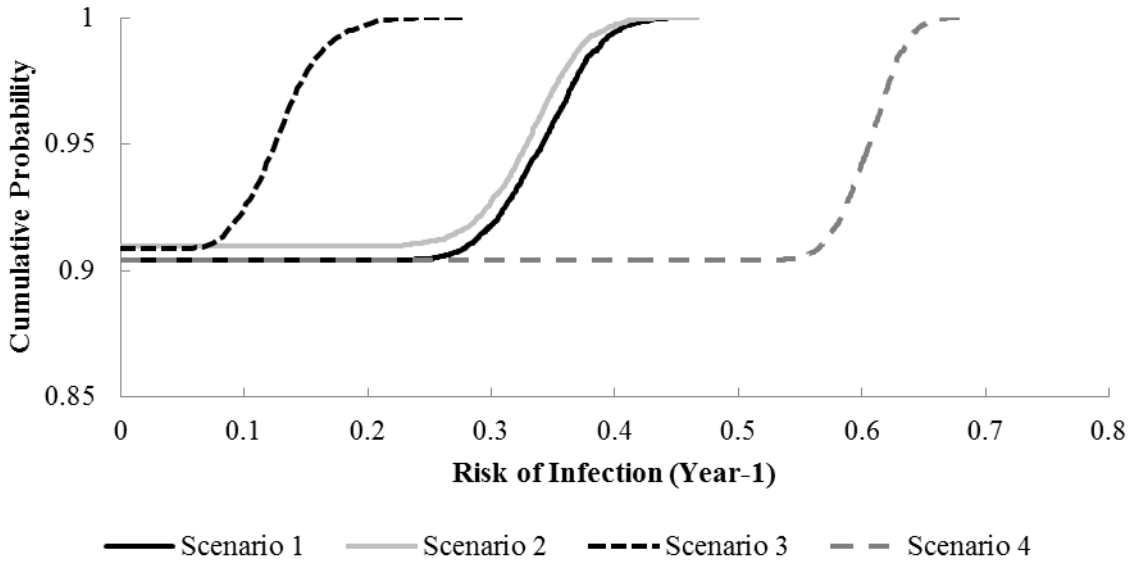


Figure 37 Risk of infection from exposure to *C. jejuni* (Concentration represented using a Poisson distribution fitted to qPCR data)

The resulting risks as shown in Figure 37 are consistent with qPCR data suggesting that for most of the population the risk of infection from exposure to *C. jejuni* in untreated graywater is expected to be zero. However, for a small portion of the population, the risks exceed acceptable limits (1 infection per 10,000 exposures (Fewtrell and Bartram, 2001)). This indicates that pathogen contamination is a valid concern because even small doses can lead to infection, thus it is suggested that proper barriers be put in place to reduce human exposure to *C. jejuni* in untreated graywater.

The mean concentration of *L. pneumophila* was higher that *C. jejuni* because the lower limit of detection was higher and one of the samples had a concentration of *L. pneumophila* that was above the detectable limit. The risks of infection from exposure to *L. pneumophila* are shown in Table 12.

Table 12 Risk of infection from exposure to *L.pneumophila* (Concentration represented using a Poisson distribution fitted to qPCR data)

Exposure Scenario	**Estimate of Risk ($Year^{-1}$)**			
	Mean	**Median**	**5th Percentile**	**95th Percentile**
#1	0.59	1.00	0.00	1.00
#2	0.58	0.99	0.00	1.00
#3	0.36	0.41	0.00	0.88
#4	0.59	1.00	0.00	1.00

The estimated risks of infection from exposure to *L.* pneumophila are unacceptably high for the majority of the population. This suggests that proper barriers should be put in place to reduce human exposure to pathogens in graywater.

Additionally the risk of infection from exposure to *L. pneumophila* in untreated graywater was estimated assuming that the sampling from 28 households represented an empirical distribution of the expected concentration of *L. pneumophila* in untreated graywater. For this scenario the distribution was input as discrete values representing the mean value for each of the eighty samples that were evaluated. This suggested a distribution in which there is a one in eighty chance that the graywater would be contaminated by *L. pneumophila,* and if contaminated it was assumed that the value would be the mean value of the triplicate qPCR reactions performed on the graywater sample that tested positive for *L. pneumophila*.

Assuming that the sampling from 28 households represents a distribution of the expected concentration of *L. pneumophila* in untreated graywater, the risk of infection from exposure to *L. pneumophila* was lower than the previous estimate that used a Poisson distribution to represent the concentration of *L. pneumophila*. Using the results from the

molecular analysis it was determined that there was a one in eighty chance that graywater would be contaminated with *L. pneumophila.* The resulting risks were then estimated and the median risk of infection was zero for each exposure scenario. The 95th percentile estimate of risk of infection was zero for each exposure scenario. The 99th percentile for the risk of infection was 1 for each exposure scenario. The results suggest that there is approximately a 99 percent probability that the risk of infection from exposure to *L. pneumophila* would be zero. However, it also suggests that 1 percent of the population reusing graywater is expected to be infected by *L. pneumophila*. This suggests, that to protect the small portion of the population from infection, proper barriers should be put in place to reduce human exposure to pathogens in graywater.

Each of the estimates provides insight into the human health risks associated with graywater reuse. However there remain uncertainties in all of the assessments of risk that were performed. The risk assessments that relied on a Poisson distribution to represent the concentration of the pathogens are limited because it is difficult to fit a probability distribution to data with many values below the detectable limits. However, using an empirical distribution is limited because it assumes that the 80 samples accurately represent the distribution of the pathogens in graywater and suggests that there are no other possibilities. Additionally, using a Poisson distribution and an empirical distribution to estimate the risks are both limited because the majority of the samples analyzed by qPCR were below detectable limits for the targeted pathogens. Using an empirical distribution assumes that all samples with a pathogen concentration below the detection limit do not contain any of the targeted pathogen which potentially underestimates the pathogen

concentration of the sample. To fit the data to a Poisson distribution required that an assumption be made to determine the pathogen concentration for the samples below the detection limit. It was assumed that all concentrations between zero and the detection limit were equally probable which potentially overestimates the pathogen concentration.

The uncertainty in the model could be reduced by increasing the number of samples, or lowering the detection limit. Increasing the number of samples would provide more data points that would provide a better indication of the expected pathogen concentrations. The detection limit is dependent on the available technology and the volume of graywater that is filtered. Increasing the volume of graywater that is filtered would decrease the lower limits of detection of the pathogen concentration providing a more accurate representation of samples with lower pathogen concentrations.

Conclusions

Using qPCR to quantify pathogens in graywater provided results that were very different than the estimates made using the pathogen concentration model. This suggests that using fecal indicators to estimate the concentration of pathogens will lead to inaccurate results. Additionally the targeted pathogens represented only a small portion of the bacteria in the collected graywater systems indicating that there is still potential that other pathogenic organisms are present in graywater.

qPCR analysis provided a conservative estimate of the concentration of pathogens in graywater which was beneficial in estimating the risks of infection from exposure to pathogens in graywater. These estimates suggested the human health risks exceeded acceptable limits. However, there remains uncertainty in the estimates of risk because it is

difficult to fit a distribution to the qPCR data in which the majority of the samples were below detectable limits. This uncertainty could be reduced by collecting more graywater samples and filtering larger volumes of graywater for qPCR analysis.

The risks that were quantified represent the risks of infection from a few targeted organisms. As previously mentioned the targeted organisms represented only a small portion of the bacteria in the system. This suggests that there is still a need for additional information regarding the microbial characteristics of graywater. Future work should include microbial community analysis in which organisms can be identified with no prior knowledge. Additionally it is important to note that qPCR quantifies organisms by identifying the DNA present in the samples. This provides important information regarding the presence of different organisms, but it does not determine the viability of the organisms present. This suggests that the estimations of risk in this report are conservative estimates because it assumes that all of the detected pathogens are viable. Future work evaluating the microbial characteristics of graywater should include evaluations of the viability of the organisms present in graywater.

CHAPTER 3: MICROBIAL COMMUNITY ANALYIS OF UNTREATED GRAYWATER

Introduction

Due to the growing demand for clean water there is a need for improvements in water resource management. Graywater is a valuable resource that could be utilized to help reduce the need for additional water sources. Graywater is defined as wastewater from bathing and washing facilities that does not contain concentrated human waste (i.e., flush water from toilets) or food waste (i.e., kitchen sink, food waste grinders) (Asano et al., 2007). The absence of human and food waste implies that graywater is relatively free of pathogens and organic matter. An estimated 50-75% of household water consumption produces graywater (Maimon et al., 2010). Due to the lower loads of pathogens and organic matter, and the abundance of graywater, there is great potential for implementing reuse systems that separate graywater from other wastewater sources. Graywater could then be utilized for non-potable end-uses such as irrigation, toilet flushing, and vehicle and home washing, among others. Reusing graywater could provide a constant water supply, which would reduce the amount of potable water that would be needed in the home and would decrease the amount of wastewater that would need to be treated (Maimon et al., 2010).

The previous chapter outlines a basic assessment of the risks of reusing graywater. Graywater samples were collected from 28 households and qPCR was performed to analyze the concentration of waterborne pathogens in graywater. It was found that 5 out of 80 samples collected tested positive for *Bacteroides*, 2 out of 80 samples tested positive for *A. hydrophila*, and 1 out of 80 samples tested positive for *L. pneumophila*. For the samples that

tested positive the targeted organisms represented only a small portion of the total bacteria in the samples. This indicates that there is the potential for other pathogens to be present in the collected samples.

The assessment in the previous chapter used qPCR to quantify pathogens in graywater. It is not feasible to use qPCR to quantify the concentration of every pathogen that may be present in the graywater. However, recent advancements in next generation sequencing technologies make it possible for metagenomic studies to be conducted to identify organisms present in environmental samples with no prior knowledge of the organism being present (Metzker, 2009). A metagenome-based approach offers a more comprehensive analysis of microbial taxonomic diversity of environmental systems (Gomez-Alvarez et al., 2012).

Additionally the risk assessment in the previous chapter neglected to account for the fate of pathogens over time. Several states have established regulations regarding the storage of graywater. In these states allowable storage times range from 24 to 72 hours depending on factors including treatment of graywater, and the end use of the stored graywater (Yu et al., 2012). Rose et al. (1991) analyzed the growth of microorganisms in stored graywater water by measuring the concentration of fecal indicators for a period of twelve days. It was found that the concentration of the fecal indicators increased in the first 48 hours and then remained relatively stable. It is clear that stored graywater can lead to microbial growth. However, there have been no molecular assays analyzing graywater during storage.

The purpose of this research was to analyze the microbial communities present in untreated graywater. Using pyrosequencing data, a more comprehensive genomic survey was

conducted on the graywater samples collected in the previous chapter. The genomic survey is represents the first time that next generation sequencing techniques have been used to evaluate the microbial communities of untreated graywater. Additionally a storage experiment was conducted to evaluate the change in microbial communities over time.

