Indicator Systems for Assessing Public Health Risk in Waters

by

Daniel Oliver Roop

A Thesis

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Master of Science

in

Environmental Engineering

by

_____

Daniel Oliver Roop

May 2012

APPROVED:

_____

Dr. Jeanine D. Plummer, Major Advisor

_____

Dr. John Bergendahl, Committee Member

Abstract

For over one hundred years, indicator organisms such as coliforms have been measured as an index of public health risk from transmission of waterborne diseases. Even so, waterborne disease outbreaks have occurred in systems with negative coliform results, many traced to viral or protozoan etiologies. Conversely, no discernible public health outcomes have occurred in systems with positive coliform results. These inconsistencies arise because coliforms, as bacteria, respond differently to environmental stressors and engineered treatment processes than protozoan and viral pathogens. Recent reviews of four decades of indicator and pathogen monitoring indicated that coliphages are more highly correlated to pathogen presence in a variety of waters than coliforms. Therefore, the goal of this research was to re-examine a variety of traditional and novel indicator systems to determine their value as indicators, either singly or as a toolbox. We collected samples of animal feces, wastewaters, source waters and treated drinking waters. Samples were collected from four geographical regions of the United States (Northeast, South, Midwest and West) to assess spatial variability and in all four seasons to assess temporal variability. Samples were monitored for total coliforms, *E. coli*, male-specific and somatic coliphages, and other physical and chemical water quality parameters including organic carbon, pH and turbidity.

The detection of coliforms and *E. coli* in this study's drinking waters suggests fecal contamination and supports the need for indicator monitoring in drinking water systems. The strength of bacterial indicators (coliforms and *E. coli*) was supported in this study by the fact that there was no seasonal variance in wastewaters or drinking waters. In addition, coliforms and *E. coli* did not vary by region in drinking waters. Male-specific and somatic coliphages proved to be promising indicators. In this study, male-specific coliphages correlated to bacterial indicators in animal feces. Both coliphages were able to survive various environmental conditions, wastewater treatment, and drinking water treatment processes. Neither of the coliphages varied by season in untreated drinking waters. An area of concern for both male-specific and somatic coliphages was the high level of non-detects. The thermotolerance of male-specific coliphages is also an area of concern for its use as a good universal indicator.

Acknowledgements

I would like to thank my Advisor, Professor Jeanine D. Plummer who provided support and guidance over the last two years. I would like to thank Abby Charest and Evan Sullivan for their assistance in the laboratory. I would like to thank Professor Sharon Long, Zong Liu and Jeremy Olstadt for the sample collection and analysis done in conjunction at the University of Wisconsin. An addition thank you to the support of my family and friends.

Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction

Indicator organisms are used in the United States as an index of public health risk from transmission of waterborne diseases. Coliforms and *E. coli* are currently regulated as indicator organisms in municipal drinking water systems. A review of the literature, however, suggests that coliforms may not be the best indicators to protect the public from water borne pathogens. Waterborne disease outbreaks have occurred in systems with negative coliform results, many traced to viral or protozoan etiologies. Conversely, no discernible public health outcomes have occurred in systems with positive coliform results. These inconsistencies arise because coliforms, as bacteria, respond differently to environmental stressors and engineered treatment processes than protozoan and viral pathogens.

Bacteriophages are increasingly being used as an indicator to confirm human fecal contamination presence in waters and have been shown to be more highly correlated to pathogen presence in a variety of waters than coliforms. Bacteriophages as indicators of human enteric viruses meet several of the criteria for ideal indicators. They resemble many of human enteric viruses in their physical structure and morphology. They are found in higher numbers than enteric viruses in wastewater and other environments, and they are more easily and rapidly detected in environmental samples than human pathogenic viruses. Two proposed indicator bacteriophages are male-specific and somatic coliphages.

The goal of this research was to re-examine a variety of traditional and novel indicator systems to determine their value As indicator organisms either singly or as a toolbox. The indicators were enumerated in samples collected in four regions of the United States in all four seasons. Samples were monitored for total coliforms, *E. coli*, male-specific and somatic coliphages, and other physical and chemical water quality parameters including organic carbon, pH and turbidity. These data were analyzed to determine correlations among parameters and to assess spatial and temporal stability of the indicators.

Chapter 2 Literature Review

The purpose of this research was to evaluate traditional and new indicator systems to determine their value as indicator organisms. There are rules and regulations in place to protect the public from contaminated waters. Current indicators include coliforms and *E. coli*, while coliphages are novel indicators of interest.

2.1 Drinking Water Regulations

The United States Environmental Protection Agency (EPA) sets and enforces drinking water regulations in the U.S. Under the Safe Drinking Water Act, the EPA advocates a multiple barrier approach to drinking water protection. The first part of the multiple barrier approach is source water protection. Source water protection includes assessing and protecting drinking water sources, protecting groundwater wells, and protecting surface water collection systems. The second part of the multiple barrier approach involves water treatment conducted by qualified operators. In addition, operators must ensure the integrity of distribution systems. Lastly, the multiple barrier approach requires water utilities to provide information to the public on the quality of their drinking water (EPA, 2011a). While there are many regulations pertaining to the chemical quality of water, including disinfection byproducts, lead, and copper, this section focuses on microbial regulations.

2.1.1 Source Water Protection

Source water protection involves preventing the pollution of groundwater, lakes, rivers, and streams that serve as sources of drinking water for local communities (EPA, 2011b). Watershed management organizations, state agencies, and federal agencies implement watershed management plans to meet water quality standards and protect water resources. Watershed management plans define and address existing or future water quality problems from both point and nonpoint sources of pollutants.

The most successful watershed management plans include participation from stakeholders, analysis and quantification of the specific causes and sources of water quality problems, identification of measurable water quality goals, and implementation of specific actions needed

to resolve such problems (EPA, 2011c). In 1987, Congress amended the Clean Water Act to establish Section 319 Nonpoint Source Management. Under Section 319, states, territories, and Indian Tribes can be awarded grant money to support a wide variety of activities including technical assistance, financial assistance, education, training, technology transfer, demonstration projects, and monitoring to assess the success of specific nonpoint source implementation projects (EPA, 1994). An example of a source water protection program is the establishment of ordinances. One such ordinance is a Groundwater Protection Overlay District, which creates land use regulations to minimize contamination of shallow aquifers and to protect and preserve existing and potential sources of drinking water. Other ordinances may call for buffers along streams or reservoirs to help reduce contaminants and runoff from entering the bodies of water.

2.1.2 Surface Water Treatment Rules

The Surface Water Treatment Rule (SWTR) was first promulgated in 1989. This rule requires two treatment technologies, filtration and disinfection, for surface waters and groundwaters under the direct influence of surface waters. Filtration removes particulate matter and disinfection inactivates potentially harmful pathogens. It is important to remove particles not only for aesthetics, but also to minimize the potential for disease transmission since pathogens can attach to particles that can then be ingested. In addition, toxic materials can exist as particles or can absorb to particles. The SWTR established requirements for pathogen reduction: 4-log removal and/or inactivation of viruses, and 3-log removal and/or inactivation of *Giardia lamblia*.

On December 16, 1998, the EPA promulgated the Interim Enhanced Surface Water Treatment Rule (IESWTR). The IESWTR builds upon the treatment technique requirements set forth in the SWTR by setting new requirements to better protect consumers against *Cryptosporidium* and other pathogens (EPA, 2011d). The purpose of IESWTR is to improve control of microbial pathogens, specifically the protozoan *Cryptosporidium* in drinking water and to address risk trade-offs with disinfection byproducts. The rule requires certain public water systems to meet strengthened filter effluent performance standards. This rule applies to all public water systems that use surface water or groundwater under direct influence of surface water, and serve 10,000 persons or more. Specifics of the IESWTR include a maximum contaminant level goal (MCLG) of zero for *Cryptosporidium*, 2-log *Cryptosporidium* removal requirements for systems that filter, strengthened combined filter effluent turbidity performance standards, individual filter

turbidity monitoring provisions and disinfection profiling and benchmarking provisions. To comply with the Stage 1 Disinfectants and Disinfection Byproducts Rule (Stage 1-DBPR), monitoring of microbial inactivation is required if changes are made to the system. In addition, the rule requires inclusion of *Cryptosporidium* in the watershed control requirements for unfiltered public water systems and covers on new finished water reservoirs. Lastly, states had to conduct sanitary surveys for all surface water systems regardless of size.

On January 14, 2002, the EPA finalized the Long Term 1 Enhanced Surface Water Treatment Rule (LT1) for smaller systems serving fewer than 10,000 people (EPA, 2011d). This rule is built upon the framework established for larger systems in the IESWTR.

The Long Term 2 Enhanced Surface Water Treatment Rule (LT2) was finalized in March, 2006. The purpose of the LT2 rule is to reduce disease incidence associated with *Cryptosporidium* and other pathogenic microorganisms in drinking waters (EPA, 2011e). The rule applies to all public water systems that use surface water or groundwater that is under the direct influence of surface water. The rule bolsters existing regulations and provides a higher level of protection by setting more stringent treatment requirements for *Cryptosporidium* in higher risk systems, which are identified through source water monitoring. In addition, the LT2 includes provisions to reduce risks from uncovered finished water storage facilities, and requires systems to maintain microbial protection as they take steps to reduce the formation of disinfection byproducts.

2.1.3 Groundwater Rule

The Ground Water Rule (GWR) was signed on October 11, 2006, published in the Federal Register on November 08, 2006, and went into effect on December 1, 2009. The GWR applies to more than 147,000 public water systems that use groundwater (as of 2003). The rule also applies to any system that mixes surface and groundwater if the groundwater is added directly to the distribution system and provided to consumers without treatment equivalent to surface water treatment. In total, these systems provide drinking water to more than 100 million consumers.

The purpose of the GWR is to reduce disease incidence associated with pathogens in groundwater systems (EPA, 2011f). The rule establishes a risk-based approach to target groundwater systems that are vulnerable to fecal contamination and includes four major components: periodic sanitary surveys, source water monitoring, corrective actions, and

compliance monitoring (EPA, 2011f). Groundwater systems that are identified as being at risk of fecal contamination must take corrective action to reduce potential illness from exposure to microbial pathogens.

2.1.4 Total Coliform Rule

The Total Coliform Rule (TCR) was published in 1989 and became effective in 1990 to control fecal contamination in drinking waters by monitoring and controlling indicator bacteria (EPA, 2011g). The TCR requires all public water systems to monitor for the presence of total coliforms in the distribution system. Total coliforms are a group of closely related bacteria that are (with few exceptions) not harmful to humans. Total coliforms are common inhabitants of ambient water and may be injured by environmental stresses (e.g., lack of nutrients) and water treatment (e.g., chlorine disinfection) in a manner similar to most bacterial pathogens and many viral enteric pathogens. Therefore, coliforms are used as an indicator of pathogens. For drinking water, total coliforms are used to determine the adequacy of water treatment and the integrity of the distribution system. The absence of total coliforms in the distribution system minimizes the likelihood that fecal pathogens are present.

The TCR requires systems to monitor for total coliforms at a frequency proportional to the number of people served. Systems which serve 1,000 people or fewer test at least once a month, while systems with 50,000 customers test at least 50 times per month and those with 2.5 million customers test at least 420 times per month. If any routine sample tests positive for total coliforms, the system must perform 3-4 repeat tests for total coliforms. If any repeat tests are positive, then the system must test the positive samples for the presence of either fecal coliforms or *E. coli*.

Revisions have been proposed to the TCR. These revisions would require public water systems that are vulnerable to microbial contamination to identify and fix problems, and establish criteria for systems to qualify for and stay on reduced monitoring, thereby providing incentives for improved water system operation. The revised TCR is expected to be finalized in 2012.

2.2 **Waterborne Disease Outbreaks**

A waterborne disease outbreak (WBDO) is a cluster of two or more infections caused by the same agent(s) and linked to the same water exposure. Outbreaks can be caused by water contaminated with pathogens (bacteria, viruses, and parasites) or chemical toxins, which can be spread through ingestion of, contact with, or breathing the contaminated water (CDC, 2008). Most cases of waterborne disease are characterized by gastrointestinal symptoms (e.g., diarrhea and vomiting) that are frequently self-limiting in healthy individuals and rarely require medical treatment. However, these same symptoms are much more serious and can be fatal for persons in sensitive subpopulations, such as young children, the elderly, and persons with compromised immune systems. The largest waterborne disease outbreak in United States history occurred in 1993 in Milwaukee, WI when over 400,000 people became ill with diarrhea when the parasite *Cryptosporidium* was found in the city's drinking water supply (CDC, 1996).

Waterborne disease outbreak statistics have been compiled in the United States since 1920. Since 1971, the US EPA, Centers for Disease Control and Prevention, and Council of State and Territorial Epidemiology have collaboratively maintained the Waterborne Disease and Outbreak Surveillance System for collecting and reporting data relating to WBDOs associated with drinking water (Craun, 2006). During 1920 to 2006, at least 1,886 outbreaks were associated with drinking water, an average of approximately 22 per year. The average annual number of WBDOs ranged from a low of 11.1 during 1951–1960 to as many as 32.4 WBDOs during 1971–1980.

A review of the most recent 10 years of data on record (1997 to 2006) shows 97 WBDOs, with nearly 10,000 individual cases (CDC, 2008). Table 2.1 summarizes the etiologies of WBDOs from 1997-2006. Bacterial agents included *Campylobacter, Escherichia coli* O157, *C. jejuni*, *Shigella* spp, *Salmonella typhimurium, Yersinia enterocolitica,* and *Escherichia coli* O145. Viral agents included hepatitis A, and norovirus G1. Parasitic agents included *Cryptosporidium* species, *Naegleria fowleri*, and *Giardia intestinals*.

Table 2.1. Waterborne Disease Outbreak Etiologies for Drinking Water and Water Not Intended for Drinking (1997-2006). (Compiled from CDC, 1999; CDC, 2001; CDC 2003; CDC, 2005; CDC, 2007)

Etiology	**Number of WBDOs**	**Number of Cases**
Bacteria	22	1790
Viruses	12	1365
Chemical / Toxin	11	85
Mixed Agent	2	1589
Parasitic	18	1596
Unidentified	32	937
TOTAL 1997-2006	**97**	**7362**

Waterborne diseases can originate from various water sources. Groundwater occurrence studies and recent outbreak data show that pathogenic viruses and bacteria can occur in public water systems that use groundwater and that people may become ill due to exposure to contaminated groundwater. Table 2.2 summarizes water sources for WBDOs from 1997 to 2006.

Table 2.2. Waterborne Disease Outbreak Sources. (1997-2006). (Compiled from CDC, 1999; CDC, 2001; CDC 2003; CDC, 2005; CDC, 2007)

Water Source	**WBDOs**	**Cases**
Ground Water	68	2288
Surface Water	7	117
Unknown / Mixed	22	4957
TOTAL 1997-2006	**97**	**7362**

As shown in Table 2.2, groundwater sources were implicated in 70% of WBDOs in the last decade. Fecal contamination can reach groundwater sources from failed septic systems, leaking

sewer lines, and by passing through the soil and large cracks in the ground. Fecal contamination from the surface may also get into a drinking water well along its casing or through cracks if the well is not properly constructed, protected, or maintained. The EPA does not believe all ground water systems are contaminated with feces, however potential exposure to microbial pathogens in groundwaters prompted the promulgation of the Ground Water Rule in 2006 (EPA, 2011f).

2.3 Traditional Indicator Systems

An indicator organism is an organism that can provide information about the health of a water body through the organism's presence, condition, or numbers (EPA, 2011h). An ideal indicator organism of pathogenic or disease risk should occur where pathogens do, occur in greater quantity and be more resistant to disinfection than pathogens. For testing purposes, the indicator should be easily isolated and enumerated. The indicator's density should relate to the degree of contamination or health hazard (Griffin *et al*., 1999). For over a century, coliform bacteria, fecal coliform bacteria, and *Escherichia coli* have been used as indicators of the microbiological safety of drinking water (Griffin *et al*. 2008). These organisms are used to indicate the possible presence of pathogens derived from human or animal waste. Some pathogens of concern in source waters include *Cryptosporidium*, *Giardia*, *E. coli* O157:H7, adenovirus, and hepatitis A virus. This section focuses on traditional indicators and their association with viral pathogens.

2.3.1 Total and Fecal Coliforms

Total coliforms are facultative anaerobes that are gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose, and produce gas and acid within 48 hours when cultured at 35^{o}C. Their lack of ability to form spores makes them susceptible to destruction by environmental conditions. Fecal coliform bacteria are non-disease causing organisms found in the intestinal tract of all warm-blooded animals. The presence of fecal coliforms in a water body indicates the presence of human or animal waste.