Materials and Methods

Collection of Samples for Genomic Survey

Samples were collected from 28 households near Raleigh, North Carolina. When possible each household provided at least 500 mL of graywater from three sources: (1) Shower/Bath; (2) Bathroom Sink; and (3) Laundry. These samples were collected using 250mL and 500 mL sterilized bottles.

From the 28 households, 29 shower/bath samples, 28 bathroom sink samples, and 23 laundry samples were collected. An attempt was made to collect samples from a variety of households with different sizes, numbers of children and numbers of pets. A summary of the households that provided graywater samples is shown in Appendix D.

After samples were collected, they were transported on ice to the NCSU Environmental Engineering Laboratory and filtered within 24 hours of collection. All samples were filtered using Millipore (Billerica, MA) 0.45μm Sterivex filter and peristaltic pump. Filtered volumes and filter times were recorded. After filtration, Sterivex filters were stored in the -20°C freezer.

DNA Extraction

Filter paper was then removed from Sterivex filters and the total DNA was extracted from filter paper using the Aluminum Sulfate method developed in the NCSU Environmental Engineering Laboratory (Hicks et. al., in preparation) as explained in Appendix C. The concentration of the extracted DNA was determined using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE). A_{260}/A_{280} and A_{260}/A_{230} ratios were also measured using the Nano Drop Spectrophotometer ND-1000 to determine the purity of the extracted DNA. A_{260}/A_{230} values close to 2.00 are desirable because it indicates that DNA is free from carbohydrate and protein contamination. A summary of the extracted DNA is shown in Appendix E.

Metagenomic Analysis

Extracted DNA was sent to the U.S. Environmental Protection Agency (EPA) for metagenomic analysis. Metagenome libraries were generated with the 454 Life Sciences GS-FLX Titanium platform (Gomez-Alvarez et al., 2012). Pyrosequencing reads were used for metagenomic analyses to determine the distribution of members of the *Bacteria* domain present in the graywater samples.

Non-metric multidimensional scaling (nMDS) and cluster analysis was also performed by EPA to identify the relationships between the different community structures of the different graywater samples. This comparison was assessed using a Bray-Curtis similarity coefficient calculated from the relative distributions of the operational taxonomic units (OTU). Additionally, an analysis of similarity (ANOSIM) based on the Bray Curtis

similarity matrix was conducted to determine the similarities between the different sources of graywater.

Description of Graywater Collected for Storage Experiment

Graywater samples were collected from five households near Raleigh, North Carolina. Three of the households that provided graywater samples had children and one household had a dog. Each household provided between 1 and 1.5 liters of graywater from the bathroom sink, laundry and shower or bath.

The total amount of graywater included approximately 5.75 L from the bathroom sink, 7.5 L from the laundry, and 6.25 L from the shower or bath for a total of approximately 19.5 L of graywater. Samples were combined to fill four plastic buckets. Each bucket contained just under 5 L of graywater. The first bucket contained only graywater from bathroom sinks, the second bucket contained graywater only from the laundry, the third bucket contained only graywater from the showers or baths, and the fourth bucket contained combined graywater from each of the previous sources mentioned. The stored graywater was covered but not sealed using a plastic lid. Samples were stored for a period of 23 days.

Sampling Procedures

Samples were collected from each bucket on days 1, 2, 4, 9, 16, and 23. Day 1 represents the day when samples were collected and placed in buckets for storage. Stored graywater was mixed gently prior to sampling and approximately 750 mL of graywater was collected at each sampling period.

Collected samples were analyzed using both traditional culture based methods and molecular techniques. Traditional culture based methods included total and fecal coliform assays. Molecular analysis included qPCR targeting several waterborne pathogens.

Total Coliform Assay

A total coliform assay was performed for each sample that was collected membrane filtration method as described in Standard Methods (Eaton and Franson, 2005). This method includes preparing m-Endo Agar LES (Fluka Analytical). Sterilized solution was prepared and poured into 60 mm petri dishes. After the agar hardened, 100 mL of collected graywater was filtered using 0.2 µm membrane filters. Membrane filters were then placed on prepared agar and incubated at 37°C for 18 to 24 hours. Initially two tenfold dilutions were performed and diluted samples were also filtered and placed on agar, to ensure that the total coliform counts could be determined. Ideal counts range from approximately 20 coliforms to 80 coliforms. Additional tenfold dilutions were performed when necessary.

Total coliform colonies were identified by a pink to dark red color with a metallic sheen. Colonies were counted and the density was determined using Equation 10. The density is reported in units of coliform forming units per 100 mL (CFU/mL).

Equation 10

$$Coliform\ Density = \frac{Counted\ Colonies\ \times 100}{Volume\ Filtered}$$

Fecal Coliform Assay

A fecal coliform assay was prepared for each sample that was collected. The fecal coliform test was performed using the membrane filtration method as described in Standard Methods (Eaton and Franson, 2005). This method includes preparing Difco M-FC Agar which was sterilized and poured into 60 mm petri dishes. After agar hardened, 100 mL of collected graywater was filtered using 0.2 μm membrane filters. Membrane filters were then placed on prepared agar and incubated at 37°C for 18 to 24 hours. Initially two tenfold dilutions were performed and diluted samples were also filtered and placed on agar, to ensure that the total coliform counts could be determined. Ideal counts range from approximately 20 coliforms to 80 coliforms. Additional tenfold dilutions were performed when necessary.

Fecal coliform colonies were identified by their various shades of blue. Colonies were counted and the density was determined using Equation 8. The density was reported in units of coliform forming units per 100 mL (CFU/mL).

Filtering for Molecular Analysis

All samples were filtered using Millipore (Billerica, MA) 0.45μm Sterivex filter and peristaltic pump. Filtered volumes and filter times were recorded. After filtration, Sterivex filters were stored in the -20°C freezer.

DNA Extraction

Filter paper was then removed from Sterivex filters and the total DNA was extracted from filter paper using the Aluminum Sulfate method developed in the NCSU Environmental Engineering Laboratory (Hicks et. al., in preparation) as explained in Appendix C. The

concentration of the extracted DNA was determined using the NanoDrop Spectrophotometer ND-1000. A_{260}/A_{280} and A_{260}/A_{230} ratios were also measured using the Nano Drop Spectrophotometer ND-1000 to determine the purity of the extracted DNA. A_{260}/A_{230} values close to 2.00are desirable because it indicates that DNA is free from carbohydrate and protein contamination. A summary of the extracted DNA is shown in Appendix F.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed to detect and quantify targeted pathogens in graywater samples. Three waterborne pathogens were targeted: *Legionella pneumophila, Campylobacter jejuni,* and *Aeromonas hydrophila.* Additionally *Bacteroides* was targeted as a fecal indicator. For the samples that tested positive for any of the four targeted organisms, a general bacterial primer was also used to quantify the bacteria in the samples.

Using qPCR methods require that standard curves are created plotting C_t values versus known concentrations of DNA. DNA from *A. hydrophila* ATCC 7966D-5, *C. jejuni* ATCC 700819D-5, *L. pneumophila* ATCC 33152D-5, and *Bacteroides vulgatas ATCC 8482D-5,* were purchased from the American Type Culture Collection (ATCC). Additionally a standard curve was developed for the general bacterial primers using Bacteroides *vulgatas ATCC 8482D-5.*

Tenfold dilution series ranging from 1 ng/µL to 1 x 10^{-9} ng/µL for *A. hydrophila,* 5 ng/µL to 5 x 10^{-9} ng/µL for *C. jejuni*, 50 ng/µL to 5 x $10^{—6}$ ng/µL, and 50 ng/µL to 5 x $10^{—4}$ for *Bacteroides* were prepared. qPCR was then performed on triplicates of the dilution series using Bio-Rad iQ5 icycler Multicolor Real-Time PCR Detection System.

The total volume of the each qPCR reaction was 25 µL. This includes 7 µL sterile water, 0.25 µL of both the forward and reverse primers (25 µM), 12.5 µL SYBR Green mix (Bio-Rad iQ SYBR Green Supermix), and 5 µL of the DNA template. Primers and cycling parameters for each of the targeted organisms are shown in Table 13.Table 13 Primers and cycling parameters for targeted organisms

Target	**Primer Sequence (5'-3')**	**Cycling Parameters**
A. hydrophila lip gene[a]	AAC CTG GTT CCG CTC AAG CCG TTG (F), TTG CTC GCC TCG GCC CAG CAG CT (R)	15 min at 95°C, 35 cycles of 60 s at 94°C, 60 s at 62°C, and 90 s at 72°C
C. jejuni mapA gene[a]	GCT AGA GGA ATA GTT GTG CTT GAC AA (F), TTA CTC ACA TAA GGT GAA TTT TGA TCG (R)	10 min at 95°C, 50 cycles of 15 s at 95°C and 30 s at 59°C
L. pneumophila mip gene[a]	GCA ATG TCA ACA GCAA (F), CAT AGC GTC TTG CATG (R)	15 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 54°C, and 60 s at 72°C
Bacteroides 16S rRNA gene[a]	AAC GCT AGC TAC AGG CTT (F), CAA TCG GAG TTC TTC GTG (R)	15 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 59°C, and 60 s at 72°C
Bacteroides 16S rRNA gene[b]	ATG GCTGTCGTCAGCT (F), ACGGGCGGTGTGTAC (R)	3 min at 50°C, 10 min at 95°C 45 cycles of 30 s at 95°C, 60 s at 50°C, and 20 s at 72°C

a-(Ahmed et al., 2008)
b-(Harms et al., 2003)

C_t values for the serial dilutions were determined and a standard curve plotting the C_t value versus the log concentration (ng/µL) of the template DNA was created for each pair of primers. A linear trendline was fit to the data to represent the relationship between the DNA concentration and the C_t values. The R^2 and the PCR efficiency was measured for each standard curve. The PCR efficiency represents how efficiently the DNA replicates. Ideally each cycle of qPCR will result in the DNA doubling. As a result a10 fold increase should occur after 3.32 cycles. Thus for a standard curve featuring 10 fold dilutions the Ct value

should increase by 3.32 for each dilution resulting in a slope of -3.32. The PCR efficiency can be calculated using equation 11.

Equation 11

$$PCR\ efficieny = 10^{-1/slope} - 1$$

Thus for perfect doubling the PCR efficiency is 100%.

qPCR was first used to screen the extracted DNA from graywater samples to determine which of the targeted organism were in each of the graywater samples. This was done by analyzing a single qPCR reaction for each of the graywater samples. The concentration of each of targeted organisms was then quantified in triplicate reactions for the samples that tested positive in the initial screening process. The C_t values were then converted to a concentration (ng/µL), which was subsequently converted to cells/mL using Equation 12.

Equation 12

$$DNA\ (fg) = \left(genome\ size\ (bp)\right) * \left(\frac{MW\ (Da)}{bp}\right) * \left(\frac{1.66 * 10^{-27}\ kg}{Da}\right) * \left(\frac{10^{18}\ fg}{kg}\right)$$

MW represents the molecular weight in Daltons. The relative weight of a base pair is 615.3830 for AT pairs and 616.3711 for GC pairs. The average molecular weight can be calculated using Equation 13.

Equation 13

$$MW(Da/bp) = (GC\ content(\%)) * 616.3711 + (AT\ content\ (\%))(\frac{MW\ (Da)}{bp})*615.3830$$

The genome size, GC content, and the AT content are shown in Table 14.

Table 14 Genome characteristics of targeted organisms

Organism	Genome Size (bp)	GC Content	AT Content	Reference
A. hydrophila ATCC 7966	4,744,448	61.5%	38.5%	Seshadri et al. (2006)
C. jejuni ATCC 700819	1,641,481	30.6%	69.4%	Parkhill et al. (2000)
L. pneumophila ATCC 33152	3,353,636	38.4%	61.6%	NCBI (2013)
Bacteroides Vulgatus ATCC 8482	4,781,702	42.0%	58.0%	Cuiv et al. (2011)

Note: Values shown for *L. pneumophila* represent the average value from several studies that have analyzed the genome sequences of various strands of *L. pneumophila*

Using Equations 12 and 13 the relative mass of each organism was found as shown in Table 15.

Table 15 Mass of targeted organisms

Organism	Mass per Cell (fg/cell)
A. hydrophila ATCC 7966	4.85
C. jejuni ATCC 700819	1.68
L. pneumophila ATCC 33152	3.43
BacteroidesVulgatus ATCC 8482	4.89

The number of cells per mL in each reaction was then found by taking the concentration (ng/µL) as determined by qPCR reactions and dividing that value by the mass per cell as shown in Table 15. The number of cells per mL in the collected graywater samples was then determined using equation 14 .

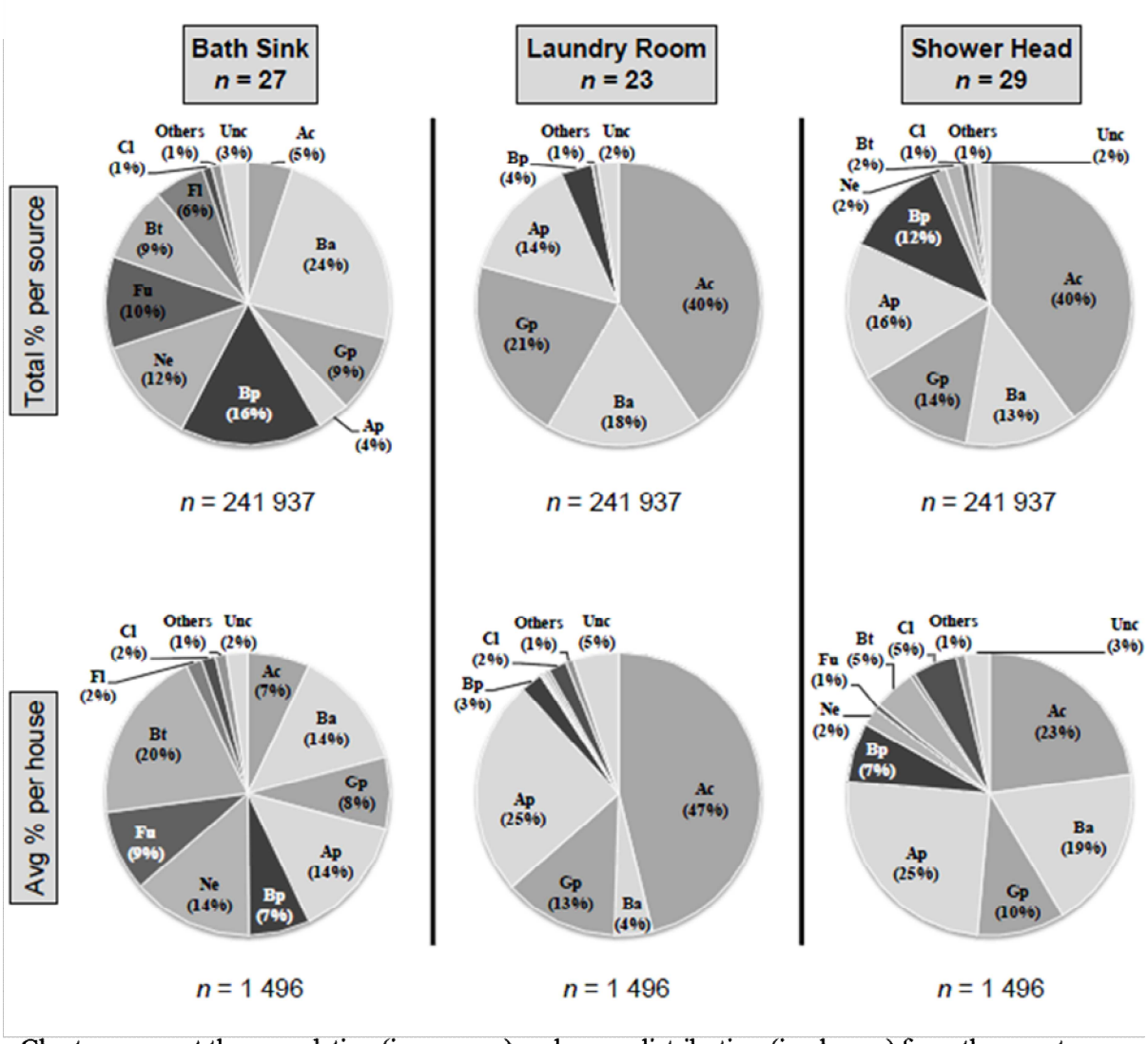
Equation 14

$$\frac{cells}{mL} = \left(DNA\ concentration\ \left(\frac{ng}{\mu L}\right)\right) * \left(\frac{100\ \mu L}{Extract}\right) * \left(\frac{Volume\ Filtered\ (mL)}{Extract}\right) * \left(\frac{10^6\ fg}{ng}\right) * \left(\frac{Cell)}{Mass\ of\ Cell\ (fg)}\right)$$

Results and Discussion

Metagenomic Analysis

There were a total of 241,937 pyrosequencing reads, with an average of 1,496 reads per sample, used in the metagenomic analyses. Pyrosequencing reads were used to determine the distribution of members of the *Bacteria* domain present in the graywater samples. The relative abundance of different taxonomic classes was identified for each of the three sources of graywater. For each source the taxonomic classes were reported as the total percent per source and the average percent per household. The results of this analysis are shown in Figure 38.



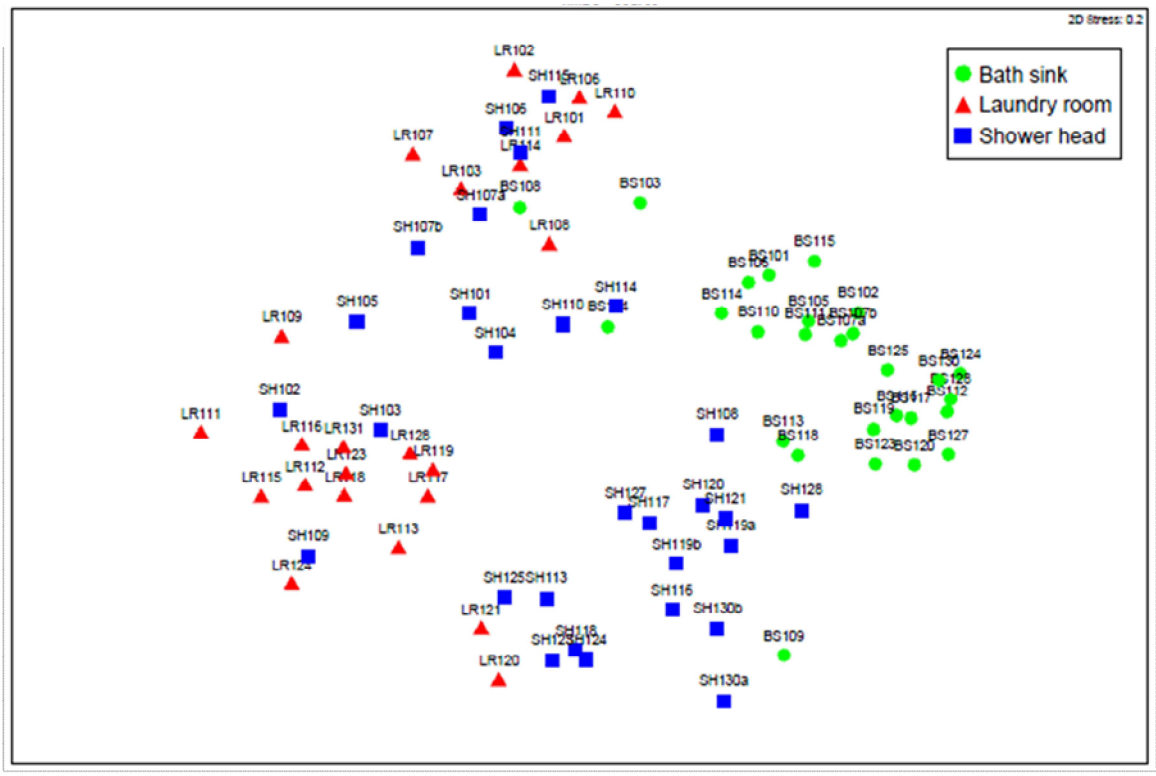
Charts represent the cumulative (i.e. source) and mean distribution (i.e. house) from three water sources. Numbers in parenthesis represent percentage of each class. Legend: Actinobacteria (Ac); Bacilli (Ba); Gammaproteobacteria (Gp); Alphaproteobacteria (Ap); Betaproteobacteria (Bp); Negativicutes (Ne); Fusobacteria (Fu); Bacteroidia (Bt); Flavobacteria (Fl); Clostridia (Cl); Others (classes each representing <1%); unclassified (Unc).

Figure 38 Distribution of the Bacteria domain as determined by taxonomic identification of partial 16S rRNA gene sequencing (at class level)

Based on this analysis *Alphaproteobacteria*, *Bacilli*, *Betaproteobacteria*, *Bacteroidia*, and *Negativicutes* are the classes of *Bacteria* that are dominant in graywater generated from

bathroom sinks. *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, and *Gammaproteobacteria*, are the most abundant classes of *Bacteria* in graywater generated from the laundry. *Actinobacteria*, *Alphaproteobacteria*, *Bacilli* and *Gammaproteobacteria* represented the majority of *Bacteria* in graywater generated from the shower/bath.

Using non-metric multidimensional scaling (nMDS) an ordination plot was created to illustrate the similarity between the different samples that were collected. The similarity was determined based on the Bray-Curtis similarity coefficients and was calculated from the presence of operating taxonomical units (OTUs). The results of this analysis are shown in Figure 39.



The analysis was based on Bray-Curtis similarity coefficients calculated from the relative distribution of OTUs.