The coliform indicator, or coliform index, was first introduced in the late 1880s (Gleeson and Gray, 1997). The approach is based on the assumption that there is a quantifiable relationship between the concentration of coliform indicators and the potential health risks from pathogens. Today, in developed countries such as the U.S. and the U.K., the practice of using coliforms and other indicator organisms is still accepted (Low, 2002).

While coliforms are used to indicate pathogen presence in water systems, there are differences in the fate of coliforms compared to viral and protozoan pathogens in surface and groundwater systems. In addition, coliforms and pathogens do not respond to the same engineered treatment processes. In natural systems, enteric viruses can survive for long periods of time and tolerate changing environmental conditions better than coliforms (Espinosa *et al*., 2009). In specific, coliform bacteria are more susceptible than enteric viruses to extremes in pH, salinity, and temperature (Fong and Lipp, 2005).

Griffin *et al*. (1999) conducted a survey to determine the concentrations of microbial fecal indicators and the presence of human pathogenic microorganisms in canal waters throughout the Florida Keys. A total of 19 sites, including 17 canal sites and 2 near-shore water sites, were assayed for total coliforms, fecal coliforms, *Escherichia coli*, *Clostridium perfringens*, enterococci, coliphages, F-specific (F^+) RNA coliphages, *Giardia lamblia*, *Cryptosporidium parvum*, and human enteric viruses (polioviruses, coxsackie A and B viruses, echoviruses, hepatitis A viruses, Norwalk viruses, and small round-structured viruses). Among other findings, they suggested that coliforms are not adequate predictors of fecal contamination and public health risks. A study in the lower Altamaha River in Georgia was conducted to identify major sources of fecal contamination. Two-liter grab samples were collected monthly from five tidally influenced stations between July and December 2002. Molecular assays targeting human enteroviruses (HEV), bovine enteroviruses (BEV), and human adenoviruses (HAdV) were used to quantify viral pathogens, and samples were also analyzed for coliform concentrations. Of the 30 water samples, 11 and 17 tested positive for HAdV and HEV, respectively. Two-thirds of the samples tested positive for either HEV or HAdV, and the viruses occurred simultaneously in 26% of samples. BEV was detected in 11 of 30 surface water samples. Analysis showed that the presence of both human and bovine enteric viruses was not significantly related to either fecal coliform or total coliform levels. Their results are similar to previous reports suggesting that fecal coliform levels cannot be used for the prediction of occurrence of human enteric viruses.

The imperfect relationship between coliform bacteria and pathogens through wastewater treatment has been known for some time. Hardwood *et al*. (2005) tested the validity of using indicator organisms (total and fecal coliforms, enterococci, *Clostridium perfringens*, and F-specific coliphages) to predict the presence of infectious enteric viruses, *Cryptosporidium*, and

Giardia. Over a one year period, multiple samplings were conducted at six wastewater reclamation facilities. Microorganisms were detected in disinfected effluent samples at the following frequencies: total coliforms, 63%; fecal coliforms, 27%; enterococci, 27%; *C. perfringens*, 61%; F-specific coliphages, ~40%; and enteric viruses, 31%. *Cryptosporidium* oocysts and *Giardia* cysts were detected in 80% and 70% of reclaimed water samples, respectively. *Cryptosporidium* was detected in 20% of the reclaimed water sources. Hardwood *et al*. (2005) found no strong correlation for any indicator-pathogen combination.

LeChevallier *et al*. (1996) conducted an 18 month survey of drinking water systems in North America to determine the factors that contribute to the occurrence of coliform bacteria in drinking water. They found a problem with coliform regrowth in drinking waters. They attributed this problem to many factors such as filtration, temperature, disinfection type, organic carbon levels, corrosion, and treatment system operational characteristics. An indicator that can be affected by so many factors may not provide an accurate indication to potential contaminant risks in waters. In a study of microbial source tracking in surface waters, Stewart-Pullaro *et al*. (2006) suggested coliforms to be inadequate indicators of viral pollution and claimed standard detection methods do not provide adequate information about pollution sources.

The literature cited above demonstrates the limitations in using coliform bacteria to adequately assess viral pathogens in source waters and finished waters, and thus to adequately determine public health risk. Waterborne diseases are known to be caused by pathogenic bacteria, viruses and protozoa. Some viruses and protozoa are more resistant to conventional water treatment than bacterial indicators (Low, 2002). Coliforms have been found present in the distribution system where no waterborne disease outbreak occurred (Geldreich and Rice, 1987). In addition, disease outbreaks and endemic waterborne disease risks have occurred in water systems that were not in violation of the 1989 Total Coliform Rule. For example, Craun *et al*. (1997) investigated reports about waterborne disease outbreaks in the United States compared to maximum contaminant limits (MCL) for coliforms in water systems that did and did not experience outbreaks. Coliforms were only detected in half of the systems and caused an MCL violation in only one fourth of them, in the months leading up to the outbreak. While the outbreak was going on, coliforms were usually, but not always, present in the systems. Similarly, a 2004 report by the CDC stated that only 10 of 17 waterborne disease outbreaks of infectious

etiologies had positive total or fecal coliform counts; more than 40% of the outbreak water samples contained acceptable coliform levels (Blackburn *et al.*, 2004).

2.3.2 *E. coli* Indicators

E. coli have long been used as an indicator of fecal pollution. *E. coli* is a sub-group of the fecal coliform group. Most *E. coli* bacteria are harmless and are found in the intestines of humans and warm-blooded animals; however, *E. coli* 0157:H7 can cause human illness if ingested (Ostroff *et al.*, 1990). Confirmation of fecal coliform bacteria or *E. coli* in a water system indicates recent fecal contamination, which may pose an immediate health risk to anyone consuming the water. *E. coli* are not normally pathogenic to humans and are present at concentrations much higher than pathogens, thus this group may be a good indicator (Scott *et al.*, 2002). In 1892, Schardinger was the first to suggest the use of *E. coli* as an indicator in water (LeClerc, 2001). Recent studies, outlined below however, have suggested that *E. coli* may not be a reliable indicator.

Borchardt *et al.* (2003) conducted a study to systematically monitor private household wells in Wisconsin for virus contamination (enteroviruses, rotavirus, hepatitis A virus (HAV), and Norwalk-like viruses (NLVs) and compared results to indicator levels. Fifty wells in seven hydrogeologic districts were sampled four times over a year, once each season. In addition to virus contamination, the researchers also tested for total coliforms, *E. coli*, fecal enterococci, F-specific RNA coliphages, nitrate, and chloride. Among the 50 wells, four (8%) were positive for viruses by reverse transcription polymerase chain reaction (RT-PCR), three wells were positive for HAV, and one well was positive for both rotavirus and NLV in one sample and enterovirus in another sample. Contamination was transient, since none of the wells was virus positive for two sequential samples. Culturable enteroviruses were not detected in any of the wells. They found indicators such as *E. coli* and total coliforms were not statistically associated with virus occurrence (Borchardt *et al.*, 2003). Likewise, Fout (2011) stated that about half of the drinking water outbreaks that have occurred in the United States since 1971 have been due to untreated groundwaters, and recent studies have shown that many groundwater systems can contain human enteric viruses in the absence of bacterial indicators. Fout (2011) analyzed data from 6 studies of groundwater quality in the United States, which totaled 1187 samples from 166 wells. He examined indicator-virus relationships in wells categorized by well vulnerability. Wells were

divided into vulnerability categories based upon US Total Coliform Rule violations (Category 1), Total Coliform Rule plus US Ground Water Rule monitoring violations (Category 2), and all other wells (Category 3). Results showed that 4–21% of the samples were positive for viruses by cultural methods. Indicators were highly effective predictors of virus occurrence for Category 1 wells as 100% of virus positive wells were also positive for indicators. In the Category 2, 88% of virus positive wells were also indicator positive wells. However, Category 3 wells had 0% of virus positive wells also containing positive indicators. Fout concluded that in drinking water from groundwater sources, indicators are very effective for vulnerable aquifers, but as aquifer vulnerability decreases, indicators become less correlated with virus presence.

2.3.3 FC/FS Ratio

One form of fecal source tracking consists of determining the ratio of fecal coliforms verses fecal streptococci (FC/FS ratio) as an indicator of the source of fecal pollution. In 1969, Geldreich and Kenner proposed that a fecal coliform: fecal streptococci ratio of four or greater may indicate human pollution, whereas ratios of two or less may indicate animal pollution (Scott *et al.*, 2002). The rationale behind the use of this method was the observation that human feces contain higher fecal coliform counts, while animal feces contain higher levels of fecal streptococci (Scott *et al.*, 2002). The validity of this methodology has been questioned because of the differential die-away rates of FC and FS, which can change the ratio over time. Feachem (1975) believed the differential die-away could strengthen the FC/FS ratio as a means of distinguishing human from non-human pollution. However, the usefulness of this ratio has been discredited. In particular, Scott *et al.* (2002) noted that the FC/FS ratio is only valid for recent (24 hour) fecal pollution. Bitton (2005) found the FC/FS ratio to be unreliable for chlorinated effluents. The American Public Health Association no longer recommends the use of the FC/FS ratio as a means of differentiating human from animal sources of pollution (Bitton, 2005).

2.4 Coliphages as an Alternate Indicator

As discussed in section 2.3, coliform bacteria and its subgroups may not be ideal for indicating viral pathogen risk. Coliphages have been investigated as possible viral indicator organisms since as early as the 1980s and may be more appropriate to monitor the fate of viruses in water (Furuse, 1987; Kazame *et al.*, 2011). Coliphages are viruses that infect coliform bacteria, but are

non-pathogenic to humans (Lee and Sobsey, 2011). They are present in human and animal feces. Some are small, icosahedral and non-enveloped viruses, making them structurally similar to many human enteric viruses. They exhibit similarities to enteric viruses regarding environmental transport and survival, however, coliphage survival characteristics vary by season and by coliphage group. In addition, coliphages may continue to replicate in surviving bacterial hosts after being shed in feces, thus exhibiting greater persistence than human enteric viruses in receiving waters (Griffin, 2008). Coliphages are classified into a number of types by their shape and nucleic acid composition (Kazama *et al.*, 2011). The two main types of coliphages are male-specific (F+ or F-specific) and somatic coliphages, both of which were evaluated in this study. Enumeration of both male-specific and somatic coliphages may be necessary to fully represent enteroviruses and other human pathogenic viruses (EPA, 2001).

2.4.1 Male-Specific Coliphages

One promising group of organisms for the indication of viral pathogens is male-specific (F-specific or F+ RNA) coliphages (Cole *et al.*, 2003). Male-specific coliphages infect coliform bacterial hosts through attachment to F-pili. F-specific coliphages can be measured in drinking waters using the enrichment method, the single agar layer method, or the filter-concentration/elution method with enumeration using *E. coli* F_{amp} or *E. coli C3000* hosts. Individual isolates can be subject to serotyping or genotyping in order to discriminate between human and non-human microbial sources. F-specific coliphages belong to two morphologically defined families, the *Leviviridae* and the *Inoviridae*. The family *Leviviridae* consists of small icosahedral viruses that contain single-stranded RNA as their genetic material. The family *Inoviridae* consists of filamentous viruses that contain single-stranded DNA as their genetic material.

F-specific coliphages appear to be present in feces and sewage, both of which are potential sources of pathogens. They also seem to be present at low levels in uncontaminated environmental settings (Cole *et al.*, 2003). A number of researchers have demonstrated that subgroups of F+ RNA coliphages via serotyping or genotyping can distinguish between inputs from human and warm-blooded animal/non-human sources. These subgroups are: Group I, non-human animals (prototype MS2); Group II, primarily human feces and occasionally pig feces (prototype GA); Group III, exclusively human (prototype Qb); and Group IV, primarily non-

human origin with rare human associations. Monitoring of F+ coliphage groups can indicate the presence and major sources of microbial inputs to surface waters, but environmental effects on the relative occurrence of different groups needs to be considered (Cole *et al*., 2003)

2.4.2 Somatic Coliphages

Somatic coliphages are DNA viruses that infect *E. coli* through attachment to specific sites on the outer cell layer, such as lipopolysaccharide (Lee and Sobsey, 2011). Somatic coliphages are the most abundant group of bacteriophages, and the methods for their detection and enumeration are the most simple, fast, and cost effective with results available in one work day. The somatic coliphage group encompasses four distinct virus families, each containing several genera. The four families are *Myoviridae, Microviridae, Siphoviridae,* and *Podoviridae*. The *Microviridae* are small, single-stranded DNA viruses. The other families are double-stranded DNA viruses of varying size, morphology and biophysical properties. It is possible that the survival of these different phages in environmental waters may differ among families and genera, with some being more persistent in water than others.

Somatic coliphages are likely to be more persistent in water than F+ RNA coliphages (Lee and Sobsey, 2011). Shin *et al*. (2005) found different inactivation kinetics of some somatic and male-specific coliphages by UV radiation. Based on their persistence, somatic coliphages may have advantages to F+ coliphages as more environmentally persistent indicators of enteric viruses in water (Lee and Sobsey, 2011).

It should be noted that when testing for somatic coliphages' concentrations, decay and temperature influence has been observed (Wu *et al*., 2010). Over a nine month period in Qinghe Beijing, Wu *et al*. (2010) monitored somatic coliphages in municipal wastewater for a wide range of temperature variations. For the monitoring period, the sewage featured an average concentration of 2.81×10^4 PFU/mL with a standard deviation of 1.51×10^4 PFU mL (4.36 ± 0.31 log). The lowest somatic coliphages concentrations were observed at the highest temperatures (July), while highest somatic coliphages concentrations were observed during the coldest sampling temperatures (December). The somatic coliphage decay was modeled by first order kinetics as shown in Equation 1, where K_d, the somatic coliphages' decay coefficient at 20^oC, was 0.28 day^{-1}.

$$C = C_o \exp(-K_d t) \qquad \text{(Equation 1)}$$

2.4.3 Coliphages as Indicators

The literature shows that relationships between coliphages, coliforms and pathogens vary under different conditions. Coliphages are as adequate an indicator of fecal pollution as coliform bacteria (O'Keefe and Green, 1989). Borrego *et al* (1990) tested the capability of coliphages as indicators of fecal pollution on the basis of their survival in two natural organic aquatic environments: river and marine. They concluded coliphages were good indicators of fecal pollution in natural waters. Similarly, Ogorzaly *et al*. (2009) conducted a study in a river located in an urbanized watershed with recognized anthropogenic influences, aimed at evaluating the relevance of direct phage genotyping by real-time PCR. They found bacterial indicators to be correlated with somatic coliphages. Previous studies have found problems with using coliphages as indicators. One issue is that bacteriophages may continue to replicate in surviving bacterial hosts after being shed in feces. More issues arise with male-specific coliphages in particular. Their infrequent presence in human feces, relative scarcity and rapid die-off rates in warm water limits the usefulness of male-specific coliphages as indicator viruses (Lee and Sobsey, 2011).

A comparison of survival of indicator viruses and enteric viruses in seawater demonstrated that while male-specific coliphages may be adequate in the wintertime, they may not be a good indicator of enteric viruses in summer months when temperatures reach 25°C as a result of different survival rates (Handzel *et al.*, 1993). Additional seawater studies, however, have reported positive correlations between enteroviruses and somatic coliphages. Moce-Llivina *et al*. (2005) examined seawater using a new procedure for detecting and counting enteroviruses based on the VIRADEN method. Viruses were quantified and a number of bacterial indicators and bacteriophages were also tested. Cultivable enteroviruses were detected in 55% of the samples and somatic coliphages outnumbered all other indicators. They concluded that somatic coliphages show a very good potential to predict the risk of viruses being present in bathing waters. Likewise, Jiang *et al*. (2007) investigated the occurrence and distribution of fecal indicator bacteria (FIB), F+ coliphage, and PCR-detectable human adenovirus and enterovirus for an entire year at 15 locations around the Newport Bay watershed. 206 samples were collected and tested in all. Their results demonstrated that FIB and coliphages have similar seasonal and freshwater-to-saltwater distribution patterns, which suggests that coliphages and FIB share

similar environmental sources. In addition, their study showed a correlation between coliphage and PCR-detectable human viral genome.