Figure 39 Non-metric multidimensional scaling (nMDS) ordination plot representing the potential relationship between samples from three graywater sources

As shown in Figure 39, the samples that are plotted close together represent samples that are similar, while samples that are plotted far apart represent dissimilarities regarding the OTUs present in the sample. As shown there is a cluster of samples collected from the bathroom sink that indicates that graywater from bathroom sinks are similar. Although there is not one distinct cluster for the laundry and shower/bath samples it appears that most of the samples are near, or similar, to other samples from the same source. Likewise, there appears to be laundry and shower/bath samples that are quite different.

The analysis of similarity (ANOSIM) was conducted on the Bray-Curtis similarity matrix that was developed for nMDS ordination plot. Global and pairwise test were conducted to evaluate the effect of the different graywater sources on the metagenomic structure. The results of this analysis are shown in Table 16.

Table 16 Results of one-way ANOSIM test based on Bray-Curtis similarity matrix derived from the distribution of microbial communities

	R Statistic	**P-Value**	**Permutation**
Global Tests	0.483	0.001	999
Pairwise Tests			
Bath Sink vs Laundry	0.739	0.001	999
Bath Sink vs Shower/Bath	0.488	0.001	999
Laundry vs Shower/Bath	0.182	0.001	999

Significance was set at $\alpha=0.05$

The results shown in Table 16 indicate a significant difference between the different sources of graywater ($P < 0.05$). This indicates that the microbial communities are significantly different for each source of graywater. This suggests that the risks of reusing the different sources of graywater are not necessarily the same and thus future evaluations should continue to evaluate each source of graywater separately.

There are many factors that could contribute to the variation between the different sources of graywater. These factors could include the route of contamination, water source, hygiene and laundry products that were used, and the presence of children or pets among others. To evaluate the similarities and differences between the different graywater samples a more extensive survey evaluating various factors should be performed and additional samples should be collected to increase the sample size.

Total Coliform Assay

Figure 40 shows both a positive and a negative result using the total coliform assay.

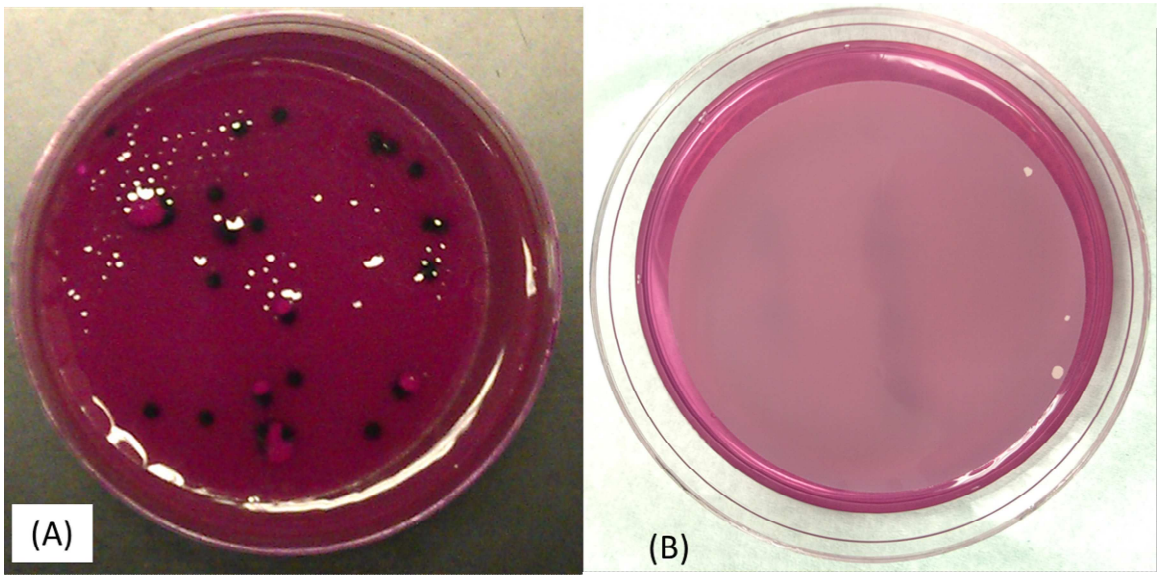


Figure 40 (A) Positive Total Coliform Result (B) Negative Total Coliform Result

The fecal coliform colonies were counted for each assay that was performed the resulting counts are shown in Figure 41.

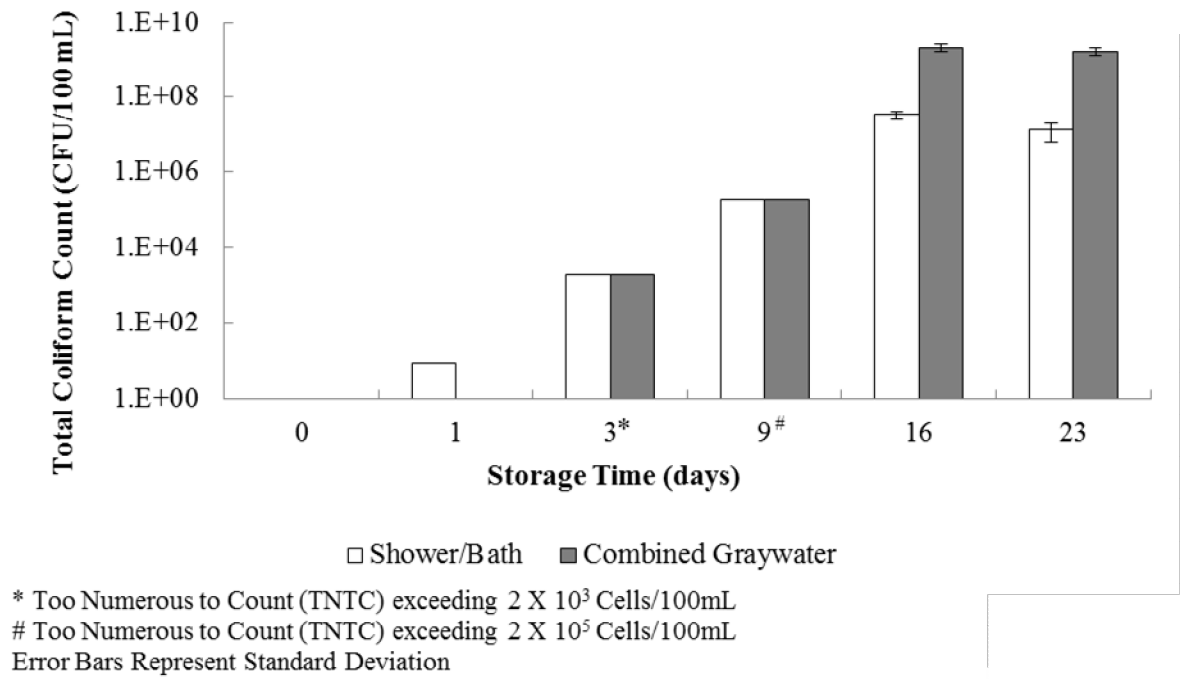


Figure 41 Total Coliforms in stored graywater

As shown in Figure 41 the initial concentration of total coliforms present in the stored graywater was below detection. However over time there was an increase in the concentration indicating bacterial growth. This was consistent with previous studies suggesting microbial growth in stored graywater (Rose et al., 1991). Total coliforms were not detected in the graywater from the bathroom sink or the laundry graywater.

Fecal Coliform Assay

Figure 42 shows both a positive and a negative result using the fecal coliform assay.

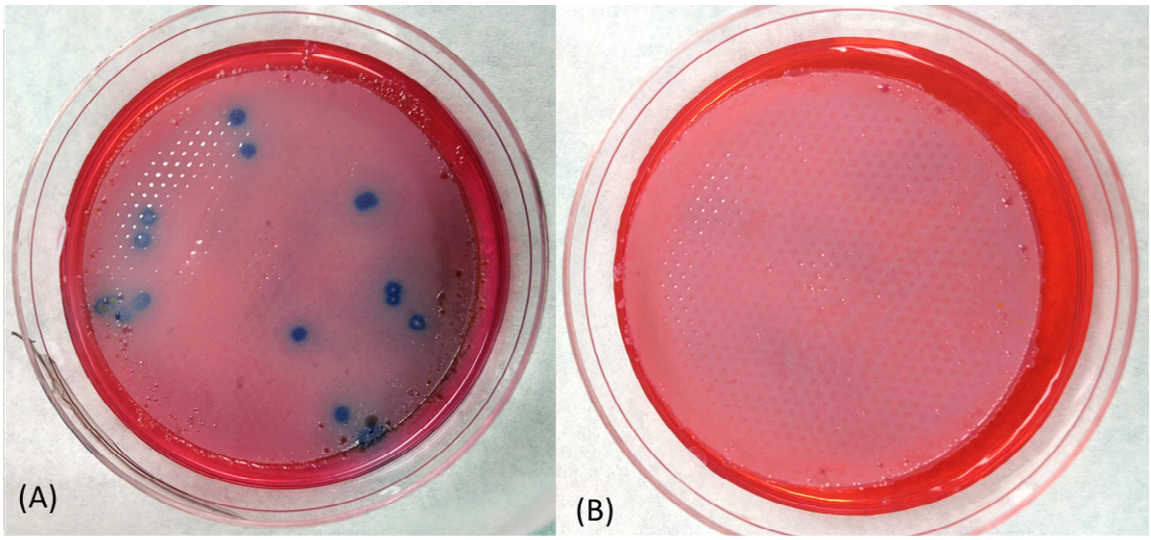


Figure 42 (A) Positive Fecal Coliform Result (B) Negative Fecal Coliform Result

The fecal coliform colonies were counted for each assay that was performed the resulting counts are shown in Figure 43.

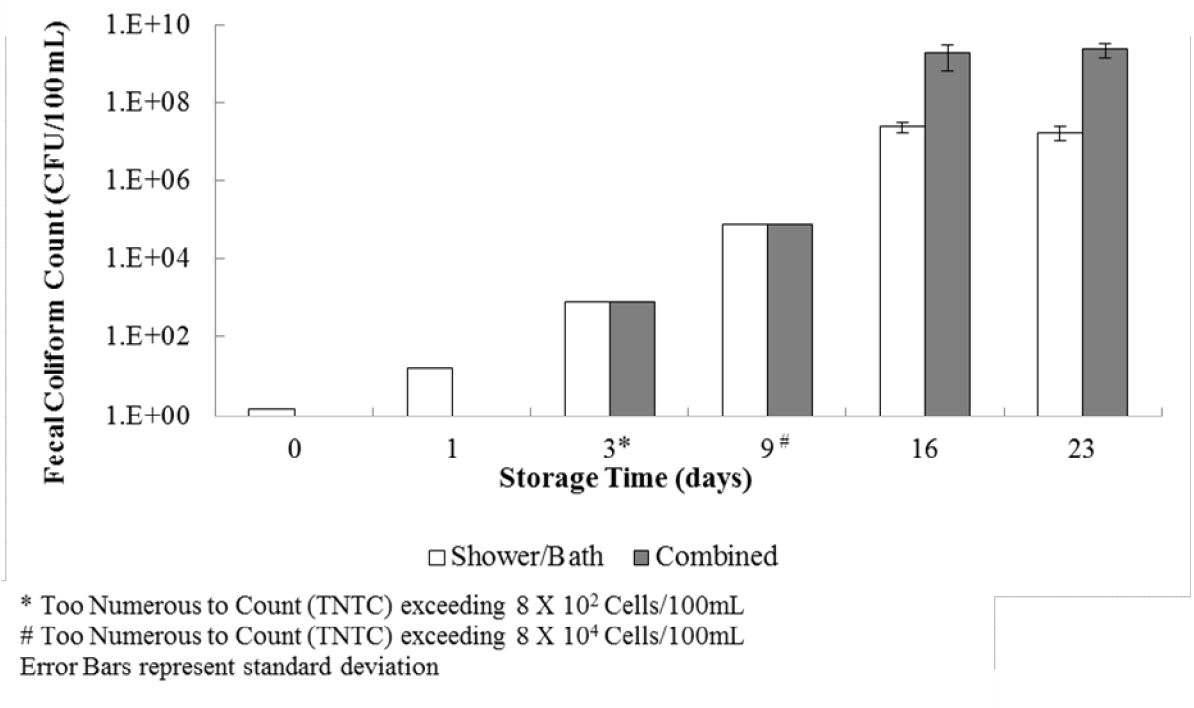


Figure 43 Fecal Coliforms in stored graywater

As shown in Figure 43 the results of the fecal coliform assay were consistent with the total coliform assays and previously published reports(Rose et al., 1991). The initial concentration of the fecal coliforms present in the stored graywater was very low. However over time there was an increase in the concentration indicating bacterial growth. Fecal coliforms were not detected in the graywater from the bathroom sink water or the laundry.

DNA Extraction

The mean concentration of the extracted DNA is shown in Figure 44.

Figure 44 Mean concentration of extracted DNA

In Figure 44 the mean concentration is represented by the white diamond and the error bars represent the standard deviation of the DNA concentration from the six samples from each type of graywater.

A_{260}/A_{280} and A_{260}/A_{230} ratios were not always close to the desirable value of 2.0. However, PCR amplification using a general bacterial primer was used to verify that DNA could still be amplified. PCR amplification yielded positive results indicating that DNA could be used for further analysis (Appendix B). A summary of the extracted DNA is shown in Appendix F.

Quantitative Polymerase Chain Reaction (qPCR)

The concentration of the targeted pathogens was quantified for each sample that was collected. A positive control containing a tenfold serial dilution of the targeted organism and a no template control was included on every plate.

The standard curve that was prepared to quantify *Bacteria* is shown in Figure 45.

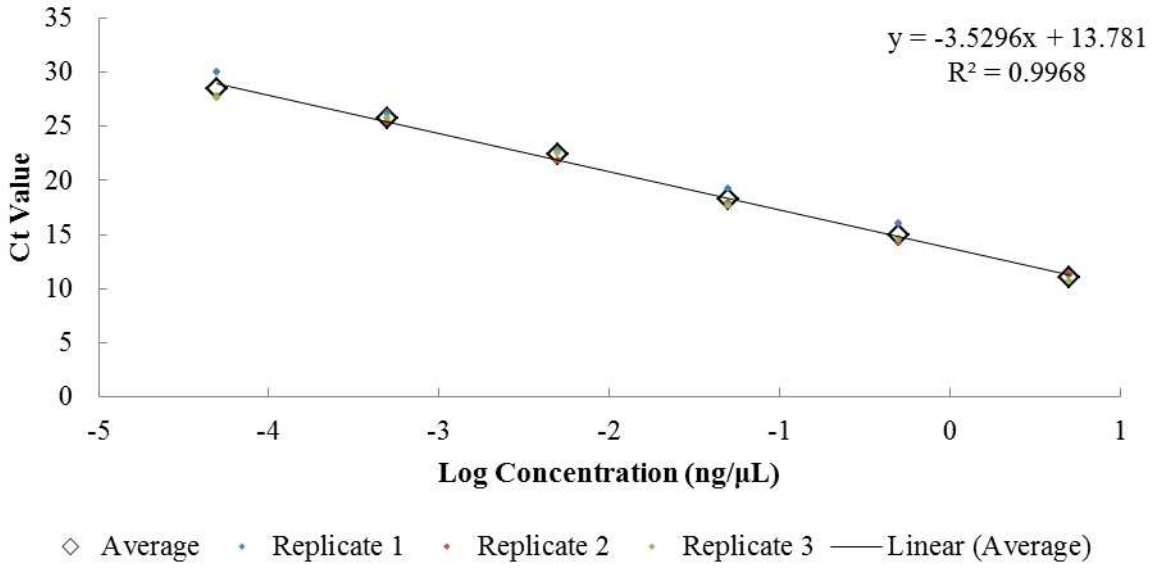


Figure 45 Standard Curve General Bacteria (PCR Efficiency=92%)

The standard curve resulted in a linear trendline with with an R^2 value greater than 0.99 indicating a high correlation between C_t Values and the DNA concentration. The efficiency of the qPCR standard curve was 92%. For each of the standard curves only the data points that represented a linear relationship were included.

qPCR targeting the four different organism was performed for each of the samples that were collected from the storage experiment. Of the samples that were collected none tested positive for *A. hydrophila, C. jejuni, L. pneumophila,* or *Bacteroides.* The concentration of the general bacteria was also determined using qPCR. The concentration of the general bacteria is shown in Figures 46 through 49. Each of these figures represents the different types of graywater analyzed in the storage experiment.