Coliphages have had mixed results as an indicator in wastewaters over the last 25 years. Borrego *et al*. (1987) conducted a study designed to test the proposal that *E. coli* specific bacteriophages might serve as universal fecal pollution indicators in water. The numerical relationship between *E. coli* and its parasitic phages was investigated in the vicinity of sewage outfalls, river water contaminated by domestic and industrial sewage discharges, and estuarine waters. Their results indicated that the coliphages were a good indicator of the presence of the pathogenic microorganisms studied, and based on nearly all the water samples tested, the results suggested coliphages to be a better indicators of fecal pollution than the classical indicator systems of the time. On the contrary, Carducci *et al*. (1999) found no relationship between coliphages and viral contamination in a study of possible indicators or viral aerosol contamination in sewage treatment plants. This was a year-long study carried out on the relationships between the presence of cytopathogenic viruses and the counts of total bacteria, fecal streptococci and somatic coliphages. Samples were collected bi-monthly from September 1995 to October 1996 at various distances from the aeration tank (aerosol source). Overall, the number of virus-positive samples was 35, of which 21 (60%) contained only reovirus, one (3%) only enterovirus, and the remaining 13 samples (37%) had enterovirus-reovirus co-infection. The results indicated total bacteria and fecal streptococci counts to be, in general, positively associated with virus presence, however, coliphage counts yielded no analogous relationship to viral contamination.

Results from Costan-Longares *et al*. (2008) support coliphages as indicators in wastewaters. They monitored four water reclamation facilities in north-eastern Spain for more than 2 years to determine the occurrence and concentrations of a set of microbial indicators and two selected pathogens. The microbial indicators were total coliforms, *E. coli*, enterococci, spores of sulphite reducing *clostridia*, somatic coliphages, F-specific RNA phages, phages infecting *Bacteroides fragilis* strain RYC2056 and phages infecting *Bacteroides tethaiotaomicron* strain GA-17. The two pathogens were cytopathogenic enteroviruses and viable *Cryptosporidium* oocysts. The indicators were evaluated through wastewater treatments. The inactivation pattern of all groups of bacteriophages tested was closer to the inactivation of enteroviruses than to the inactivation of the conventional bacterial indicators tested. Based on their statistical analysis they found the

number of bacteriophages to be able to predict both the presence and concentrations of enteroviruses. Costan-Longares *et al.* concluded that a combination of both bacterial and bacteriophage indicators seems to be the best choice for ensuring the microbial quality of reclaimed water. The presence of indicator phages higher than a certain threshold in water samples may indicate the presence of viruses (Lucena and Jofre, 2010). They believe the fate of bacteriophages in natural-water environments and their outcome in water and sludge treatments resemble those of human-pathogenic viruses.

Based on a review of the literature, coliphages seem to have characteristics that made them good indicators. They are positively correlated with bacterial indicators in varying water types, and they resemble many human enteric viruses in their physical structure, morphology, and ability to survive in the environment. However, temperature can play a role in coliphage survival and growth outside the gastrointestinal tract. Coliphages in some studies did not correlate with bacterial indicators. In addition, studies have recommended coliphages not be used as an indicator of viral pathogens.

Chapter 3 Methods

The purpose of this research was to evaluate traditional and new indicator systems to determine their value as indicator organisms in drinking waters. To meet this goal, feces, wastewater samples and drinking water samples were collected from four different regions in the United States over a 24 month period. They were analyzed for indicator concentrations as well as physical and chemical water quality. This chapter provides information on the sampling protocols. Second, this chapter discusses the analytical procedures used for characterizing the samples. Lastly, the chapter details the statistical analyses that were performed on the data.

3.1 Experimental Design

Currently, pathogen risk in drinking waters is assessed by measuring coliform bacteria indicators. In this research, the value of both coliforms and coliphages as indicator organisms was determined, and correlations between indicators and other water quality parameters were evaluated. Data were collected from drinking waters, including untreated surface waters, untreated groundwaters, and treated drinking water systems. Data were also collected from domestic wastewater samples which contain human fecal matter. Lastly, data were collected from various domestic animal feces in order to compare indicators in human vs. non-human animals. The following sections describe the procedures for collecting and transporting samples, while section 3.2 details the laboratory methods used to analyze each sample.

3.1.1 Sampling Overview

Samples were collected from four geographical regions in the United States (Northeast, South, Midwest, and West) in order to assess spatial variability. Wastewater, drinking water and fecal samples came from volunteer samplers in Massachusetts, Florida, Wisconsin, Colorado, North Carolina, Nevada and Washington. Fecal samples were collected at farms or private residences, while wastewater and drinking water samples were obtained from municipal treatment facilities.

To assess temporal variability, samples were collected in multiple seasons. Fecal and wastewater sampling was conducted from June 2010 to April 2011. A total of 12 sampling events were

completed over these 11 months with fecal and wastewater samples collected in each region in the spring, summer, and winter. Drinking water sampling was conducted from May 2011 to March 2012. A total of 16 sampling events were completed such that the facility in each region was sampled once in each season.

3.1.2 Fecal Sampling Protocol

Fresh fecal samples were collected from horses, cows, chickens, pigs, sheep, dogs, rabbits, and donkeys. Animals were monitored by the sampler, and then immediately after defecation, the feces were collected. A summary of the fecal samples is provided in Table 3.1.

Table 3.1. Fecal Sampling Overview

Region	**Sampling Dates**	**Location**	**Animals**
North-east	Summer (June 2010) Fall (Sept. 2010) Winter (Jan. 2011)	Private Residence, Brookfield, MA	Chicken
		Private Farm, Brookfield, MA	Horse
		Private Residence, Littleton, MA	Rabbit, Dog
		Private Farm, Littleton, MA	Cow, Horse
South	Summer (July 2010) Winter (Jan. 2011) Spring (March 2011)	Private Facility, Ft. Pierce, FL	Chicken, Cow, Goat, Horse, Dog, Sheep
Mid-west	Spring (June 2010) Summer (Aug.t 2010) Winter (Feb. 2011)	Houfe Farm, Edgerton, WI	Cow
		Private Residence, Madison, WI	Dog
		Private Residence, Lodi, WI	Dog
West	Summer (Aug. 2010) Winter (Feb. 2011) Spring (April 2011)	Private Farm, Boulder, CO	Rabbit, Horse, Donkey, Sheep, Llama

Fecal samples were collected with sterile spatulas (Fisherbrand, Fisher Scientific Catalog Number 14-375-253) and placed in sterile specimen cups (Fisherbrand, Fisher Scientific Catalog Number 14-375-147) labeled with the sample information. The entire fecal sample or half a specimen cup's worth of feces was collected, whichever was less. The specimen cups were then

capped and placed in a ziplock bag. The sealed ziplock bag was then wrapped in bubble wrap (to prevent samples from freezing) and placed in a cooler with ice packs. Each sampling event included five to seven different fecal samples.

Once all fecal samples were collected, the cooler was transported to WPI by vehicle (for Northeast samples) or overnight shipping (for all other regions). At WPI, approximately 2 grams of feces was removed from each sample and placed into a sterile container for analysis. The remaining fecal matter in the specimen cups was shipped to the Wisconsin State Laboratory of Hygiene (WSLH) in Madison, WI in a cooler with ice packs for further analysis.

3.1.3 Wastewater Sampling Protocol

Wastewater was collected as grab samples from each of the wastewater treatment facilities, which are shown in Table 3.2. Two raw and two final (pre-disinfection) effluent wastewater samples were collected for each sampling event. The samples were collected in 1 L autoclaved Nalgene sample bottles (Nalgene, Fisher Scientific Catalogue Number 02-893D) which were filled to the shoulder and tightly capped. Each bottle was labeled with the sampling information. The bottles were wrapped in bubble wrap and placed in coolers with ice packs immediately after sampling. The bubble wrap prevented the samples from freezing.

Table 3.2. Wastewater Sampling Overview

Region	Sampling Dates	Treatment Facility	
		Name	Location
Northeast	Summer (June 2010) Fall (September 2010) Winter (January 2011)	Withheld	Massachusetts
South	Summer (July 2010) Winter (January 2011) Spring (March 2011)	Withheld	Florida
Midwest	Spring (June 2010) Summer (August 2010) Winter (February 2011)	Withheld	Wisconsin
West	Summer (August 2010) Winter (February 2011) Spring (April 2011)	Withheld	Washington

Once all wastewater samples were collected, the cooler was transported to WPI by vehicle (for Northeast samples) or overnight shipping (for all other regions). One raw and one final wastewater sample were removed from the cooler and placed in the refrigerator to await analysis. The two remaining filled 1 L sample bottles were wrapped in bubble wrap and shipped in a cooler with ice packs to the WSLH for further analysis.

3.1.4 Drinking Water Sampling Protocol

Drinking waters were collected as grab samples from each of the drinking water system facilities (see Table 3.3). Samples were collected before treatment (raw), after various treatment processes, and within the distribution system at each of the facilities. For each sample, three sampling containers were filled: one autoclaved carboy (Nalgene, Thermo Scientific Catologue Number 2235) filled to a the 20 L mark and two 1 L autoclaved Nalgene sample bottles (Nalgene, Fisher Scientific Catologue Number 02-893D), each filled to the neck. The 20 L sample was later concentrated while the 1 L samples were analyzed as collected. Each carboy or

bottle was labeled with the sampling information. The 1 L bottles were wrapped in bubble wrap and placed in coolers with ice packs immediately after sampling.

Table 3.3. Drinking Water Sampling Overview

Region	Sampling Dates	Treatment Facility		Samples
		Name	Location	
North-east	Spring (May 2011)	Withheld	Massachusetts	Raw Ozonated Filtered Chlorinated Distribution system
South	Summer (Aug. 2011)	Withheld	North Carolina	Raw Filtered Finished Distribution system
Mid-west	Summer (July 2011)	Withheld	Wisconsin	Raw Chlorinated Distribution system
West	Spring (June 2011) Summer (Aug. 2011)	Withheld	Nevada	Raw Filtered Distribution (groundwater) Distribution (surface water)

Once all samples were collected, they were transported back to the nearest laboratory facility for concentration of the 20 L samples using Hollow Fiber Ultrafiltration (HFUF) (see 3.1.4.1). In addition to the treatment facility samples, 20 L of a reagent grade water sample from the laboratory facility (where HFUF was performed) was also concentrated. Once samples were concentrated, the cooler was repacked with concentrated and original samples and transported to WPI via overnight shipping (with the exception of Northeast samples, which were concentrated

at WPI). One set of samples was retained at WPI for analysis, and the duplicate set of samples was shipped in a cooler with ice packs to the WSLH for further analysis.

3.1.4.1 Hollow Fiber Ultrafiltration

The 20 L drinking water samples were concentrated using Hollow Fiber Ultrafiltration (HFUF). The purpose of this procedure is to concentrate large volumes (10-100 L) of drinking water in order to concentrate microbial contaminants to allow detection of low levels of these organisms. This method has been tested for efficacy with bacteria (*E. coli* and enterococci), viruses (coliphage, adenovirus, norovirus), and parasites (aerobic endospores as a surrogate, *Cryptosporidium*, and *Giardia*) (EPA Method 1600).

The HFUF method used in this research is a hybrid of the method developed by WSLH for preparedness response and the EPA method applied for QA/QC criteria development. HFUF was completed by Zong Liu (Ph.D. Candidate, University of Wisconsin at Madison) or Jeremy Olstadt (Laboratory Assistant, WSLH), who traveled to each sampling location to perform the concentration procedure on-site. Detailed steps for this procedure are provided in Appendix A. The procedure concentrated each 20 L sample to a final volume of approximately 400 mL, which is a concentration by a factor of approximately 50 (50X). Exact concentration factors were provided by the WSLH after each sampling event.

3.2. Indicator Organism Enumerations

Indicator organism enumerations were performed on all fecal, wastewater, and drinking water samples. All enumerations were performed using aseptic techniques. Everything used was either autoclaved or purchased pre-sterilized prior to use. Table 3.4 presents the instruments and methods used to quantify each indicator.

Table 3.4: Indicator Organism Enumeration

Parameter	Instrument	Method Description	Number
Total Coliforms	Quanti-Tray IDEXX Corp (Idexx, Westbrook, ME)	Colilert Enzyme Substrate Test	SM 9223
E. coli	Quanti-Tray IDEXX Corp (Idexx, Westbrook, ME)	Colilert Enzyme Substrate Test	SM 9223
Male Specific Coliphage	NA	Single or Double layer	EPA 1601 & 1602
Somatic Coliphages	NA	Single or Double layer	EPA 1601 & 1602

*SM = Standard Methods

3.2.1 Total Coliforms and *E. coli*

Total coliforms and *E. coli* were enumerated for all fecal, wastewater, and drinking water samples in accordance with Standard Method 9223, the enzyme substrate test (APHA *et al.*, 2005). This method was completed using Colilert® (IDEXX, Westbrook, ME), a commercially available enzyme-substrate liquid-broth medium that allows the simultaneous detection of total coliforms and *E. coli*. The test can be performed in multiple tube, multiple well, or presence absence format. In this research, the multiple well format was used (Quanti-Tray®, IDEXX, Westbrook, ME).

Table 3.5 presents the various dilutions and concentrations used for the samples. Depending on the sample, coliforms and *E. coli* were determined for samples as collected (no concentration or dilution, denoted as 10^0), with dilution, or with concentration. All samples were 100 mL in volume and all tests were conducted in duplicate. For feces, 1 gram of feces was weighed in a 50 mL sterile centrifuge tube. A portion of buffered water (see section 3.2.1.1) from a dilution bottle with 99 mL buffered water was poured into the centrifuge tube. The tube was capped and shaken to resuspend the fecal matter. The contents of the centrifuge tube were poured back into the

dilution bottle to create the 10^0 fecal resuspension (1 gram feces in total volume of 100 mL). The fecal resuspension was placed at 4°C for 4 hours to allow the feces to disperse. Then, a dilution series was created as shown in Table 3.5. The 10^{-2} dilution was created by transferring 1 mL of the 10^0 suspension into a dilution bottle with 99 mL dilution water, and so forth.

For wastewater, the sample as collected was the 10^0 sample. Therefore, 100 mL of the sample as collected was transferred to a 250 mL bottle to be used for enumerating coliforms and *E. coli*. As with the feces, a dilution series was created as shown in Table 3.5. For drinking water, 100 mL of the sample as collected was the 10^0 sample. The HFUF sample was concentrated by approximately 50 times (50X). For coliforms and *E. coli*, a 5X concentrated sample was created by combining 10 mL of the 50X sample with 90 mL of buffered water.

Table 3.5: Dilutions and Concentrations Used For Coliform and *E. coli* Enumerations

Sample	**Dilutions**
Feces	10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}
Wastewater	10^0, 10^{-2}, 10^{-4}, 10^{-6}
Drinking Water	10^0, 5X, 25X Concentrated

Once the samples, dilutions and concentrations were prepared, one Colilert® packet was added to each 100 mL sample and then vigorously shaken. The sample and reagent mixture was then poured into a Quanti-Tray® and sealed in an IDEXX Quanti-Tray Sealer. The Quanti-Tray was then incubated for 24 hours at 35°C $\pm$ 0.5°C. A yellow color indicates positive for total coliforms and fluorescence under UV light (Entela, UVL-23RW, Upland, CA) in a dark room indicates positive *E. coli* presence. There are 49 large cells and 48 small cells. Positive cells were counted and then compared to an MPN table to determine the Most Probable Number of total coliforms and *E. coli* in each sample per 100 mL. Results from duplicate tests were averaged and the values were adjusted to account for the dilution or concentration of the sample to determine the MPN/100 mL in the original sample as collected.

3.2.1.1 Buffered Water

Buffered water was used for the dilution series. Buffered water is a solution that neither prohibits nor enhances growth of microorganisms. Buffered water was made according to Standard Method 9050c.1a (APHA *et al.*, 1995), by diluting 5 mL of stock magnesium chloride and 1.25 mL of stock phosphate buffer up to 1 L of E-pure water. The stock magnesium chloride was made by dissolving 20.275 g of $MgCl_2 \cdot 6H_2O$ to a total volume of 250 mL of E-pure and the stock phosphate buffer was made by suspending 8.5 g of KH_2PO_4 up to 125 mL of E-pure. If necessary, pH was adjusted to 7.2 $\pm$ 0.5 with sodium hydroxide.