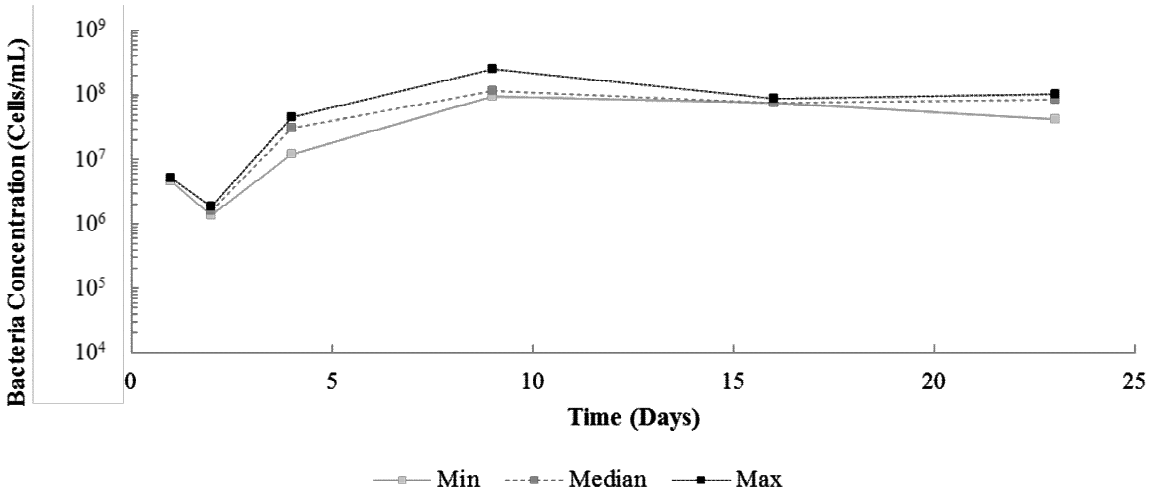


Figure 46 Concentration of bacteria in bathroom sink water stored over time

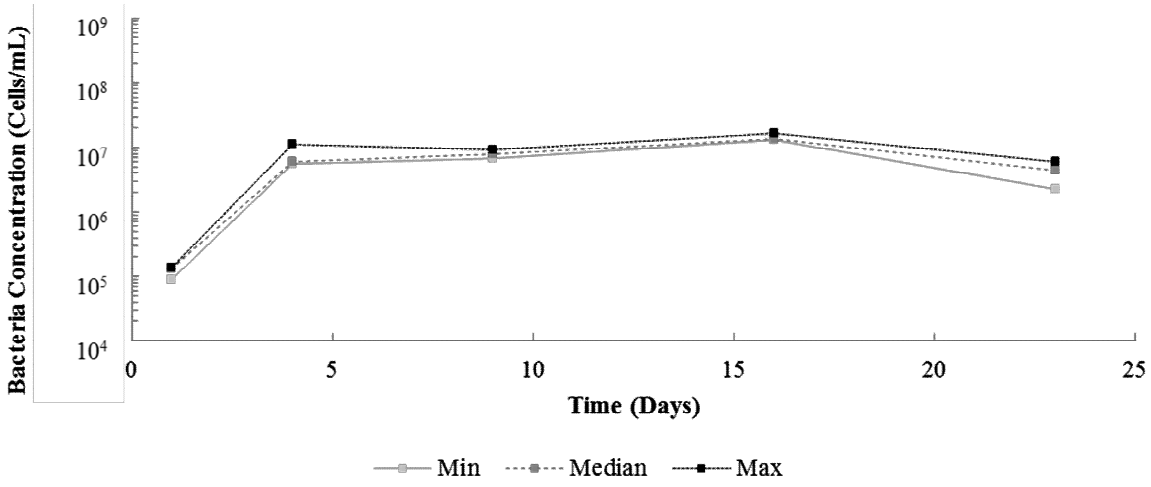


Figure 47 Concentration of bacteria in laundry water stored over time

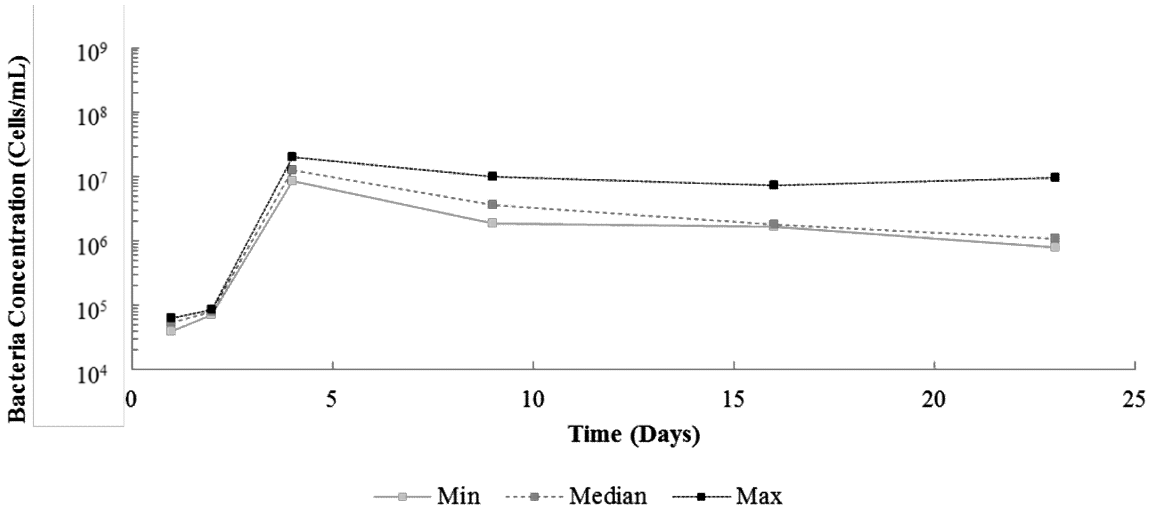


Figure 48 Concentration of bacteria in shower/bath water stored over time

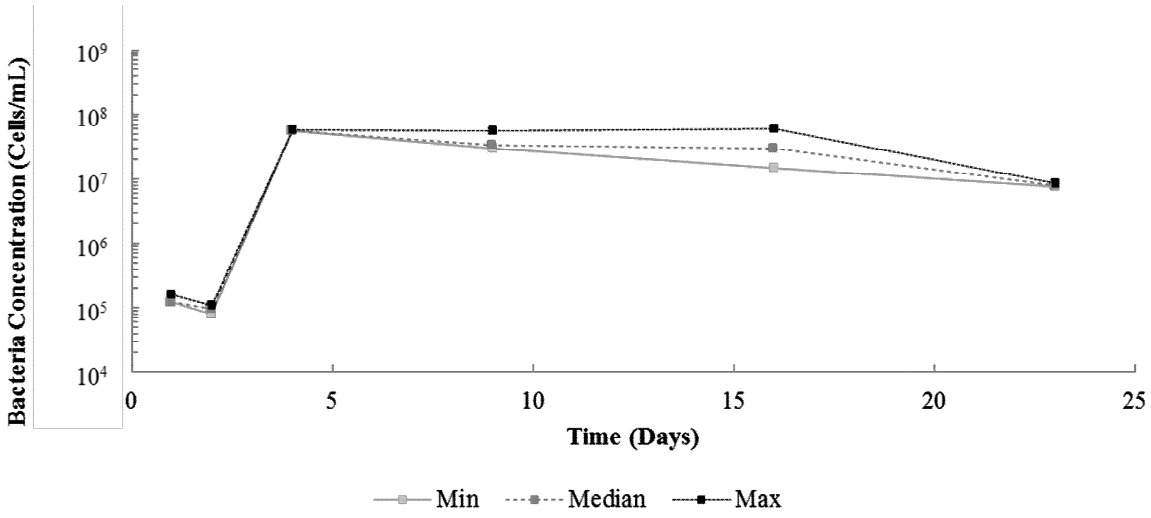


Figure 49 Concentration of bacteria in combined graywater stored over time

The concentration of bacteria in the different types of graywater resulted in trends that indicate bacterial growth within the first 4 days. Each of the different types of water had an initial concentration of bacteria that ranged from a mean of 5.21×10^4 to 4.81×10^6 cells

per mL and then resulted in an increase in bacteria in the first 4 days. After the initial growth the bacterial growth stopped and concentrations remained relatively stable. These results are consistent with the coliform assays where there was initial increase in coliform forming bacteria and then the concentrations of bacteria began to stabilize. Graywater typically has a high concentration of easily degraded organic matter that is favorable to bacterial growth (Ottoson and Stenstrom, 2003). However, in the storage experiment there was a finite amount of organic matter. After the organic matter was consumed by the bacteria in the stored graywater, the growth began to slow. It is expected that over time the concentration would begin to decline due to the lack of food available for the bacteria.

Conclusions

This was the first metagenomic analysis of graywater using next generation sequencing techniques. In this analysis the microbial communities were identified at the taxonomic class level. Further analysis can be directed to the taxonomic classes that were present. Additionally the different sources of graywater were determined to be significantly different based on the operating taxonomic units present in collected samples. This suggests that the microbial communities are significantly different for each source of graywater indicating the human health risks associated with reusing graywater could vary from source to source. Thus, human health risks associated with graywater reuse should be evaluated separately for each source of graywater.

The storage experiment was consistent with previous reports (Rose et al., 1991) indicating that stored graywater can provide an environment that is suitable for bacterial growth. In the storage experiment there was an initial increase in the concentration of

bacteria in the stored graywater. The concentration of bacteria then began to stabilize likely due to the finite amount of organic matter in the graywater. However, for most graywater reuse scenarios a constant supply of graywater will pass through storage containers. This suggests that there will also be a continuous supply of organic matter for the bacteria present in the stored graywater. Thus it is recommended that graywater water not be stored for more than 24 hours. This coincides with many state regulations that suggest that graywater should be stored for less than 24 to 72 hours (Yu et al., 2012).

CHAPTER 4: SUMMARY AND CONCLUSIONS

Graywater is a valuable resource that can be utilized to reduce the amount of potable water needed in the home. However, reusing graywater presents human health risks that must be properly managed. The human health risks were evaluated in this report by quantifying the microbial characteristics of graywater and then assessing the risks of reusing graywater.

qPCR was used to quantify the concentration of pathogens in untreated graywater. Very few samples tested positive for the targeted pathogens. No graywater samples tested positive for *C. jejuni*, one out of eight tested positive for *L. pneumophila*, and two out of eighty tested positive for *A. hydrophila*. The concentration of the pathogens represented only a small portion of the bacteria present in the graywater indicating a need for further analysis of the microbial communities present in graywater. Additionally it needs to be acknowledged that this study quantified pathogens based on the DNA present in graywater and did not account for the viability of the pathogens present. This method provides a conservative estimate because it is not known if all of the pathogens present in the samples are active.

A preliminary risk assessment showed that using fecal indicators to estimate the concentration of pathogens in graywater introduces uncertainty into the risk assessment model. Quantifying pathogens in graywater using qPCR reduces the uncertainty in the model because it eliminates the need to estimate the pathogen concentration using fecal indicators.

The results of the qPCR were used to estimate the human health risks associated with reusing untreated graywater. The estimated risk of infection resulted in estimates that varied from the estimates in the preliminary risk assessment. However, for at least a portion of the population the risk of infection from exposure to *C. jejuni* and *L. pneumophila* exceeded

acceptable limits suggesting that proper barriers be put in place to reduce human exposure to pathogens in untreated graywater.

This was the first metagenomic analysis of graywater using next generation sequencing techniques. In this analysis the microbial communities were identified at the taxonomic class level. Further analysis can be directed to the taxonomic classes that were present. Additionally the different sources of graywater were determined to be significantly different based on the operating taxonomic units present in collected samples. This suggests that the microbial communities are significantly different for each source of graywater indicating the human health risks associated with reusing graywater could vary from source to source. Thus, human health risks associated with graywater reuse should be evaluated separately for each source of graywater.

Evaluating the microbial community shifts during the storage of graywater it was found that graywater can provide an environment that is conducive to microbial growth. Thus it is recommended that graywater not be stored for more than 24 hours.

CHAPTER 5: FUTURE WORK

This project provided valuable information about the human health risks associated with graywater reuse. However, there remains many important questions that need to be answered. Future work about the human health risks associated with graywater reuse should include:

- **Microbial Community Analysis:** This project provided the first genomic survey of graywater. Metagenomic analysis identified different taxonomic classes present in graywater. Future research should continue to utilize next generation sequencing techniques to identify potential pathogenic organisms present in graywater.
- **Pathogen Viability Analysis:** This project utilized DNA present in graywater to identify and quantify pathogens. This method identifies the presence of the targeted organisms. However, it does not determine the viability or the activity of the pathogens present. This affects the risk assessment because pathogens that are no longer active pose little or no human health risks. Future work should include analysis of the RNA present in graywater samples because this can provide insight into the viability of the organisms present.

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APPENDICES

Appendix A Instruction packet given to households providing graywater samples

NC STATE UNIVERSITY

Jory Wahlen
Campus Box 7908
Raleigh, NC 27695-7908
801-668-7886
jbwahlen@ncsu.edu

Dear Participant:

We are seeking your participation in research conducted at North Carolina State University. This research is focused on determining the risks of graywater reuse. Graywater for this study is defined as wastewater that excludes wastewater from toilets and kitchen sinks. Graywater contributes 50-80% of the total household wastewater and therefore if reused could contribute greatly to reducing water consumption nationally and globally. Potential reuses of graywater include toilet flushing, irrigation, vehicle washing as well as many other potential uses. Despite the great potential of reusing graywater, very little is known regarding the characteristics and public health risks. The purpose of this research is to determine the microbial characteristics of graywater. This will be accomplished by collecting graywater samples from at least 30 households. From each of these 30 households we will obtain samples from three graywater sources: (1) Bath/Shower, (2) Bathroom Sink and (3) Washing Machine. The samples will then be analyzed to determine pathogens present in graywater.

You can assist us in this research by collecting and providing graywater samples. If participating, you will be asked to provide a sample from each of the household sources mentioned in the previous paragraph. Materials will be provided as well as detailed instructions explaining the proper sampling procedures. Your participation in this research allows us to analyze graywater from a diverse population, helping us to better understand the characteristics of graywater.

Proper precautions are taken to decrease the risks for you as a participant. The health risks associated with participation in this study are a result of your exposure to graywater samples during collection. However, these risks can be decreased by proper hygiene. After you have collected samples it is important that you wash your hands. Your privacy will also be respected to ensure that personal information remains private.

If concerns exist please feel free to contact me at jbwahlen@ncsu.edu, or 801-668-7186. Thank you for your participation.

Sincerely,
Jory Wahlen
Graduate Research Assistant
North Carolina State University

Participant Procedures

Included Materials:
Participants will be provided with the following materials:
6- 250 mL sterilized bottles

Sampling Procedures:
Samples from three sources will be collected:
1. Bath/Shower
2. Bathroom Sink
3. Washing Machine

Sampling procedures for each of these sources will be conducted as follows:

Bath/Shower:
1. Before bathing/showering, plug drain
2. Bath/Shower as usual
3. After bathing/showering use 250 mL sterilized bottle (labled SH-____a and SH-____b) to collect sample from the used bath/shower water. This can be done by submerging the bottle in the bath/shower water and then removing bottle. When collecting sample ensure that bottle is full. If it is difficult to completely fill bottle, a clean cup may be used to collect bath/shower water, the bath/shower water can then be poured into the bottle.
4. After sample is collected be sure to properly wash hands

Note: The desired volume of bath/shower water is at least 500 mL. To ensure that bath/shower samples represent the entire household, we ask that each member of the household fill a 250 mL bottle. If the household has more than two individuals, additional bottles can be provided. Likewise if the household has less than two individuals, we can provide larger bottles to ensure that at least 500 mL of bath/shower water is collected from each household.