3.2.1.2 Positive and Negative Controls for Fecal Coliforms and E. coli

One positive and one negative control were completed for total coliforms and *E. coli* for each sampling event. For the positive control, *E. coli* (ATCC #11775) was cultured in the laboratory. First, tryptic soy broth (TSB; Bacto # 211825, Sparks, MD) was prepared by weighing 3 grams of tryptic soy broth powder and dissolving it into 100 mL of E-pure water. 50 mL of TSB was added to a labeled shaker flask and autoclaved. After cooling, the positive control shaker flask was inoculated with one loopfull of frozen (-70^{o}C) *E. coli* stock. The flask was then incubated at 35^{o}C on a rotating platform at 100 revolutions per minute for 12 – 16 hours. After incubation, 1 mL from the flask was added to 99 mL of dilution water in a dilution bottle. This positive control was enumerated using the multiple well procedure described in section 3.2.1. For the negative control, a dilution bottle with 100 mL of buffered water was processed using the multiple well procedure.

3.2.2 Coliphages

Male-specific and somatic coliphages were enumerated using the single agar and double agar layer methods (EPA methods 1601 and 1602, respectively). The single agar layer method was used for quantifying coliphages in wastewater and fecal samples, while the double agar layer method was used for drinking water samples. Prior to each sampling event, the following cultures and solutions were prepared: overnight *E. coli* cultures (F_{amp} and CN-13), tryptic soy

agar (TSA), $MgCl_2$, phosphate buffered solution (PBS), antibiotics (100X Naladixic acid and 100X streptomycin/ampicillin), and titered stocks of MS2 and ΦX174 coliphages (prepared once during research project). On the day of sampling, 4 hour log phase *E. coli* F_{amp} and *E. coli* CN-13 hosts from overnight *E. coli* were prepared. Detailed instructions for preparation of these cultures and reagents are provided in Appendix B.

3.2.2.1 Coliphage Enumeration in Fecal and Wastewater Samples

Coliphages were enumerated in fecal and wastewater samples using the single agar layer procedure. Fecal resuspensions and dilutions were made in PBS (see Table 3.6). 1 g of feces was weighed and added to a 10 mL centrifuge tube containing 9 mL PBS. Since feces is reported as pfu/gm feces, plating 1 mL of this resuspension is equivalent to plating 0.1 gm feces. Therefore, this is designated as the 10^{-1} dilution. 1 mL of this 10^{-1} dilution is added to 9 mL of PBS in a centrifuge tube to create the 10^{-2} dilution. This process was continued to 10^{-3} for all animals, and to 10^{-4} for dogs and 10^{-5} for chickens. For wastewater samples, 10 mL of the wastewater as collected was poured into a 10 mL centrifuge tube for the 10^{0} dilution. Then, 1 mL of this 10^{0} dilution was added to 9 mL of PBS in a centrifuge tube to create the 10^{-1} dilution. This process continued to 10^{-4} for raw wastewater, and to 10^{-2} for final effluent.

Table 3.6: Target Fecal and Wastewater Dilutions

Sample	**Target Dilutions**					
Wastewater (raw)			10^{-2}	10^{-3}	10^{-4}	
Wastewater (final)	10^{0}	10^{-1}	10^{-2}			
Sourcewater	10^{0}	10^{-1}	10^{-2}			
Feces*		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}

*Dilution to 10^{-4} for dog feces and 10^{-5} for chicken feces only

Coliphages were enumerated in 100 mm plates. Six plates were made for each dilution for each sample – three to enumerate male-specific coliphages and three to enumerate somatic coliphages. The plaque forming units quantified on the triplicate plates were averaged together. The

following describes how to prepare each plate. First, 1 mL of the sample was aseptically pipetted onto the 100 mm plate. Then, on a different spot on the plate so as not to splash the sample, 0.5 mL of 4 hour log phase *E. coli* F_{amp} (for male-specific coliphage enumeration) or CN-13 (for somatic coliphage enumeration) host was pipetted on and then the plate was tilted to mix the sample and *E. coli* together. The sample and host were allowed 1-2 minutes for the coliphage to absorb to the host. During this time, the agar, which was previously autoclaved and placed in a 48°C waterbath to maintain the temperature, was prepared. 3.125 mL of 4M $MgCl_2$ and 2.5 mL of 100X streptomycin/ampicillin (for male-specific coliphage enumeration) or 100X naladixic acid (for somatic coliphage enumeration) were pipetted into a bottle containing 250 mL TSA by running the solutions down the side of the bottle into the agar. Once the antibiotic was added to the agar, it must be used (plated) within ten minutes. After the 1 – 2 minute time had elapsed, 10 – 12 mL of TSA was pipetted onto each plate in an empty spot so as not to splash the sample. After the addition of agar, the plate was swirled to thoroughly mix the agar, sample and *E. coli.*

The plates were allowed to sit undisturbed with their covers slightly askew for approximately 5 minutes. Once the agar had solidified, the covers were closed. The plates were stacked upside down, wrapped and sealed in baggies, and placed in an incubator. The plates were incubated at 36°C for 18 – 24 hours. After 18 – 24 hours, the number of plaques on each plate was counted and recorded. As noted earlier, triplicate plates were completed for each sample. The results from the three plates were averaged.

In addition to the samples, the following controls were also prepared:

- agar negative controls: one plate of agar only for each bottle of agar used in plating;
- *E. coli* positive control: one plate of agar and *E. coli* F_{amp} host (positive host control for male-specific coliphages) and one plate of agar and *E. coli* CN-13 (positive host control for somatic coliphages);
- coliphage positive control: one plate of agar, host and stock MS2 coliphage (for male-specific coliphage) and one plate of agar, host and stock ΦX174 phage solution (for somatic coliphage); and

- matrix spike: two plates of agar, host, stock MS2 coliphage and sample (for male-specific coliphage) and two plates of agar, host, stock ΦX174 coliphage and sample (for somatic coliphage), using random samples/dilutions/concentrations for each sampling event.

3.2.2.2 Coliphage Enumeration in Drinking Water Samples

Coliphages in drinking water samples were enumerated using the double agar layer procedure. This procedure was performed in 150 mm plates in which a 100 mL sample was distributed among 5 plates and the sum of all plaque forming units on these 5 plates was added together. For each sample, the following samples were prepared for plating (note that the full set of samples was prepared for male-specific coliphage enumeration, and a second full set of samples for somatic coliphage enumeration):

- 10^0 replicate A: 100 mL of sample as collected, measured into a 250 mL screw cap bottle;
- 10^0 replicate B: prepared as replicate A;
- 25X HFUF concentrate: 50 mL of HFUF concentrated sample and 50 mL PBS into sterile 250 mL screw cap bottle; and
- 5X HFUF concentrate: 10 mL of HFUF concentrated sample into and 90 mL of PBS into sterile 250 mL screw cap bottle.

In addition, the following samples were prepared per sampling event (again, with one full set for male-specific coliphage enumeration and a second for somatic):

- two 25X HFUF with matrix spikes: prepared as 25X HFUF sample, with MS2 or φX added;
- Positive control: 100 mL PBS in a 250 mL screw cap bottle with MS2 or φX added;
- Agar negative control: 15 mL PBS in a 50 mL centrifuge tube;
- Host control: 15 mL PBS in a 50 mL centrifuge tube; and
- Temperature control: 100 mL PBS in a 250 mL screw cap bottle (for temperature monitoring; not to be plated).

First, 4M $MgCl_2$ was added to each of the bottles (0.5 mL) and tubes (0.075 mL). Then, the sample bottles and tubes were placed into a 48°C water bath, with a temperature probe inserted into the "temperature control" bottle. The bottles/tubes were shaken for approximately 5 minutes, until the temperature of the temperature control reached 36°C. The sample bottles were removed from the bath and *E. coli* was added to all bottles/tubes except the agar negative control. 10 mL of log-phase *E. coli* F_{AMP} (for male-specific coliphages) or 10 mL of log-phase CN-13 *E. coli* (for somatic coliphages) was added each bottle, and 1.5 mL of the appropriate *E. coli* was added to the "host control" centrifuge tube. No *E. coli* were added to the agar negative control. Once the *E. coli* had been added, the petri dishes must be plated within 20 minutes. The sample bottles and centrifuge tubes were placed back into the 48°C water bath and shaken until the temperature in the "temperature control" bottle reached 43°C $\pm$ 1°C. Once this temperature was obtained, the sample bottles/tubes were transferred to a separate 43°C water bath.

While in the 43°C water bath, the agar was prepared with the addition of antibiotics. For male-specific coliphages, 2.0 mL of 100X streptomycin/ampicillin was added for every 100 mL 2X TSA. For somatic colihpages, 2.0 mL of 100X naladixic acid was added for every 100 mL 2X TSA. For example, 6.0 mL of antibiotics was added per 300 mL 2X TSA or 17 mL per 850 mL 2X TSA. Antibiotics were added along the inside of the agar bottle to reduce the formation of bubbles, and then the agar bottle was mixed by gentle rocking. Once the antibiotics were added to the agar, plating must occur within 10 minutes, otherwise the antibiotics will degrade. Agar was poured into each sample bottle or centrifuge tube such that the contents of each bottle doubled. The bottles and tubes were gently inverted and turned to mix while trying to avoid the formation of bubbles. The contents of each bottle were poured equally onto five 150 mm plates, using the entire solution. For the "host" and "agar" tubes, the entire solution was poured onto one plate. The petri dish covers were left askew for approximately 5 minutes while the agar hardened. Once hardened, the dishes were covered, inverted, stacked, bagged and sealed, and incubated for 16 – 24 hours. After 16 – 24 hours, the plaque forming units were counted. The total plaque for count for each sample is the sum of the 5 plates.

3.3 Physical and Chemical Laboratory Analytical Procedures

The wastewater and drinking water samples were analyzed for physical and chemical parameters as shown in Table 3.7. The parameters included turbidity, pH, and total and dissolved organic carbon.

Table 3.7: Physical and Chemical Laboratory Tests

Parameter	**Instrument**	**Method**	
		Description	**Standard Method No.**
Turbidity	Hach 2100N (Hach Company, Loveland, CO)	Nephelopmetric Method	2130
pH	Fischer Scientific AB15 (Fisher Scientific, Pittsburgh, PA)	Electrochemical Method	4500-H^+
Total and dissolved organic carbon	Shimadzu TOC-5000A (Shimadzu, Colombia, Maryland)	High-Temperature Combustion Method	5310B

3.3.1 Turbidity

Turbidity was measured on wastewater and drinking water samples (as collected) using a Hach Model 2100N Laboratory Turbidimeter (Loveland, CO) and in accordance with Standard Method 2130 (APHA *et al.*, 1995). First, samples were allowed to warm to room temperature. For each sample, the sample bottle was gently inverted several times and the samples were poured into a clean, oiled turbidity vial. The turbidity vial was filled to the white line and capped. The vial was gently inverted several times and placed into the turbidimeter (making sure to align the white arrow on the sample cell to the white line on the turbidimeter). After waiting 15 seconds, the digital readout on the turbidimeter was observed for 30 seconds and an average reading determined. Two replicate vials were filled and measured for each sample. The

turbidimeter was calibrated every 4 months with Stable Cal Calibration standards of less than 0.1, 20, 200, 1000, and 4000 ntu (Hach Calibration Standards, Catalog Number 226621-05).

3.3.2 pH

The pH of the wastewater and drinking water samples (as collected) were measured with a Fisher Scientific AB15 pH meter (Pittsburgh, PA) in accordance with Standard Method 4500-H^+(APHA *et al.,* 2005). On each day of use, the pH meter was calibrated before use with 4, 7, and 10 pH buffers. Sample water was poured into a small clean beaker from the sample bottle after inverting several times. The pH probe was then placed in the sample and the value read from the digital readout of the calibrated pH meter. Two replicate samples were poured and measured for each sample.

3.3.3 Total and Dissolved Organic Carbon

Total and dissolved organic carbon concentrations (TOC and DOC) were measured for the wastewater and drinking water samples (as collected) in accordance with Standard Method 5310B (APHA *et al.,* 2005). All of the glassware used for the total and dissolved organic carbon analyses was acid-washed by soaking in a 20% sulfuric acid bath for a minimum of one hour and then rinsing 3 times with E-pure water. Samples were preserved for TOC and DOC analysis on the day they were received. For TOC, samples were poured into 40 mL acid washed glass vials and preserved to a pH of 2 with 40 µL of 6 N HCl. The samples were then capped with screw caps with TFE lined septa and stored at 4ºC for a maximum of 2 weeks before analysis. For DOC, samples were filtered through a glass fiber filter (Whatman GF/F filter with 0.7 µm retention, Cat. No. 1825-025). The filters were pre-washed with 20-30 mL of E-pure water. Then, the sample was passed through the filter. The first 5-10 mL of the sample was discarded and the next 30 mL filtered into a 40 mL acid washed glass vial. The samples were then preserved to a pH of 2 in the same manner as the TOC samples, and capped and stored at 4ºC for up to 2 weeks before analysis.

3.3.3.1 Standard Preparation

The TOC and DOC of the water samples were measured with a Shimadzu TOC-5000A Analyzer (Shimadzu Corp., Kyoto, Japan). The TOC analyzer utilizes a three point calibration curve made with potassium hydrogen phthalate. First, a stock primary standard of 1000 mg/L was prepared: 0.75 grams of potassium hydrogen phthalate was dried in a 103-110ºC oven for 30 minutes and cooled in a desiccator for an additional 30 minutes. Following the cooling process, 0.5314 grams of the dried potassium hydrogen phthalate was weighed using an analytical balance, and added to a 250 mL volumetric flask filled half way with E-pure water. The solution was swirled until the chemical was dissolved. The volume in the flask was then brought up to the mark with E-pure water. The stock primary solution of 1000 mg/L was put in a brown glass bottle and stored at 4ºC for a maximum of 4 weeks.

Second, an intermediate standard of 100 mg/L was made. To make the intermediate standard, 10 mL of the primary stock standard was transferred using a volumetric pipette into a 100 mL volumetric flask half filled with E-pure water. The volume in the flask was then bought up to the mark with additional E-pure water. The intermediate standard of 100 mg/L was stored at 4ºC for a maximum of 2 days.

The working standards used in the calibration curve depended on the sample being analyzed, as shown in Table 3.8. For each working standard, a 100 mL flask was filled half way with E-pure water and 100 µL of 6 N HCl was added to each flask. Then, the appropriate volume of intermediate standard needed to create the working standard was calculated using Equation 3-1.

$$Cintermediate \times Vinterdemtiate = Cworking \times Vworking \quad \text{(Equation 3-1)}$$

In Equation 3-1, C is the concentration in mg/L and V is the volume in mL. For example, for 100 mL of a 10 mg/L working standard, 10 mL of the intermediate standard was used:

$$Cintermediate \times Vinterdemtiate = Cworking \times Vworking$$

$$V_{intermediate} = \frac{C_{working} \times V_{working}}{C_{intermediate}}$$

$$V_{intermediate} = \frac{10\frac{mg}{L} \times 100\ mL}{100\frac{mg}{L}} = 10\ mL$$

This calculated volume of the intermediate standard was added to the working standard flask. Lastly, the volume in each flask was brought up to mark with E-pure water.

Table 3.8: Working Standards for TOC/DOC Analysis

Sample	**Standards for Calibration (mg/L)**
Raw wastewater	Curve 1: 100, 50, 0 Curve 2: 20, 10, 0
Final wastewater	Curve 1: 50, 20, 0 Curve 2: 10, 5, 0
Drinking water	Curve 1: 50, 20, 0 Curve 2: 10, 5, 0

3.3.3.2 TOC/DOC Quantification

Once all of the working standards were prepared, the auto-sampler cells for the Shimadzu TOC-5000A were filled. Each standard and sample was inverted three times, poured into an autosampler vial, and then the vial was covered with parafilm and plastic Shimadzu lids. The standards were placed in the inner ring of the autosampler rack from highest to lowest. Multiple calibration curves were produced and the instrument selected the best curve for determining the concentration of each sample. The samples were placed in the outer ring of the autosampler rack. Quality control was established by placing two working standards with known concentrations in with the sample vials to verify accurate measurements.

All standards and samples were sparged for three minutes before analysis to remove any carbon dioxide and then analyzed three to five times. The standards and samples were measured a minimum of three times, after which the standard deviation (SD) and coefficient of variation (CV) were calculated. If the values were not in the desired range (200 for standard deviation and 2.0% for coefficient of variation) after the third measurement, then another measurement was taken. Measurements were taken until three values had an SD or CV in the desired range or until 5 measurements were taken (and the three measurements with the lowest SD or CV were used).

3.4 Statistical Analysis

Two statistical methods were utilized for analyzing data collected from the sampling sites: correlation analysis and analysis of variance. Correlation analyses were performed on the individual water quality parameters to identify relationships between them. ANOVA was completed to determine differences between different regions and differences between season with each water quality parameter.

3.4.1 Correlation Analysis

Correlation analyses were done using the Microsoft Excel data analysis tool pack. The data analysis yields a rho value output which is a correlation coefficient representing the linear relationship between the data pairs. Correlation coefficient values range from –1.00 to +1.00, where the negative sign indicates an inverse correlation. Zero indicates no correlation and 1 indicates complete linear correlation.

The rho-value is used to determine whether a correlation is statistically significant. The two factors that determine whether a rho value shows statistical significance is the confidence level used and the number of data pairs that the rho value is generated from. A 95% confidence level was used, which is commonly used for research. This is a P-value of 0.05. For example, a statistically significant correlation is a P-value of 0.05, and a highly significant correlation would

be ≥0.005. If the absolute value of the calculated rho value is greater than or equal to the table value from the appendix (Appendix C), there is a statistically significant correlation.

3.4.2 ANOVA Analysis

The analysis of variance (ANOVA), also known as the F-test, is a method to determine the variation of the means of a group of data or variables to evaluate statistical significance. ANOVA analyses were done utilizing the Microsoft Excel data analysis tool pack. The ANOVA test assumes a null hypothesis, which states that there is no difference between the data within a data set. If the analysis is found to be statistically significant, then the null hypothesis is rejected for the alternative hypothesis. The alternative hypothesis states that the means of the data in the data set are different. Similar to the correlation analysis, a 95% confidence level (P-value $\leq$ 0.05) was considered to be statistically significant. The ANOVA analysis was performed with the data segregated by region to determine differences in water quality between the different locations. Then the analysis was repeated with the data organized by season to assess seasonal differences in water quality.

Chapter 4 Results

Animal feces, wastewaters, and drinking water samples processed and the data was analyzed. The collected results included physical, chemical, and bacteriological quantifications. The results were statistically run for correlations and analysis of variance.

4.1 Quality Assurance and Quality Control

Appropriate steps were taken to ensure quality throughout the sampling process. Positive and negative controls were utilized for quality control in all coliform, *E. coli*, and coliphage testing. For physical and chemical water quality analysis, Standard Methods protocals were followed. Instruments were appropriately calibrated according to the manufacturer's recommendation, and standard curves were prepared accordingly. When testing for organic carbon concentrations, all glassware was sulfuric acid washed prior to use. For microbiological work, all labware was either autoclaved prior to use, or purchased pre-sterilized. All quality assurance and quality control results were acceptable.

4.2 Fecal Samples

Fecal samples were collected in three seasons over the course of 11 months. A total of 76 samples from eight different animal types were collected and tested for traditional bacterial indicators (coliforms and *E. coli*) and coliphages (male-specific and somatic). Full results are provided in Appendix D. The following sections present a summary of the results and statistical analysis of the data.

4.2.1 Indicator Organism Concentrations

A summary of low and high values for the bacterial indicators and coliphages in fecal samples is shown in Table 4.1. All indicators were below detection levels in some fecal samples. It is well established that healthy ruminants harbor *E. coli* and thus coliforms in their gastrointestinal tract (Grauke *et al.*, 2002). It is therefore unusual to not detect coliforms and *E. coli* in fecal matter from chickens and cows. These results are likely attributed to a collection or sampling error. There was one fecal sample from one cow (collected 6-10-2010, WI) with coliforms and *E. coli* below detection limits. The two additional cow fecal samples collected on 6-10-2010 from

Wisconsin had counts on the order of 10^4 and 10^7 cfu/g, which suggest a collection or processing error for the zero count cow fecal sample. Similarly, there was one chicken fecal sample with coliforms and *E. coli* below detection limits (4-12-2011, WI). The other chicken fecal sample collected on this day had 100 cfu/g for coliforms and *E. coli*. In contrast, all other chicken feces had coliforms and *E. coli* in the range of $5.7x10^4$ to $5.0x10^8$ cfu/g. These results suggest the chicken fecal samples from 4-12-2011 may have been compromised during collection or processing.

Table 4.1 Indicator organism concentrations in fecal samples

Animal	Coliforms (cfu/g)		*E. coli* (cfu/g)		Male-specific Coliphage (pfu/g)		Somatic Coliphage (pfu/g)	
	Low	High	Low	High	Low	High	Low	High
Chicken	BDL	$6.1x10^8$	BDL	$3.4x10^8$	BDL	$2.0x10^6$	BDL	$>2.5x10^7$
Cow	BDL	$2.6x10^7$	BDL	$1.1x10^7$	BDL	$1.5x10^4$	BDL	$8.4x10^4$
Dog	$5.4x10^4$	$1.0x10^8$	$6.6x10^4$	$1.0x10^8$	BDL	$1.7x10^2$	BDL	$1.8x10^4$
Horse	$3.1x10^2$	$5.0x10^4$	$3.1x10^2$	$7.6x10^2$	BDL	$7.6x10^2$	BDL	$1.0x10^5$
Donkey	305	$3.6x10^5$	305	$1.8x10^4$	BDL	$2.9x10^4$	BDL	$2.4x10^2$
Goat	$1.1x10^6$	$1.2x10^7$	$1.1x10^6$	$1.1x10^7$	BDL	$2.7x10^1$	BDL	$1.8x10^2$
Llama*	$1.1x10^7$		$1.1x10^7$		$5.2x10^4$		$1.8x10^2$	
Rabbit	$3.7x10^3$	$2.6x10^5$	$1.6x10^2$	$2.6x10^5$	BDL	$4.8x10^4$	BDL	$3.0x10^5$
Sheep	$1.5x10^5$	$4.1x10^6$	$1.4x10^5$	$3.4x10^6$	BDL	$4.8x10^1$	BDL	$1.1x10^4$

BDL = Below Detection Limit
*Only one llama fecal sample collected

Coliphages were detected in approximately half of the fecal samples. 42 of 76 samples were below detection limits for male-specific coliphages and 33 of 76 were below detection limits for somatic coliphages. These results are supported by previous research. Long *et al.* (2005) collected and measured coliphages in 36 grazing and agricultural animal fecal samples from different geographical locations in different seasons in a study of the potential role of male-specific coliphages as delineators of sources of surface water microbial pollution. All grazing animal fecal samples were below the detection limit of 3.0 pfu/g for F-specific coliphages. For somatic coliphages, some fecal samples were below detection limits for cows, horses, sheep and pigs. Calci *et al.* (1998) found a high percentage of horse, cow, and sheep feces to have male-

specific coliphage concentrations below 10 pfu/g. However, most of the 11 animal types in their study shed relatively low numbers of male specific coliphages, despite all of these animals harboring male specific coliphage.

For samples with detectable levels of indicator organisms, coliforms and *E. coli* ranged from the hundreds of cfu per gram in horses to 10^8 per gram in chickens. Coliphages also had a wide range, from the hundreds of pfu/g in horses and dogs, to much higher values in chickens (greater than $2.5x10^7$ pfu/g). These results are consistent with coliphage levels in feces found in previous studies. Leclerc *et al.* (1999) analyzed fecal samples from dogs, sheep, goats, ducks, geese, chickens, cows, hogs and horses and found male-specific coliphage counts ranged from $8.6x10^2$ pfu/g (dog) to $1.9x10^7$ pfu/g (horses). Long *et al.* (2005) found somatic coliphage counts to be as high as $3.6x10^6$ pfu/g for cow feces and 1.9×10^7 pfu/g for horse feces.

In addition to microbial measures, pH was measured on fecal resuspensions (10^{-2} dilution). The pH levels in fecal samples ranged from 5.18 (chicken) to 8.93 (rabbit). The greatest pH range was in chicken feces (5.18 – 8.57), followed by cow feces (5.23 – 8.24). Horse feces had the smallest pH range (6.49 – 7.19). There did not appear to be seasonal or regional trends. Rollins *et al.* (1984) found animal species with similar diets to have similar fecal pH.

4.2.2 Correlation Analysis

A correlation analysis was conducted on the fecal sample data to determine associations between the indicator organisms. Results that were below detection limits were set to 0 for this analysis. There were six possible correlations. A critical rho value of 0.226 was required for a statistically significant correlation at the 95% confidence level and 0.295 at the 99% confidence level. The calculated rho values are shown in Table 4.2. Coliforms and *E. coli*, male-specific coliphages and coliforms, and male-specific coliphages and *E. coli* were correlated at the 99% confidence level. As *E. coli* are a subset of coliforms, it was expected that they would be correlated to the highest degree of confidence. Male-specific and somatic coliphages attach themselves and infect coliform bacteria, therefore, the positive correlation between male-specific coliphage and bacterial indicators is expected (Cole *et al.*, 2003). In contrast, somatic coliphages were not correlated to any of the other indicator organisms, suggesting differences between male-specific and somatic coliphages. Somatic coliphages were not detected in 43% of fecal samples, which

might explain the lack of correlation. However, male-specific coliphages also had many non-detects, but had positive correlations. The findings in this study are contradicted by the results of other studies. Baldini and Brezina (2008), in a study of somatic coliphages as indicators of fecal contamination in estuarine waters, found a statistically significant correlation between *E. coli* and somatic coliphage. Ibarluzea *et al*. (2007) found the correlation between somatic coliphage and bacteriological indicators to be moderate. For fecal samples with detectable coliphages, the concentrations of somatic coliphages in this study tended to be greater than the concentrations of male-specific coliphages. In most cases, when both coliphages were detected in a fecal sample, the concentration of somatic coliphages was 1 to 3 orders of magnitude greater than male-specific coliphages. Similarly, Lee and Sobsey (2011) and Brion *et al*. (2001) found somatic coliphages to be present in greater numbers than male specific coliphages, which they attributed to better environmental persistence.

Table 4.2 Rho values for correlation analysis of fecal samples. Critical rho value for 95% confidence = 0.226 (Italicized). Critical rho value for 99% confidence = 0.295 (Bold italicized)

Indicator	Coliforms	*E. coli*	Male-specific Coliphage	Somatic Coliphage
Coliforms	1			
E. coli	***0.973***	1		
Male-specific Coliphage	***0.583***	***0.583***	1	
Somatic Coliphage	0.093	0.071	-0.013	1

4.2.3 ANOVA

Sixteen separate analysis of variance (ANOVA) tests were conducted. First, analyses were run to determine if there were statistical differences in each indicator based on animal type, considering all animals tested. Then differences in indicators were assessed based on animal type, season and region, considering the four animals for which the most samples were collected (chicken, cow, dog, horse). Results for the first analyses considering all 76 samples and 8 animal types are shown in Table 4.3. Coliform and *E. coli* levels were statistically different by animal.

For coliforms, fecal samples from donkeys had the lowest average (1.2×10^5 cfu/g) while chicken had the highest (1.4×10^8 cfu/g). The same was true for *E. coli*, with donkeys and chickens averaging 6.2×10^3 and 7.1×10^7 cfu/g, respectively. There was no statistical difference based on animal type for either of the coliphages. This could indicate that coliphage concentrations are not affected by animal types they are harbored in and excreted from. Cole *et al*. (2003) found male-specific coliphages differed for different animal types in that cattle and swine contained coliphages more frequently than waterfowl and companion animals. However, swine and waterfowl were not tested in this study.

Table 4.3 Analysis of variance on fecal samples from all 8 animal types; chicken, cow, dog, donkey, goat, horse, rabbit, sheep. Statistical difference at the 95% confidence level by animal indicated by bold p-value.

Indicator	**P-value**	**Lowest average value**	**Highest average value**
Coliforms	**0.013**	Donkey	Chicken
E. coli	**0.043**	Donkey	Chicken
Male-specific Coliphage	0.513	NA	NA
Somatic Coliphage	0.192	NA	NA

Of the 76 fecal samples, 63 were collected from chicken, cows, dogs, and horses. As these four animals had the most sampling data, the ANOVA analysis were re-run considering only these types. As shown in Table 4.4, coliforms, *E. coli*, and somatic coliphages were all statistically different by animal. For coliforms, fecal samples from cows had the lowest average (2.6×10^6 cfu/g) while chicken feces had the highest (1.4×10^8 cfu/g). The same was true for *E. coli*, with cow feces averaging 1.78×10^6 cfu/g and chicken feces averaging 7.1×10^7 cfu/g. There was no statistical difference based on animal type for the male-specific coliphages. For somatic coliphages, fecal samples from dog had the lowest average (1.3×10^3 cfu/g) while fecal samples from chicken had the highest (3.2×10^6 cfu/g). The analysis with four animal types indicates that somatic coliphages are statistically different by animal type.

Table 4.4 Analysis of variance on fecal samples considering chicken, cow, dog, horse. Statistical difference at the 95% confidence level by animal indicated by bold p-value.

Indicator	P-value	Lowest average value	Highest average value
Coliforms	**0.002**	Cow	Chicken
E. coli	**0.008**	Cow	Chicken
Male-specific Coliphage	0.150	NA	NA
Somatic Coliphage	**0.038**	Dog	Chicken

Additional analyses on the fecal sample results were conducted using the data from the four most frequently sampled animals. First, ANOVA was used to determine if there were differences in indicator levels by season (Table 4.5). At the 95% confidence level, there were no statistically significant differences in any of the indicators by season for each animal. Fecal samples were collected fresh immediately after defecation of the animal and thus represent conditions in the gastrointestinal tract, which may not be affected by season.

Table 4.5 Analysis of variance on season for fecal samples considering chicken, cow, dog, horse. Statistical difference at the 95% confidence level by animal indicated by bold p-value.

Indicator	Seasonal ANOVA P-Values			
	Chicken	Cow	Dog	Horse
Coliform	0.698	0.452	0.181	0.587
E. coli	0.568	0.591	0.185	0.625
Male Specific Coliphage	0.128	0.511	0.475	0.475
Somatic Coliphage	0.512	0.535	0.487	0.386

ANOVA was also used to determine if there were differences by geographical region for the four most sampled animals (Table 4.6). At the 95% confidence level, coliforms and *E. coli* varied by region in chicken feces, and both male-specific and somatic coliphages varied by region in cow feces. In dog feces, male-specific coliphages varied by region.

Table 4.6. ANOVA Regional P-Values for fecal samples. Difference at 95% confidence indicated in bold.

Indicator	**Regional ANOVA P-Values**			
	Chicken	Cow	Dog	Horse
Coliform	**0.001**	0.292	0.123	0.303
E. coli	**0.008**	0.355	0.347	0.141
Male Specific Coliphage	0.349	**0.001**	**0.027**	0.190
Somatic Coliphage	0.650	**0.048**	0.060	0.887

4.2.4 Non detects

The frequency of detection is important in considering the usefulness of a potential indicator organism. As shown in Table 4.7, coliforms and *E. coli* were detected in the vast majority of fecal samples. This is expected since healthy ruminants harbor *E. coli* and thus coliforms in their gastrointestinal tract (Grauke *et al.*, 2002). As previously discussed, the non-detects were likely due to sampling errors. For coliphages, however, about 50% of the fecal samples were below detection limits. This high level of non-detects is supported by the literature. Calci *et al.* (1998) reported that more than 53% of chickens in their study shed <10 pfu/g of male-specific coliphages. Likewise, Jones and Johns (2009) believe that certain coliphage properties prevent a full coliphage recovery from the fecal sample. They observed male-specific coliphage non detects to range from 30 to 96% in pig, cattle and poultry fecal samples. Jones and Johns (2009) feel the 1 g of fecal material used in the coliphage enumeration process is insufficient. Male-specific coliphages were detected in only 9 of 25 fecal samples when the sample size was 1 g;

however, when the sample size was increased to 10 g, male-specific coliphages were detected in 16 of 25 samples. The frequency of non-detect coliphages is a concern for its use as an indicator.

Table 4.7. Frequency of non-detects in fecal samples.

Quantity	Coliforms	*E. coli*	Male-specific Coliphage	Somatic Coliphage
# of fecal samples	76	76	76	76
# of positive results	73	71	34	43
# of non-detects	3	5	42	33
% of non-detects	3.9	6.6	55.3	43.4

4.3 Wastewater Results

Wastewater samples were collected from four wastewater treatment plants in three seasons over the course of 11 months. A total of 25 raw and final wastewater samples were collected and tested for physical and chemical water quality parameters, traditional bacterial indicators (coliforms and *E. coli*), and coliphages (male-specific and somatic). Full results are presented in Appendix E. The following sections present a summary of the results and statistical analysis of the data.