Bathroom Sink:
1. Before washing, plug drain
2. Wash hands, face, brush teeth etc. as usual
3. After washing, use 250 mL sterilized bottle (labeled BS-____a and BS-____b) to collect sample from the used bathroom sink water. This can be done by submerging the bottle in the bathroom sink water and then removing bottle. When collecting sample ensure that bottle is full. If it is difficult to completely fill bottle, a clean cup may be used to collect the water from the bathroom sink, the bathroom sink water can then be poured into the bottle.
4. After sample is collected be sure to properly wash hands

Note: The desired volume of bathroom sink water is at least 500 mL. To ensure that bathroom sink samples represent the entire household, we ask that each member of member of the household fill a 250 mL bottle. If the household has more than two individuals, additional bottles can be provided. Likewise if the household has less

than two individuals, we can provide larger bottles to ensure that at least 500 mL of bathroom sink water is collected from each household.

Washing Machine:

1. Begin laundry as usual
2. During wash cycle when washing machine is full of water, stop machine and use 250 mL sterilized bottle (labeled LR-____a) to collect sample. This can be done by submerging the bottle in the laundry water and then removing bottle.
3. During rinse cycle when washing machine is full of water, stop machine and use 250 mL sterilized bottle (labeled LR-____b) to collect sample. This can be done by submerging the bottle in the laundry water and then removing bottle.
4. After sample is collected be sure to wash hands.

Storage:
After a sample has been collected, please place bottle containing sampled graywater in refrigerator.

Transportation:
Samples are to be filtered within 24 hours; therefore Jory Wahlen should be contacted upon completion of sampling. Jory can be contacted at 801-668-7186 or jbwahlen@ncsu.edu. He will arrange to pick up and transport samples.

Participant Survey

General Questions

Laundry Sample? Yes ☐ No ☐

Bathroom Sink? Yes ☐ No ☐

Bath/Shower? Yes ☐ No ☐

Household Size: _____

Number of Adult Males: _____

Number of Adult Females: _____

Number of Children: _____

Number of Pets: _____

Type of Pets: _____

_____

Laundry Questions

Wash Cycle? Yes ☐ No ☐

Rinse Cycle? Yes ☐ No ☐

Detergent? Yes ☐ No ☐

Bleach? Yes ☐ No ☐

Did Children Contribute to Laundry: Yes ☐ No ☐

Number of Individuals Contributing to Laundry: _____

Other Considerations Regarding Laundry Sample:

_____

_____

_____

_____

_____

_____

_____

Bathroom Sink Questions

Number of Individuals Contributing to Bathroom Sink Sample: _____

Did Children Contribute to Bathroom Sink Sample? Yes ☐ No ☐

Teeth Brushing? Yes ☐ No ☐

Hand Washing? Yes ☐ No ☐

Face Washing? Yes ☐ No ☐

Shaving? Yes ☐ No ☐

Other Considerations Regarding Bathroom Sink Sample:

_____

_____

_____

_____

Bath/Shower Questions

Number of Individuals Contributing to Bath/Shower Sample: _____

Did Children Contribute to Bath/Shower Sample? Yes ☐ No ☐

Bath? Yes ☐ No ☐

Shower? Yes ☐ No ☐

Other Considerations Regarding Bath/Shower Sample:

_____

_____

_____

_____

_____

Appendix B Optimization of Sampling Procedures

Before samples were collected the sampling, filtering, and DNA extraction protocols needed to be optimized. Graywater samples were filtered so that the microorganisms could be concentrated and then the DNA could be extracted from the filter paper. Initially it was proposed that the samples would be filtered using 0.2µm membrane filter and a vacuum pump as shown in Figure 50.

Figure 50 Thermo Scientific Nalgene® Polysulfone Filter Holder

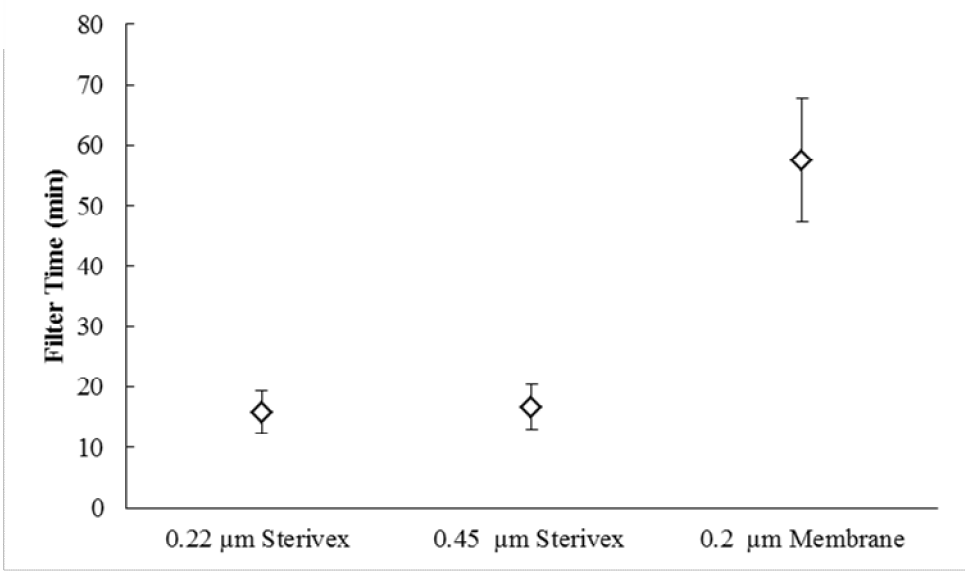
Despite the fact that this method resulted in positive DNA extraction, other filtering methods where analyzed because this method required long filtering times. The other filtering methods that were examined were using a 0.4µm membrane filter and using an inline cartridge filters.

Using 0.4µm filter paper initially provided optimistic results as the first 250mL were filtered within 3 minutes, however the pores soon became clogged and the next 150mL took over 1 hour to filter. This indicated that for 500mL, 0.4µm filters and 0.2µm filters would result in similar filtering times.

The other method that was considered was the use of inline cartridge filters. For this method a peristaltic pump was used to pass samples through a Millipore Sterivex inline cartridge filter. Sterivex filters are shown in Figure 51.

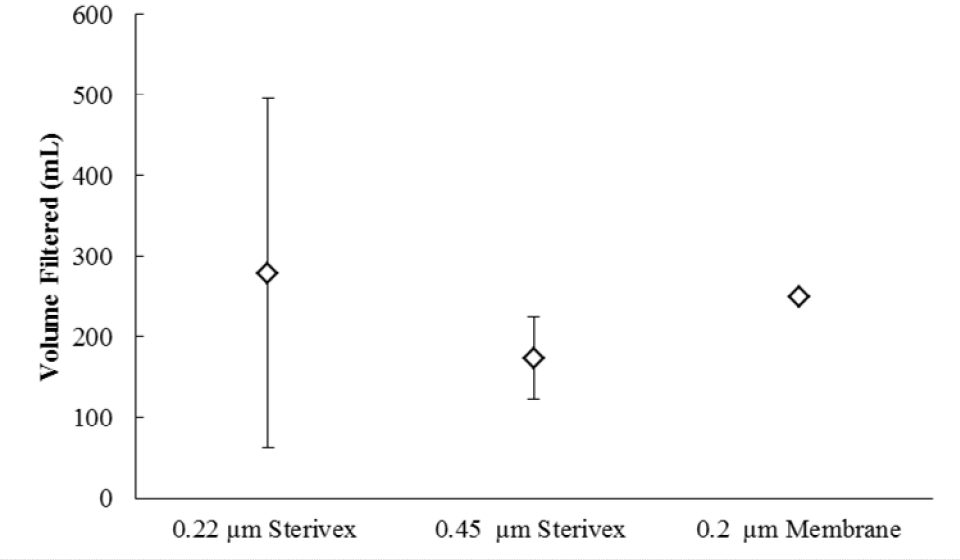
Figure 51 Sterivex Inline Cartridge Filter

Both 0.22μm and 0.45μm Sterivex filters where analyzed. To determine the feasibility of using Sterivex filters, filtering times and volumes where compared for each filtering method. Results are shown in Figures 52 and 53 respectively.



Note: Error Bars Represent Standard Deviation

Figure 52 Mean Filtering Times for Each Filtering Method



Note: Error Bars Represent Standard Deviation. No error bars for 0.2 µm membrane filter because 250 mL was filtered for each sample filtered by 0.2 µm membrane

Figure 53 Mean Filtered Volume for Each Filtering Method

As shown, the filtering times are much lower for the Sterivex filters. The filtered volumes shown do not appear to have a quantifiable advantage for either method. It is expected that as the number of samples that are filtered increase, the range of the volumes filtered by each of these methods will be similar.

Due to shorter filtering times, the Sterivex filters were further analyzed to determine the DNA yield, quality, and success of PCR amplification. This analysis was based on the comparison of the same sample filtered with a 0.22µm Sterivex filter and a 0.45µm Sterivex filter as shown in Table 17.

Table 17 Comparison of 0.22µm and 0.45µm Sterivex Filters

	0.22 µm Sterivex Filter	**0.45 µm Sterivex Filter**
Sample	SH-4	SH-6
Type	Shower	Shower
Volume Filtered	600 mL	200 mL
DNA Concentration	11.59 ng/µL	5.59 ng/µL

The 0.22µm Sterivex filtered a larger volume and yielded a higher concentration of DNA. The DNA that was extracted from the two samples was amplified by PCR using universal bacterial primers 8f and 1492r. The extracted DNA was visualized using gel electrophoresis as shown in Figure 54. Gel electrophoresis is performed to verify the presence of the gene that is targeted during PCR. Gel electrophoresis separates DNA strands based on the length. A DNA ladder is used a positive control to identify the expected lengths.