4.3.1 Indicator organism concentrations

Coliforms and coliphages were tested as indicators. The results are shown in Table 4.8. For the wastewater samples, bacterial indicators (total coliform and *E. coli*) were up to 10^8 per 100 mL in raw wastewater, but decreased by 2-3 orders of magnitude through treatment – with levels as low as the hundreds per 100 mL in final wastewater prior to disinfection. These numbers are consistent with previous studies. In a study of coliforms and bacteriophages in wastewater, Claydong *et al.* (2001) found total coliform levels in raw domestic wastewater ranged from $4.3x10^6$ to $1.1x10^8$ MPN/100 mL, and after treatment coliform levels decreased by more than 95%. For coliphages, raw wastewater had up to 10^5 plaque forming units per 100 mL, but as low as below detection limits in the final wastewater (prior to disinfection). Other studies have found similar results for coliphages, with concentration levels in raw wastewater ranging from 10^4 to

10^6 pfu/ 100 mL (Calci *et al.*, 2008). The fact that coliphages are tolerant to wastewater treatment makes them suitable indicators of fecal contamination (Espinosa *et al.*, 2009).

Table 4.8 Concentrations of indicator organisms in raw and final wastewater.

Sample	Value	Coliforms		Coliphages	
		Total (cfu/100 mL)	*E. coli* (cfu/100 mL)	Male Specific (pfu/100 mL)	Somatic (pfu/100 mL)
Raw	Low	$6.6x10^5$	$3.5x10^4$	$2.2x10^3$	$7.3x10^2$
	Average	$2.6x10^7$	$2.8x10^6$	$9.5x10^4$	$7.2x10^4$
	High	$1.0x10^8$	$7.3x10^6$	$3.0x10^5$	$1.6x10^5$
Final	Low	$9.0x10^2$	$1.2x10^2$	BDL	$1.7x10^2$
	Average	$1.3x10^5$	$1.7x10^4$	$2.2x10^2$	$4.5x10^4$
	High	$9.8x10^5$	$8.9x10^4$	$7.6x10^2$	$5.1x10^5$

4.3.2 Physical and Chemical Wastewater Characteristics

Several physical and chemical water quality parameters were measured for each wastewater sample. Figure 4.1 summarizes the turbidity, pH, TOC, and DOC values for all raw and final wastewater samples. Turbidity in the raw wastewater was typically in the hundreds, with levels as high as 900 ntu. These levels were reduced through treatment, as observed in the final wastewater samples with turbidities in the ones or tens ntu. No major differences were observed in pH, which ranged from 6.15 to 7.62. TOC and DOC were also reduced through treatment. The raw TOC ranged from 42.3 mg/L to 194 mg/L, while final wastewater ranged from 6.15 mg/L to 24.1 mg/L. The raw DOC ranged from 29.4 mg/L to 97.3 mg/L, while final wastewater DOC levels ranged from 6.04 mg/L to 15.6 mg/L. These physical and chemical data are consistent with the literature. Raw wastewater turbidity can range from less than 1 ntu to thousands of ntu (Hargesheimer *et al.*, 2002). According to Davis and Masten (2009) the typical pH range for untreated domestic wastewater is 6.5 to 8.5; the majority of sampled raw and final results fell within this range. Outside of this range, aquatic organisms can become physiologically stressed. Typical untreated wastewater organic carbon levels range from 50 to 300 mg/L (Davis and Masten, 2009).

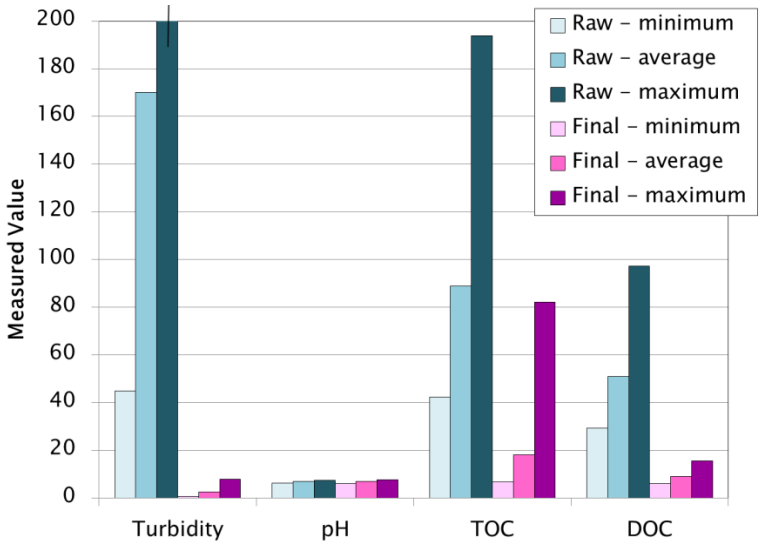


Figure 4.1. Physical and chemical wastewater characteristics.

4.3.3 Correlation Analysis

Correlation analysis was conducted on the wastewater sample data to determine associations between water quality parameters and indicator organisms. First, the data were analyzed considering all 25 wastewater samples. There were 36 possible correlations for all wastewater results. For a statistically significant correlation at the 95% confidence level, a critical rho value of 0.396 was required, while a critical rho value of 0.505 was required for the 99% confidence level. The calculated rho values are shown in Table 4.9. At the 95% confidence level, four correlations were found to be statistically significant. At the 99% confidence level, seven correlations were found to be statistically significant. All indicators except somatic coliphages exhibited some statistical correlation with another parameter; however, there were no correlations between the bacterial indicators and the coliphages.

Table 4.9 Correlation analysis rho values for wastewater samples . Critical rho value for 95% confidence = 0.396 (italicized). Critical rho value for 99% confidence = 0.505 (Bold italicized)

	Turb	pH	TOC	DOC	Coliform	*E. coli*	Male-Specific coliphages	Somatic coliphages
Turb	1							
pH	-0.022	1						
TOC	0.269	-0.178	1					
DOC	*0.485*	-0.154	***0.824***	1				
Coliforms	***0.740***	-0.151	*0.419*	***0.761***	1			
E. coli	***0.658***	-0.154	***0.511***	***0.827***	***0.928***	1		
Male-Specific coliphages	0.385	0.050	0.388	*0.446*	0.358	*0.427*	1	
Somatic coliphages	0.190	0.184	0.216	0.236	0.219	0.231	0.059	1

These data were then separated into 2 groups: raw wastewater (13 samples) and final wastewater (12 samples). Correlation analyses were run for each group separately. For the raw wastewater samples the critical rho value for a statistically significant correlation at the 95% and 99% confidence levels were 0.602 and 0.735 respectively. The calculated rho values are shown in Table 4.10. Somatic colilphages correlated with total and dissolved organic carbon at the 95% confidence level. In addition, *E. coli* correlated with DOC at the 95% confidence level. Previous work by Otterholt and Charnock (2011) also found a strong correlation between organic carbon levels and *E. coli* presence in an investigation of the microbiological quality of five leading brands of Norwegian bottled still waters. Throughout this study, organic carbon levels have shown correlations with both bacterial and coliphage indicators.

Table 4.10. Raw only wastewater rho values. Critical rho value for 95% confidence = 0.602 (Italicized). Critical rho value for 99% confidence = 0.735 (Bold underlined italicized)

	Turb	pH	TOC	DOC	Coliforms	*E. coli*	Male-Specific coliphages	Somatic coliphages
Turb	1							
pH	-0.001	1						
TOC	-0.114	-0.084	1					
DOC	0.277	-0.301	*0.642*	1				
Coliform	*0.703*	-0.212	0.118	***0.753***	1			
E. coli	*0.603*	-0.269	0.049	*0.673*	***0.932***	1		
Male-Specific coliphages	0.104	0.214	-0.232	-0.181	0.125	0.098	1	
Somatic coliphages	0.398	-0.193	*0.684*	*0.689*	0.490	0.509	-0.134	1

Next, correlation analysis was run for the 12 final wastewater samples. For a statistically significant correlation at the 95% confidence level, a rho value of 0.591 was required, while a rho value of 0.777 was required for 99% confidence. As shown in table 4.11, at the 95% confidence level, nine correlations were found to be statistically significant. At the 99% confidence level, five correlations were found to be statistically significant. As previously discussed, the correlation between coliforms and *E. coli* is expected. All indicators except somatic coliphages exhibited some statistical correlation. Most notable is the correlation between male-specific coliphage and coliforms at the 95% confidence level, and the correlation between male-specific coliphage and *E. coli* at the 99% confidence level. Previous work by Claydong *et al.* (2001) also found a significant correlation between male-specific coliphage and total coliforms. The lack of correlation between somatic coliphage and other indicators has been observed in previous studies. Imamovic *et al.* (2010) found negative or very low correlation coefficients when comparing bacterial indicators (*E. coli* strains or coliforms) with somatic coliphages ($r = -0.10$ to 0.41).

Table 4.11. Correlation analysis rho values for final wastewater samples. Critical rho value for 95% confidence = 0.591 (Italicized). Critical rho value for 99% confidence = 0.777 (Bold italicized)

Parameter	Turb.	pH	TOC	DOC	Coliforms	*E. coli*	Male-specific Coliphage	Somatic Coliphage
Turbidity	1							
pH	0.144	1						
TOC	0.035	0.431	1					
DOC	*0.596*	0.461	0.099	1				
Coliforms	0.429	0.029	0.061	***0.821***	1			
E. coli	***0.827***	0.119	0.035	***0.855***	***0.855***	1		
Male-specific Coliphage	*0.733*	0.003	0.029	*0.751*	*0.697*	***0.832***	1	
Somatic Coliphage	0.031	0.271	0.138	-0.154	-0.038	-0.065	-0.240	1

4.3.4 ANOVA

Twenty four separate analysis of variance (ANOVA) tests were conducted. The data were grouped into all wastewater samples, raw wastewater only, and final wastewater only. The results for seasonal variances are presented in Table 4.20. At the 95% confidence level, there were no differences in any indicators by season. Similarly, Long *et al.* (2005) found no seasonal trend in male-specific coliphage densities in wastewaters. Season may not have played a role in indicator concentrations in wastewater because the sewage is typically transported to the

treatment facility in subsurface closed piping systems where atmospheric temperatures will have little effect.

Table 4.12 Seasonal Analysis of Variance for wastewater samples. Statistical difference at 95% confidence level by season indicated by bold p-value.

Indicator	Seasonal ANOVA P-Values		
	All Wastewater	**Final Wastewater**	**Raw Wastewater**
Coliform	0.969	0.616	0.884
E. coli	0.925	0.893	0.968
Male Specific Coliphage	0.299	0.732	0.054
Somatic Coliphage	0.577	0.421	0.442

Regional analysis of variance results are presented in Table 4.13. There were no differences in any indicators by region at the 95% confidence level for all wastewater data and for raw wastewater data. *E. coli* varied by region in final wastewater at the 95% confidence level. Regional effects on *E. coli* have been shown in other studies. Parveen *et al.* (2006) found region to play a moderately significant role in the resistance of *E. coli* to certain antibiotics.

Table 4.13. Regional Analysis of Variance for wastewater samples. Statistical difference at 95% confidence level by region indicated by bold p-value.

Indicator	Regional ANOVA P-Values		
	All Wastewater	**Final Wastewater**	**Raw Wastewater**
Coliform	0.465	0.313	0.467
E. coli	0.362	*0.003*	0.210
Male Specific Coliphage	0.866	0.069	0.824
Somatic Coliphage	0.236	0.446	0.103

4.4 Drinking Water Results

Drinking water samples were collected from four drinking water treatment plants in four seasons over the course of ten months. A total of 70 treated and untreated water samples from both ground and surface sources were collected and tested for physical and chemical water quality parameters, traditional bacterial indicators (coliforms and *E. coli*), and coliphages (male-specific and somatic). Full results are presented in Appendix F. The following sections present a summary of the results and statistical analysis of the data.

4.4.1 Indicator Organism Concentrations

A summary of low, average and high values for the bacterial indicators and coliphages in all drinking water samples is shown in Table 4.14. The low values for both untreated and treated coliforms and coliphages were below detection. Untreated water sources had total coliforms up to 1450 cfu/mL, but these levels were reduced through treatment by one or more orders of magnitude. The greatest untreated *E. coli* count was 9.18 cfu/100mL in drinking water, and decreased by 1 order of magnitude or less through treatment. These numbers are consistent with average coliform and *E. coli* levels found by previous studies (Frankenberger, 2012; LeChavellier *et al*, 1996). Interestingly, average and high male-specific coliphage values increased slightly from untreated to treated samples from 2.02 to 2.49 pfu/ 100mL. This can likely be explained by the fact that the *high treated* sample was collected in the spring from New England, while the *high untreated* sample was collected in the winter from the New England, indicating seasonal variability on coliphage concentrations. Calci *et al*. (2008) suggested that wastewater treatment plants are the principal contributors of male-specific coliphages to source waters. The high untreated somatic coliphage level was 4.88 pfu/100mL while the treated high was 1.0 pfu/100mL. Average somatic coliphages were reduced by 1 order of magnitude through treatment from 10^{-1} to 10^{-2} pfu/100mL. Stewart-Pullaro *et al*. (2006) found similar coliphage levels in source waters.

Table 4.14. Drinking water indicator concentrations.

Sample	Value	Coliforms		Coliphages	
		Total (cfu / 100 mL)	*E. coli* (cfu / 100 mL)	Male Specific (pfu / 100 mL)	Somatic (pfu / 100 mL)
Untreated	Low	BDL	BDL	BDL	BDL
	Average	183	1.24	20.2	0.552
	High	1450	9.18	227	4.88
Treated	Low	BDL	BDL	BDL	BDL
	Average	6.12	0.407×10^{-1}	24.9	0.092
	High	258	2.70	1020	1.0

4.4.2 Physical and Chemical Drinking Water Characteristics

Several physical and chemical water quality parameters were measured for each drinking water sample. Figure 4.2 summarizes the turbidity, pH, TOC, and DOC values for all drinking water samples. Turbidity in untreated drinking waters averaged 2.5 ntu, with levels as high as 9.7 ntu. These levels were reduced through treatment, with average treated drinking water turbidity less than 1 ntu and a high of 3.9 ntu. No major differences were observed in pH through treatment, which ranged from 6.03 to 8.89. TOC and DOC were also reduced through treatment. The untreated TOC ranged from 0.26 mg/L to 7.42 mg/L, while treated drinking water ranged from 0.04 mg/L to 7.77 mg/L. The untreated DOC ranged from 0.68 mg/L to 7.03 mg/L, while treated drinking water DOC levels ranged from 0.11 mg/L to 4.21 mg/L. These physical and chemical data are consistent with the literature (LeChevallier *et al.*, 1991; EPA, 2012)

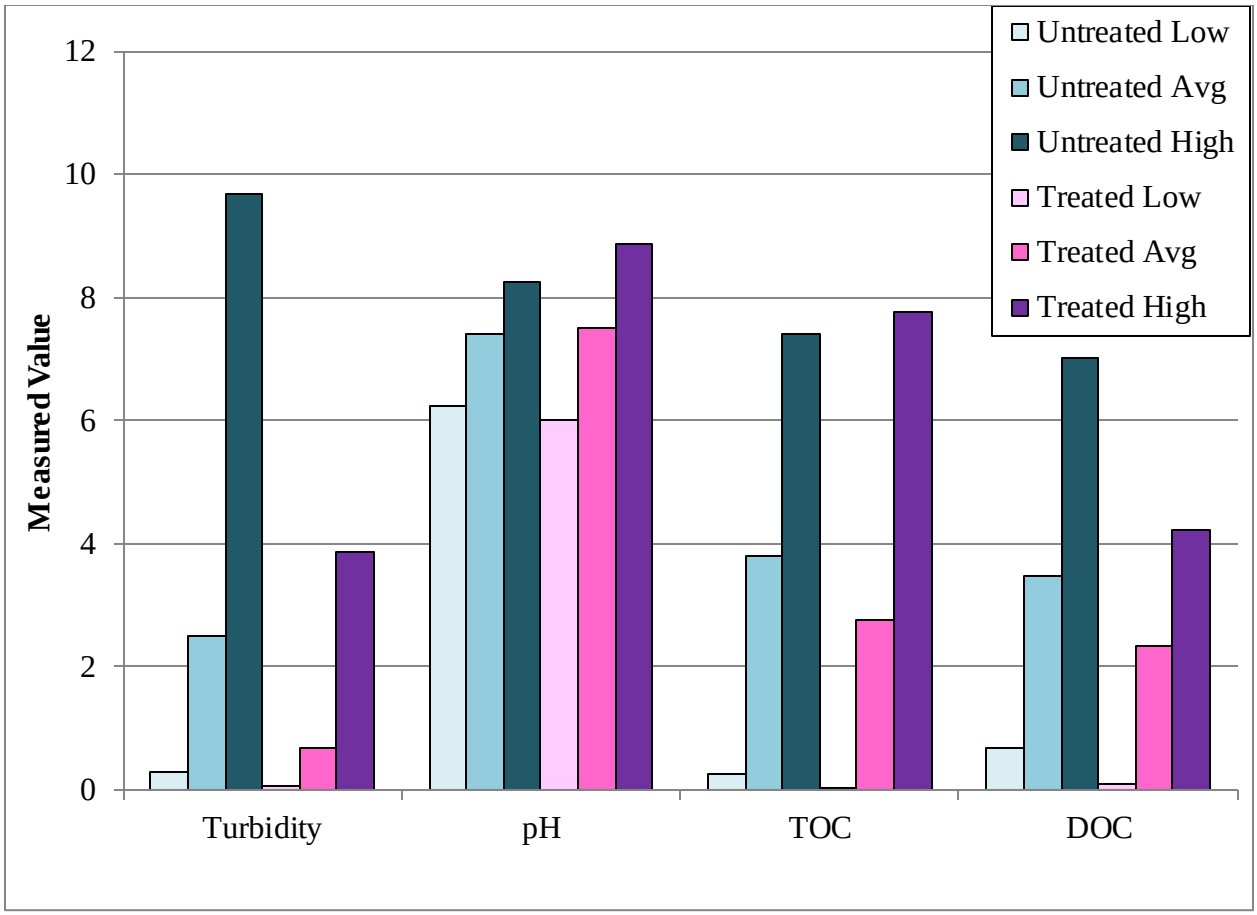


Figure 4.2. Physical and chemical drinking water levels

4.4.3 Correlation Analysis

A correlation analysis was conducted on the drinking water sample data to determine associations between water quality parameters and indicator organisms. First, the data were analyzed considering all 70 drinking water samples. There were 28 possible correlations. For a statistically significant correlation at the 95% confidence level, a critical rho value of 0.235 was required, while a critical rho value of 0.307 was required at the 99% confidence level. Due to instrument malfunction, fewer TOC and DOC sample values were obtained, thus the critical rho values for a statistically significant correlation at the 95% and 99% confidence levels were 0.305 and 0.395 for TOC and DOC, respectively. The calculated rho values are shown in Table 4.15. At the 95% confidence level, 10 correlations were found to be statistically significant, while 8 correlations were found to be statistically significant at the 99% confidence level. All indicators

exhibited some statistical correlations. Male-specific coliphages were correlated with pH at the 95% confidence level. Somatic coliphages were correlated with TOC and DOC at the 99% confidence level. In addition to somatic coliphages, DOC was correlated at the 99% confidence level with turbidity and TOC. There were no correlations between the bacterial indicators and the coliphages. The correlations between organic carbon and coliforms has been observed before. Boualam *et al.* (2002) studied the growth and culturability of coliform bacteria as a function of organic carbon levels in surface water through the treatment process. They observed a positive relationship between dissolved organic carbon and coliform bacteria culturability in treated drinking water from surface water sources.

Table 4.15. Correlation analysis rho values for all drinking water samples. Critical rho value for 95% confidence = 0.235 (italicized). Critical rho value for 99% confidence = 0.307 (bold italicized). For TOC/DOC 95% and 99% confidence levels were 0.305 and 0.395 respectively.

	Turb	*pH*	*Coliforms*	*E. coli*	*Male-specific coliphages*	*Somatic coliphage*	*TOC*	*DOC*
Turbidity	1							
pH	***-0.364***	1						
Coliforms	0.047	*-0.285*	1					
E. coli	***0.561***	-0.162	***0.487***	1				
Male-specific coliphage	0.191	*-0.296*	-0.095	-0.089	1			
Somatic coliphage	-0.179	0.168	0.078	-0.134	-0.140	1		
TOC	***0.347***	0.267	0.016	-0.211	0.171	***0.459***	1	
DOC	***0.591***	0.061	0.081	-0.229	0.114	***0.521***	***0.659***	1

The data were then separated into two groups: untreated drinking water (21 samples) and treated drinking water (49 samples). Correlation analyses were run for each group separately. For the

untreated drinking water samples, the critical rho value for a statistically significant correlation at the 95% and 99% confidence levels were 0.428 and 0.526, respectively. For TOC and DOC the critical rho values for a statistically significant correlation at the 95% and 99% confidence levels were 0.506 and 0.665 for TOC and DOC respectively. The calculated rho values are shown in Table 4.16. At the 95% confidence level, 4 correlations were found to be statistically significant. At the 99% confidence level, 3 correlations were found to be statistically significant. Most notable are the correlations between somatic coliphages and coliforms, and between somatic coliphages and *E. coli*. This was the only observed correlation between somatic coliphages and any bacterial indicator in this study. Nieuwstad *et al*. (1988) also observed a strong correlation between the somatic coliphages and fecal coliform bacteria. They studied the removal of microorganisms from wastewater by activated sludge and precipitation processes and found somatic coliphages to be an indicator of fecal contamination. Similarly Lucena *et al*. (2010) found somatic coliphages to be correlated with fecal coliforms and suggested that detection and counting of one bacterial indicator and somatic coliphages would be more informative about the presence of pathogens in fresh waters than the enumeration of two bacterial indicators.

Table 4.16. Correlation analysis rho values for untreated drinking water samples. Critical rho value for 95% confidence = 0.428 (itlalicized). Critical rho value for 99% confidence = 0.526 (bold italicized). For TOC/DOC 95% and 99% confidence levels were 0.506 and 0.665 respectively.

	Turbidity	pH	Coliforms	*E. coli*	Male-specific colipage	Somatic phage	TOC	DOC
Turbidity	1							
pH	-0.281	1						
Coliforms	0.165	-0.163	1					
E. coli	-0.003	-0.002	***0.722***	1				
Male-specific colipage	-0.250	-0.082	-0.173	0.286	1			
Somatic phage	-0.056	0.195	***0.714***	***0.839***	0.005	1		
TOC	0.288	-0.050	0.220	-0.151	-0.274	0.033	1	
DOC	*0.638*	-0.113	0.449	0.326	-0.382	0.500	0.264	1

Next, a correlation analysis was run for the 49 treated drinking water samples (Table 4.17). For a statistically significant correlation at the 95% confidence level, a rho value of 0.282 was required, while a rho value of 0.366 was required for 99% confidence. For TOC and DOC the critical rho values for a statistically significant correlation at the 95% and 99% confidence levels were 0.356 and 0.459, respectively. One correlation was found at the 99% confidence level, between TOC and DOC. At the 95% confidence level there were six additional correlations: pH with turbidity, male-specific coliphages with turbidity and TOC, somatic coliphages with TOC,

and DOC with coliforms and *E. coli*. Again, a correlation between organic carbon and coliforms was present as was found by LeChevallier *et al*. (1991) and Boualam *et al*. (2002).

Table 4.17. Correlation analysis rho values for treated drinking water samples. Critical rho value for 95% confidence = 0.282 (itlalicized). Critical rho value for 99% confidence = 0.366 (bold italicized). For TOC/DOC 95% and 99% confidence levels were 0.356 and 0.459 respectively.

	Turbidity	pH	Coliforms	*E. coli*	Male-specific colipage	Somatic phage	TOC	DOC
Turbidity	1							
pH	*-0.335*	1						
Coliforms	-0.059	-0.011	1					
E. coli	-0.119	-0.013	0.116	1				
Male-specific colipage	*0.289*	-0.250	-0.020	-0.078	1			
Somatic phage	0.216	0.090	-0.068	-0.198	-0.071	1		
TOC	0.265	0.234	-0.249	-0.249	*0.421*	*0.361*	1	
DOC	0.341	0.193	*-0.368*	*-0.369*	0.282	0.341	***0.594***	1

4.4.4 ANOVA

Twenty eight separate analysis of variance (ANOVA) tests were conducted. These were done to determine if there were statistical differences in each indicator based on season, region and source. The data were grouped into all drinking water samples, untreated drinking water only, and treated drinking water only. The results for seasonal variances are presented in Table 4.18. At the 95% confidence level, *E. coli* varied by season in treated drinking waters. *E. coli* are a

commonly used indicator, and seasonal variance is not reflective of an ideal indicator. This may have occurred because warmer temperatures can increase survival times and lead to increased fecal indicator densities (Plummer and Long, 2007). This is supported by work done by Ouyang and Isaacson (2006), who assessed seasonal variations in surface water quality; they found temperature to play a role in *E. coli* concentrations. Male-specific coliphages also varied by season in *all* and *treated* drinking waters. These findings are supported by previous work. Cole *et al.* (2003) found male-specific coliphages from waters to be significantly influenced by season. They suggested higher male-specific coliphage inactivation rates in warmer months, and thus a greater likelihood of having male-specific coliphage-positive samples during these months. Likewise, coliphage levels in Arkansas surface waters showed seasonal variance (Dryden *et al.*, 2006). It is interesting, therefore, that the temperature differences between regions were not more significant. There were no statistical differences in any indicator by region. The results for regional variances are presented in Table 4.19.

The variation in male-specific coliphages by season warrants concern for its use as an indicator. Nappier *et al.* (2006) proposed male-specific coliphages as more reliable indicators of human viral pathogens than traditional indicators because they are similar to human enteric viruses in their physical structure, composition, survivability in the environment, and persistence in treatment processes. Much of the United States experiences seasonal changes, which could create inconsistent male-specific coliphage indicator readings as a result of seasonal differences.

Table 4.18. Seasonal Analysis of Variance for drinking water samples. Statistical difference at 95% confidence level by season indicated by bold p-value.

Indicator	Seasonal ANOVA P-Values		
	All Drinking Water	**Untreated Drinking Water**	**Treated Drinking Water**
Coliform	0.315	0.303	0.585
E. coli	0.518	0.350	**0.025**
Male Specific Coliphage	**0.016**	0.356	**0.015**
Somatic Coliphage	0.662	0.377	0.197

Table 4.19. Regional Analysis of Variance for drinking water samples. Statistical difference at 95% confidence level by region indicated by bold p-value.

Indicator	Regional ANOVA P-Values		
	All Drinking Water	**Untreated Drinking Water**	**Treated Drinking Water**
Coliform	0.064	0.518	0.058
E. coli	0.665	0.139	0.490
Male Specific Coliphage	0.249	0.425	0.349
Somatic Coliphage	0.136	0.726	0.219

Chapter 5 Discussion and Recommendations

5.1 Discussion

The detection of indicator bacteria in untreated drinking waters shows the contribution of fecal matter in source waters and reinforces the need for an adequate treatment processes for producing microbiologically safe water in public supply systems. This study evaluated traditional bacterial indicators (coliforms and *E. coli*) and viral indicators (male-specific and somatic coliphages), as indicators of public health risk in waters. An ideal indicator should be similar to potentially harmful pathogens in their physical structure, composition, morphology, survivability in the environment, and persistence in treatment processes (Nappier *et al.*, 2006).

First, coliforms and *E. coli* were evaluated as indicators in animal feces, wastewaters and drinking waters. Both coliforms and *E. coli* were detected in the large majority of fecal samples and in all wastewaters. In drinking waters, there were detects in lower concentrations. Neither bacterial indicator varied by season in wastewaters and drinking waters, nor did they vary by region in drinking waters. Coliforms and *E. coli* were detected in treated wastewaters, however, samples were collected prior to final disinfection and therefore inactivation was not assessed. In drinking waters, some samples were disinfected while others were not. Thus, comparisons of indicators in these samples depends on the treatment processes.

With regard to ideal indicator characteristics, this study revealed areas of concern for bacterial indicators. *E. coli* showed regional variance in final wastewaters. In addition, both bacterial indicators varied by animal type and region in fecal samples.

This study revealed coliphages to be a promising indicator of fecal contamination and ultimately public health risk. Male-specific coliphages were correlated with coliforms and *E. coli* in fecal samples, suggesting fecal contamination as the source of male-specific coliphages in waters. Both male-specific and somatic coliphages showed no regional variance in drinking water and wastewater samples. Somatic coliphages showed no seasonal variance in wastewaters. In addition, no seasonal variances for untreated drinking waters were observed for either coliphage. Like the bacterial indicators, some coliphages survived the wastewater and drinking water treatment processes examined in this study.

The ability of coliphages to survive various environmental conditions in different regions and seasons without variance suggests it may be a good indicator. However, this study revealed some concerns about the using coliphages as indicators. Most significantly was the level of non-detects in fecal samples for both male-specific and somatic coliphages (~50%). A good indicator should have a greater detection frequency, such as coliforms and *E. coli*, which had a high detection frequency (~90%) in fecal samples. Drinking water samples were concentrated up to 25X in this study. Concentrating samples to a higher degree could increase detection of coliphages. A concern with male-specific coliphages in this study was its thermotolerance; male-specific coliphages varied by season in *all* drinking water and *treated* drinking water samples. It was interesting that somatic coliphages only correlated with bacterial indicators in untreated drinking water samples, not in wastewaters or fecal samples. This is surprising due to the gastrointestinal origins of somatic coliphages.

An interesting correlation that kept occurring in this study was that of organic carbon with the bacterial and coliphage indicators. In wastewater samples, DOC correlated with coliforms, *E. coli*, and both coliphages. In *all* drinking water samples, organic carbon correlated with both bacterial and coliphage indicators. At the 99% confidence level, both TOC and DOC positively correlated with coliforms and *E. coli*. Testing for organic carbon, particularly DOC, could be a good physical indicator test in regions where microbiological tests may be too costly or time consuming.

This study has demonstrated that traditional bacterial indicators and coliphages have many qualities of ideal indicators. Areas of concern were addressed for both indicator types in this study and in a review of the literature. It is recommended that male-specific and somatic coliphages be analyzed in additional to coliforms and *E. coli* to test for fecal contamination in waters. Similar to this conclusion, Espinosa *et al.* (2009) found coliphages to be complementary or equivalent to other indicators, and suggested coliphages be included as fecal pollution indicators. In some cases bacterial indicators have shown no correlation with enteric viral genomes, while male-specific and somatic coliphages did in surface waters downstream from a wastewater treatment plant (Skraber *et al.*, 2004). Their findings support the need for coliphages to be added to the indicator suite for assessing public health risk in waters.

5.2 Future Work

The indicator systems studied in this research were compared to each other and to physical and chemical water quality characteristics. The next step is to compare indicator occurrence to viral pathogen data for fecal samples, wastewaters, and drinking waters. Recent research suggests that coliphages reflect the general survival characteristics of enteric viruses (Espinosa *et al.*, 2009). Future work at WPI and the University of Wisconsin will enumerate and analyze norovirus, adenovirus, and torque teno virus (TTV) in fecal samples, wastewaters, and drinking waters. TTV is a newly proposed indicator of viral pathogen presence, and a thorough analysis is needed to determine its value assessing public health risk from viral pathogens.

Somatic coliphages were found in water samples in this study, however, only in untreated drinking water samples. According to the correlation analysis, they did not appear to be of fecal origin. Future work should concentrate samples to greater levels in water samples before testing for male-specific and somatic coliphages. This may reveal correlations between somatic coliphages and bacterial indicators, and may reduce the number of non-detects for both male-specific and somatic coliphages.

References

American Public Health Association, American Water Works Association, Water Pollution Control Federation. Standard Methods for the Examination of Water and Wastewater, 19th Edition (1995). Washington, D.C.: American Public Health Association.

Baldini, M.D., and Brezina, S.S. Detection of somatic coliphages as indicators of fecal contamination in estuarine waters. Revista Argentina de Microbiología (2008) 40: 72-74

Bitton, Gabriel. Microbial indicators of faecal contamination: application to microbial source tracking. Report submitted to the Florida Stormwater Association 719 East Park Avenue, Tallahassee, 32301.(2005)

Blackburn, Brian G., Craun, Gunther G., Yoder, Jonathan S., Hill, Vincent, Calderon, Rebecca L., Chen, Nora, Lee, Sherline H., Levy, Deborah A., Beach, Michael J.. Surveillance for Waterborne-Disease Outbreaks Associated with Drinking Water---United States, 2001--2002. *MMWR.Surveillance summaries* 53.8 (2004): 23. Web.

Borchardt, Mark A., Bertz, Phil D., Spencer, Susan K., Battigelli, David A.. Incidence of Enteric Viruses in Groundwater from Household Wells in Wisconsin. *Applied and Environmental Microbiology* 69.2 (2003): 1172-80. Web.

Borrego, J. J.Coliphages as an Indicator of Faecal Pollution in Water. their Survival and Productive Infectivity in Natural Aquatic Environments. *Water research (Oxford)* 24.1 (1990): 111. Web.