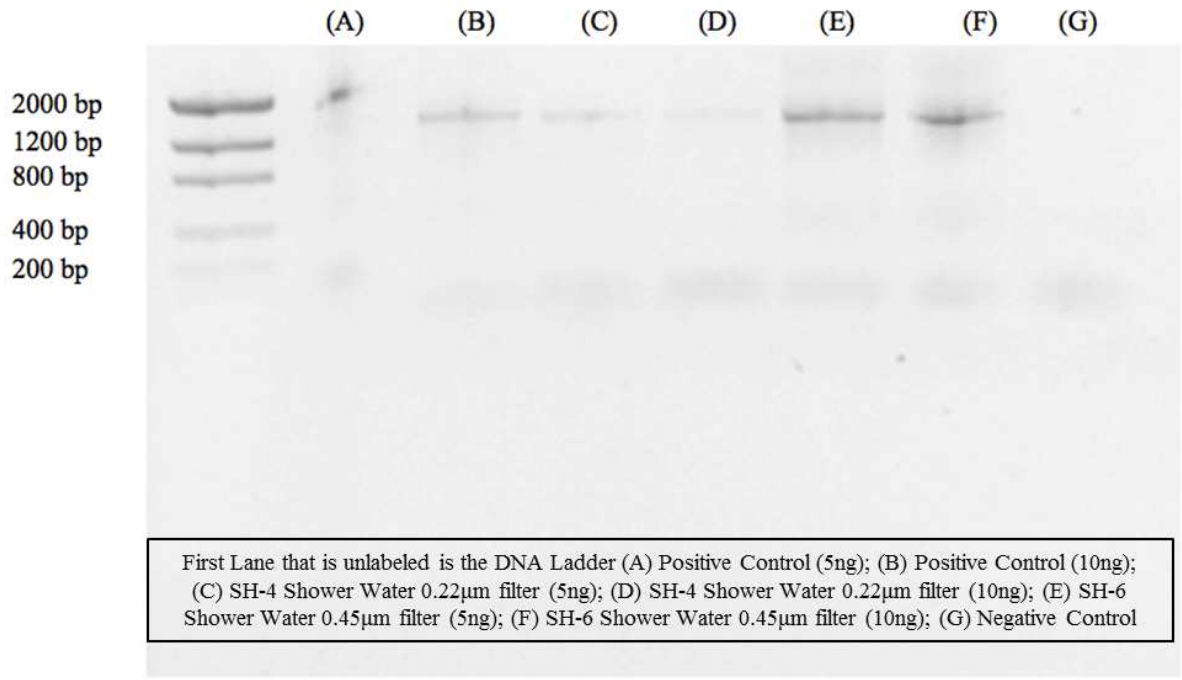


Figure 54 PCR Results Using a Universal Bacterial Primer

Figure 54 shows that lanes A through F contain a positive band that falls between the 2000 bp and 1200 bp. The bands representing samples filtered by 0.45µm Sterivex filters appear darker than the bands representing samples filtered by 0.22µm Sterivex filters. This suggests that there filtering with 0.45µm Sterivex filter produces higher quality PCR product. It was thus determined that the 0.45µm Sterivex filters should be used for filtering collected samples. This conclusion was made despite the fact that the 0.22µm filters were able to filter larger volumes and yield higher DNA concentrations because as shown in Figure 54 the 0.45µm Sterivex filter resulted in a more distinct PCR product.

Appendix C Laboratory DNA Extraction Method (Aluminum Sulfate Method)

Materials Needed:

1) Humic acid removal solution: 100 mM NaH_2PO_4; 100 mM aluminum sulfate (pH 6.0)

2) Lysis solution: 100mM NaCl, 500mM Tris, 10% w/v sodium dodecyl sulfate; 1% sodium pyrophosphate (pH 9.0)

3) 100% Isopropanol

4) 70% Ethanol

5) 7.5M Ammonium Acetate

6) TE Buffer (pH 8.0)

7) Zirconium beads (100um; previously baked at 200^{o}C for 2hours)

8) pH microelectrode (note: Do not use electrode until you have been trained to do so)

Method:

1) Humic substances are precipitated prior to cell lysis by addition of a low pH aluminum sulfate solution.For groundwater filters, add 800µl of humic acid solution to a 250 ml filter. (sit 15 min)
2) Vortex and measure pH. If needed, adjust to 6.5 by addition of HCl. Record pH.
3) Raise pH to 9.0-9.5 with sodium hydroxide (NaOH) and vortex briefly. Be careful not to go above pH 10.0.
4) Add 0.25g acid washed zirconium beads (100 µm diameter).
5) Add 400µl of Lysis Solution
6) Bead beat at max speed 1 minute

7) Centrifuge tubes for 5 minutes at 13,200 rpm and transfer clean supernatant. Centrifuge again if necessary to remove debris.
8) Remove protein by adding 0.5 volume of 7.5M ammonium acetate.
9) Invert to mix. Incubate on ice for 10 minutes.
10) Centrifuge tubes for 5 minutes at 13,200 rpm and transfer supernatant. Centrifuge again if necessary to remove white precipitate.
11) Add 1 volume of 100% isopropanol to supernatant and invert to mix.
12) Incubate at room temperature 5 minutes.
13) Centrifuge tubes for 5 minutes at 13,200 rpm and carefully decant supernatant.
14) Add 1 ml of 70% ethanol to DNA pellet. Vortex briefly
15) Centrifuge tubes for 5 minutes at 13,200 rpm and carefully remove all ethanol using a pipette.
16) Allow pellet to air dry (~5 minutes). Do not over dry.
17) Resuspend pellet in 100 µl of TE buffer (pH 8.0)

Appendix D Summary of Households Providing Graywater Samples

Household	Size	Children	Pets	Type of Pet
1	2	0	0	N/A
2	2	0	0	N/A
3	2	0	1	Cat
4	1	0	0	N/A
5	2	0	0	N/A
6	2	0	1	Dog
7	5	3	2	Dog and Cat
8	2	0	2	Cats
9	1	0	0	N/A
10	1	0	0	N/A
11	1	0	1	Cat
12	2	0	0	N/A
13	1	0	0	N/A
14	2	0	0	N/A
15	4	2	2	Dog and Cat
16	3	1	1	Dog
17	4	2	0	N/A
18	3	1	0	N/A
19	4	2	0	N/A
20	5	3	0	N/A
21	3	1	1	Dog
23	3	1	0	N/A
24	2	0	0	N/A
25	2	0	0	N/A
27	6	4	1	Dog
28	4	2	0	N/A
30	3	1	0	N/A
31	3	1	0	N/A

Appendix E Summary of extracted DNA

Type	Household	Average Concentration (ng/µL)	Average 260/280	Average 260/230
BS	1	18.73	1.66	70.59
BS	2	38.48	2.02	15.56
BS	3	0.71	3.67	-0.05
BS	4	26.61	1.56	1.24
BS	5	31.45	1.66	2.00
BS	6	9.27	1.58	-0.80
BS-A	7	29.85	1.97	-9.54
BS-B	7	27.69	1.97	3.15
BS	8	11.87	1.65	-2.98
BS	9	176.23	2.06	2.36
BS	10	38.46	1.58	1.12
BS	11	9.61	1.79	-0.58
BS	12	63.18	2.00	5.56
BS	13	2.01	1.12	-0.08
BS	14	12.06	1.46	-0.94
BS	15	16.02	1.64	-1.80
BS	16	23.85	1.65	-5.87
BS	17	55.09	2.01	-1.44
BS	18	9.30	1.48	-0.70
BS	19	5.39	1.57	-0.26
BS	20	4.86	1.49	-0.28
BS	21	11.01	1.44	-1.29
BS	23	11.91	1.67	-1.03
BS	24	9.17	1.75	-0.43
BS	25	16.09	1.65	-0.99
BS	26	7.05	1.58	20.07
BS	27	27.44	1.92	-4.28
BS	28	32.81	1.61	1.73
BS	29	5.45	1.84	-0.73
BS	30	20.71	1.79	-17.88

BS – Indicates graywater collected from the bathroom sink

Appendix E continued

Type	Sample	Average Concentration (ng/µL)	Average 260/280	Average 260/230
LR	1	19.47	1.50	1.53
LR	2	6.26	1.60	-0.37
LR	3	10.39	1.54	-1.20
LR	4	-	-	-
LR	5	-	-	-
LR	6	50.77	1.57	0.79
LR	7	10.10	1.72	-0.56
LR	8	55.44	1.50	0.75
LR	9	281.51	2.04	2.35
LR	10	92.06	1.49	0.73
LR	11	15.82	1.63	4.39
LR	12	2.81	8.02	-0.11
LR	13	7.59	1.41	-0.72
LR	14	12.95	1.37	-4.11
LR	15	12.65	1.61	-1.41
LR	16	3.68	1.23	-0.22
LR	17	7.14	1.54	-0.51
LR	18	3.38	1.04	-0.17
LR	19	10.89	1.39	-1.34
LR	20	9.71	1.54	-1.09
LR	21	4.99	1.28	-0.29
LR	22	6.47	1.36	-5.75
LR	23	4.05	1.21	-0.19
LR	24	1.27	1.14	-0.05
LR	25	-	-	-
LR	26	3.44	2.04	-1.28
LR	27	-	-	-
LR	28	14.08	1.38	-4.78
LR	29	3.99	1.61	-0.32
LR	30	-	-	-
LR	31	20.66	1.66	2.98

LR – Indicates graywater collected from the laundry

Appendix E continued

Type	Household	Average Concentration (ng/µL)	Average 260/280	Average 260/230
SH	1	7.58	1.41	-0.88
SH	2	1.25	0.78	-0.05
SH	3	9.64	1.40	-1.38
SH	4	4.21	1.96	-0.26
SH	5	8.54	1.47	-6.88
SH	6	5.13	1.65	-0.31
SH-A	7	5.19	1.61	-0.23
SH-B	7	5.40	1.75	-0.44
SH	8	6.94	2.03	-0.40
SH	9	204.34	2.08	2.55
SH	10	9.29	2.37	-0.77
SH	11	9.98	1.60	-0.95
SH	12	-	-	-
SH	13	1.97	0.93	-0.09
SH	14	10.73	1.39	-2.38
SH	15	2.77	1.08	-0.12
SH	16	1.98	0.87	-0.08
SH	17	50.33	1.48	0.77
SH	18	2.44	1.01	-0.10
SH-A	19	5.18	1.43	-0.24
SH-B	19	4.83	1.27	-0.22
SH	20	12.76	1.37	-2.00
SH	21	3.68	1.50	-0.16
SH	22	1.59	0.80	-1.62
SH	23	9.10	1.41	-0.69
SH	24	1.75	0.62	-0.05
SH	25	5.08	1.32	-0.29
SH	26	1.54	-4.66	-0.14
SH	27	9.79	1.41	-0.85
SH	28	24.51	1.47	1.50
SH	29	2.70	2.42	-0.15
SH	30	6.73	1.34	-0.46

SH – Indicates graywater collected from shower/bath

Appendix F Summary of extracted DNA from graywater storage experiment

Type	Date	Average Concentration (ng/µL)	Average 260/280	Average 260/230
BS	1/30/2013	17.69	1.75	-1.61
BS	1/31/2013	15.59	1.65	-1.44
BS	2/2/2013	273.28	2.07	2.65
BS	2/8/2013	323.29	2.02	2.44
BS	2/15/2013	142.24	1.99	2.99
BS	2/22/2013	130.27	1.95	2.76
LR	1/30/2013	4.28	1.60	-0.23
LR	2/2/2013	89.52	2.15	5.89
LR	2/8/2013	123.65	1.89	1.67
LR	2/15/2013	119.76	1.82	1.46
LR	2/22/2013	133.38	1.78	1.31
SH	1/30/2013	7.77	1.41	-0.59
SH	1/31/2013	5.90	1.41	-0.33
SH	2/2/2013	94.37	2.06	4.23
SH	2/8/2013	38.48	1.65	1.15
SH	2/15/2013	53.25	1.69	1.07
SH	2/22/2013	74.26	1.52	0.74
CO	1/30/2013	6.87	1.83	-0.48
CO	1/31/2013	6.60	1.54	-0.47
CO	2/2/2013	250.41	2.11	2.45
CO	2/8/2013	175.52	1.98	1.98
CO	2/15/2013	122.04	1.94	2.35
CO	2/22/2013	171.47	2.01	2.37

BS-Indicates graywater samples from bathroom sink
LR-Indicates graywater samples from laundry
SH-Indicates graywater samples from shower/bath
CO-Indicates graywater samples from combined graywater