Boualam, M., Mathieu, L., Fass, S., Cavard, J., Gatel, D..Relationship between Coliform Culturability and Organic Matter in Low Nutritive Waters. *Water research* 36.10 (2002): 2618-26. Web.

Calci, Kevin. R., Burkhardt, William III, Watkins, William D., Rippey, Scott R.. Occurrence of Male-Specific Bacteriophage in Feral and Domestic Animal Wastes, Human Feces, and Human-Associated Wastewaters. *Applied and Environmental Microbiology* 64.12 (1998): 5027. Web.

Carducci, A., Gemelli, C., Cantiani, L.,Casini, B., Rovini, E., Assessment of Microbial Parameters as Indicators of Viral Contamination of Aerosol from Urban Sewage Treatment Plants. *Letters in applied microbiology* 28.3 (1999): 207-10. Web.

Centers for Disease Control and Prevention. Water Related Data and Statistics Page. Accessed November, 2011. < http://www.cdc.gov/healthywater/statistics/surveillance/index.html>

Claydong, P. Bacteriophages, Coliform and Fecal Coliform Bacteria in Wastewater. *Advances in water and wastewater treatment technology : molecular technology, nutrient removal, sludge reduction and environmental health* 21 (2001): 119. Web.

Cole, Dana, Long, Sharon C., Sobsey, Mark D.. Evaluation of F+ RNA and DNA Coliphages as Source-Specific Indicators of Fecal Contamination in Surface Waters. *Applied and Environmental Microbiology* 69.11 (2003): 6507-14. Web.

Costán-Longares, Ana, Montemayor, M., Payaan, A., Mendez, J., Mujeriego, R., Lucena, F..Microbial Indicators and Pathogens: Removal, Relationships and Predictive Capabilities in Water Reclamation Facilities. *Water research* 42.17 (2008): 4439-48. Web.

Craun, Gunther F. Waterborne outbreaks reported in the United States. *Journal of water and health (1477-8920)*, (2006): 4(suppl 2), p.19.

Davis, Mackenzie and Masten, Susan. Principles of Environmental Engineering and Science. McGraw-Hill Companies, Inc., New York, NY. 2009.

Dryden, S. K., Ramaswami, B., Giammar, D.E., Angenent, L.T.. A Rapid Reverse Transcription-PCR Assay for F+ RNA Coliphages to Trace Fecal Pollution in Table Rock Lake on the Arkansas–Missouri Border. *Water research* 40.20 (2006): 3719-24. Web.

Espinosa, Ana A. C. Comparative Study of Enteric Viruses, Coliphages and Indicator Bacteria for Evaluating Water Quality in a Tropical High-Altitude System. *Environmental Health* 8.1 (2009): 49. Web.

Feachem, R. An improved role for faecal coliform to faecal streptococci ratios in the differentiation between human and nonhuman pollution sources. (1975) Water Res. 9:689–690

Fong, Theng-Theng, Dale W. Griffin, and Erin K. Lipp. Molecular Assays for Targeting Human and Bovine Enteric Viruses in Coastal Waters and their Application for Library-Independent Source Tracking. *Applied and Environmental Microbiology* 71.4 (2005): 2070-8. Web.

Fout, Shay S. Enteric Virus and Indicator Occurrence in Groundwater Sources of Public Drinking Water. *Epidemiology (Cambridge, Mass.)* 22.1 (2011): S156. Web.

Frankenberger, Jane, E. coli and Indiana Lakes and Streams, Perdue Univedrsity, Website<https://engineering.purdue.edu/SafeWater/watershed/ecoli.html>. Print.

Geldreich EE, Rice EW, Eugene, W.W.. Occurrence, significance, and detection of *Klebsiella* in water systems. *J. Am. Water Works Assoc.* 74–80 (1987)

Gleeson C, Gray N. 1997. The Coliform Index and Waterborne Disease. London (1997): E & FN Spon.194 pp.

Grauke, L. L. J. Gastrointestinal Tract Location of Escherichia Coli O157:H7 in Ruminants. *Applied and Environmental Microbiology* 68.5 (2002): 2269-77. Web.

Griffin, Dale W., Gibson III, Charles J., Lipp, Erin K., Paul III, John H., Rose, Joan B., Riley, Kelley. Detection of Viral Pathogens by Reverse Transcriptase PCR and of Microbial Indicators by Standard Methods in the Canals of the Florida Keys. *Applied and Environmental Microbiology* 65.9 (1999): 4118-25. Web.

Griffin, Jennifer S., Long, Sharon, C., Plummer, Jeanine D.. "Torque Teno Virus: An Improved Indicator for Viral Pathogens in Drinking Waters." *Virology journal* 5.1:112 (2008): 112. Web.

Handzel, T.R., Green, R.M., Sanchez, C., Chung, H., Sobsey, Mark D.. Improved specificity in detecting F-specific coliphages in environmental samples by suppression of somatic phages. Water Science Technology 27, 123–131. 1993.

Harwood, Valerie J., Levine, Audrey D., Scott, Troy M., Chivukula, Jerzy L., Farrah, Samuel R., Rose, Joan B..Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health Protection. *Applied and Environmental Microbiology* 71.6 (2005): 3163-70. Web.

Ibarluzea, Jesús J. Somatic Coliphages and Bacterial Indicators of Bathing Water Quality in the Beaches of Gipuzkoa, Spain. *Journal of water and health* 5.3 (2007): 417. Web.

Imamovic, L. L. Quantification of Shiga Toxin-Converting Bacteriophages in Wastewater and in Fecal Samples by Real-Time Quantitative PCR. *Applied and Environmental Microbiology* 76.17 (2010): 5693-701. Web.

Irshad, Mohammad. *Torque Teno Virus Infection in Hemodialysis Patients in North India*. 42 Vol. , 2010. Web.

Jones, Tineke H., and Michael W. Johns. Improved Detection of F-Specific RNA Coliphages in Fecal Material by Extraction and Polyethylene Glycol Precipitation. *Applied and Environmental Microbiology* 75.19 (October 1, 2009): 6142-6. Web.

Kazama, Shinobu, Tameike, Name, Nakagawa, Naoko, Otaki, Masahiro. A Fate Model of Pathogenic Viruses in a Composting Toilet Based on Coliphage Inactivation. *Journal of Environmental Sciences* 23.7 (2011): 1194-8. Web.

LeChevallier, M. W., W. Schulz, and R. G. Lee. Bacterial Nutrients in Drinking Water. *Applied and Environmental Microbiology* 57.3 (1991): 857-62. Web.

LeChevallier, M. W., N. J. Welch, and D. B. Smith. Full-Scale Studies of Factors Related to Coliform Regrowth in Drinking Water. *Applied and Environmental Microbiology* 62.7 (1996): 2201-11. Web.

LeChevallier, M. W. Bacterial Nutrients in Drinking Water. *Applied and Environmental Microbiology* 57.3 (1991): 857. Web.

Leclerc, H., Advances in the Bacteriology of the Coliform Group: Their Suitability as Markers of Microbial Water Safety. *Annual Review of Microbiology* 55.1 (2001): 201. Web.

Leclerc, H., Edberg, S., Pierzo, V., Delattre, J.M.. Bacteriophages as Indicators of Enteric Viruses and Public Health Risk in Groundwaters. *Journal of applied microbiology* 88.1 (2000): 5-21. Web.

Lee, Hee S. and Sobsey, Mark D.. Survival of Prototype Strains of Somatic Coliphage Families in Environmental Waters and when Exposed to UV Low-Pressure Monochromatic Radiation Or Heat. *Water research* 45.12 (2011): 3723-34. Web.

Long, Sharon C., El-Khoury, Samar S., Oudejans, Sjon J.G., Sobsey, Mark D.. Assessment of Sources and Diversity of Male-Specific Coliphages for Source Tracking. *Environmental Engineering Science* 22.3 (2005): 367-77. Web.

Low, Chian. Appropriate Microbial Indicator Tests for Drinking Water in Developing Countries and Assessment of Ceramic Water Filters. Master of Engineering thesis. Department of Civil and Environmental Engineering, Massachusetts Institute of Technology. Cambridge, MA. (May 13, 2002)

Lucena, Fransisco and Jofre, Juan. Potential use of Bacteriophages as Indicators of Water Quality and Wastewater Treatment Processes. *Bacteriophages in the Control of Food - and Waterborne Pathogens.*, 2010. 103-118. *OnlineLibrary*. Web.

Mocé-Llivina, Laura, Francisco Lucena, and Juan Jofre. Enteroviruses and Bacteriophages in Bathing Waters. *Applied and Environmental Microbiology* 71.11 (November 2005): 6838-44. Web.

Nappier, Sharon P., Aitken, Michael D., Sobsey, Mark D.. Male-Specific Coliphages as Indicators of Thermal Inactivation of Pathogens in Biosolids. *Applied and Environmental Microbiology* 72.4 (April 2006): 2471-5. Web.

Nieuwstad, Th J., Mulder, E.P., Havelaar, A.H., Olphen, Van M.. Elimination of Micro-Organisms from Wastewater by Tertiary Precipitation and Simultaneous Precipitation Followed by Filtration. *Water research* 22.11 (1988): 1389-97. Web.

Ogorzaly, Leslie, Tissier, Adeline, Bertrand, Isabelle, Maul, Armand, Gentzer, Christoper. Relationship between F-Specific RNA Phage Genogroups, Faecal Pollution Indicators and Human Adenoviruses in River Water. *Water research* 43.5 (2009): 1257-64. Web.

O'Keefe, Brian and Green, John. Coliphages as indicators of faecal pollution at three recreational beaches on the Firth of Forth Wat. Res., 23 (1989), pp. 1027–1030

Ostroff, Steven, M., Griffin, Patricia M., Tauxe, Robert V., Shipman, Larry D., Greene, Katherine D., Wells, Joy G., Lewis, Jay H., Blake, Paul A., Kobayashi, John M.. A statewide outbreak of Escherichia coli O157:H7 infections in Washington State. Am J Epidemiol (1990); 132:239-47.

Otterholt, Eli, and Colin Charnock. Microbial Quality and Nutritional Aspects of Norwegian Brand Waters. *International journal of food microbiology* 144.3 (2011): 455-63. Web.

Ouyang, Zhiming and Isaacson, Richard. Identification and Characterization of a Novel ABC Iron Transport System, fit, in *Escherichia coli*. American Society for Microbiology. Infect. Immun. December 2006 vol. 74 no. 12 6949-6956

Parveen, S., Lukasik, J., Scott, T.M., Tamplin, M.L., Portier, K.M., Shepped, S., Braun, K., Farrah, S.R. Geographical Variation in Antibiotic Resistance Profiles of Escherichia Coli Isolated from Swine, Poultry, Beef and Dairy Cattle Farm Water Retention Ponds in Florida1. *Journal of applied microbiology* 100.1 (2006): 50-7. Web.

Plummer, Jeanine D., and Sharon C. Long. Monitoring Source Water for Microbial Contamination: Evaluation of Water Quality Measures. *Water research* 41.16 (2007): 3716-28. Web.

Rollins, Dale, Fred C. Bryant, and Roxanne Montandon. Fecal pH and Defecation Rates of Eight Ruminants Fed Known Diets. *The Journal of Wildlife Management* 48.3 (1984): pp. 807-813. Web.

Hrudey, S.E., Payment, P., Huck, P.M., Gillham, R.W.,Hrudey, E.J. *A fatal waterborne disease epidemic in Walkerton,Ontario: comparison with other waterborne outbreaks in the developed world*Print.2007

Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Kukasik, Jerry. Microbial Source Tracking: Current Methodology and Future Directions. *Applied and Environmental Microbiology* 68.12 (2002): 5796-803. Web.

Skraber, S., B. Gassilloud, and C. Gantzer. Comparison of coliforms and coliphages as tools for assessment of viral contamination in river water. Appl. Environ. Microbiol. 70:3644-3649. (2004)

Shin, G. A. Low Pressure Ultraviolet Inactivation of Pathogenic Enteric Viruses and Bacteriophages. *Journal of environmental engineering and science* 4 (2005)Web.

Stewart-Pullaro, J. F+RNA Coliphage Typing for Microbial Source Tracking in Surface Waters. *Journal of applied microbiology* 101.5 (2006): 1015-26. Web.

U.S. Environmental Protection Agency, Office of Water: Source Water Protection. http://www.epa.gov/safewater/protect.html (Accessed October 2011a)

U.S. Environmental Protection Agency Water Page: Source water protection. http://water.epa.gov/infrastructure/drinkingwater/sourcewater/protection/index.cfm (Accessed October 2011b)

U.S. Environmental Protection Agency, Office of Water: Setting Standards for Safe Drinking Water. http://www.epa.gov/safewater/standard/setting.html (Accessed March 2011c)

U.S. Environmental Protection Agency Water Page: Interim Enhanced Surface Water Treatment Rule. http://water.epa.gov/lawsregs/rulesregs/sdwa/ieswtr/ (Accessed October 2011d)

U.S. Environmental Protection Agency Water Page: Long Term 2 Enhanced Surface Water Treatment Rule (LT2). http://water.epa.gov/lawsregs/rulesregs/sdwa/lt2/index.cfm (Accessed October 2011e)

U.S. Environmental Protection Agency Water Page: Groundwater Rule (GWR). http://water.epa.gov/lawsregs/rulesregs/sdwa/gwr/index.cfm (Accessed October 2011f)

U.S. Environmental Protection Agency Water Page: Total Coliform Rule (TCR) http://water.epa.gov/lawsregs/rulesregs/sdwa/tcr/ (Accessed October 2011g)

U.S. Environmental Protection Agency Water Page: National Beach Guidance and Required Performance Criteria - Appendix 1C1: Indicator Organisms. http://water.epa.gov/grants_funding/beachgrants/app1c.cfm (Accessed October, 2011h)

U.S. Environmental Protection Agency Office of Water. Method 1601: Male-specific (F+) and Somatic Coliphage in Water by Two-step Enrichment Procedure. EPA 821-R-01-030. Washington, D.C. 20460. April, 2001.

World Health Organization. *Water Quality : Guidelines, Standards and Health - Risk Assessment and Management for Water Related Infectious Disease*. Eds. L. Fewtrel and J. Bartram. Albany, NY, USA: World Health Organization, 2001. Web.

Wu, Jinling, Haitao, Li, Xia, Huang. Indigenous Somatic Coliphage Removal from a Real Municipal Wastewater by a Submerged Membrane Bioreactor. *Water research* 44.6 (2010): 1853-62. Web.

Appendix A

Hollow Fiber Ultrafiltration for Concentration of Microorganisms in Drinking Water Samples

Version: November 26, 2007

Revised: August 28, 2011

The purpose of this procedure is to concentrate large volumes (10-500L) of drinking water in order to concentrate microbial contaminants to allow detection of low levels of these organisms. This method has been tested for efficacy with bacteria (*E. coli* and enterococci), viruses (coliphage, adenovirus, norovirus), and parasites (aerobic endospores as a surrogate, *Cryptosporidium*, and *Giardia*). This method is a hybrid of the one developed by WSLH for preparedness response and EPA's method applied for QA/QC criteria development.

Media and Reagents

5% newborn calf serum (or fetal bovine serum)

95 mL sterile cell culture water

5 mL calf serum

prepare day of use

(this is enough for 1 filter)

1000X NaPP solution

10 g sodium polyphosphate

100 mL sterile cell culture water

in sterile container, heat in 65ºC waterbath

to dissolve (may need to be warmed overnight)

Store at RT for up to 3 months

10% Sodium thiosulfate

100 g sodium thiosulfate

1000 mL sterile cell culture water

Autoclave, 15 minutes, 121ºC

Store at RT

Filter Pre-Wash Solution

1L sterile cell culture water

1mL 1000X NaPP

(this is enough for 1 filter)

*use within 24hrs of preparation

Filter Post-Wash Solution

1L sterile cell culture water

0.1mL TWEEN 80

1mL 1000X NaPP

0.01mL Antifoam Y-30

(this is enough for 1 filter)

use within 24hrs of preparation

Apparatus and Materials (in order of assembly)

Main system

- 20 or 50L carboy or cubitainer (Fisher 02-960-20B)
- Two Reducing (tubing) connectors (Fisher 22-235-73A)
- Three 10mL pipets (Fisher 13-678-14A)
- Two 36in lengths and one 24in length of MasterFlex Silicone Tubing (platinum) L/S 36 (Cole-Parmer Instrument Co order # 96410-36)
- MasterFlex I/P High Performance Pump Head, PPS Housing/SS Rotor model EW77600-62 (Cole Parmer EW-77600-62)
- Pump Drive
- Two #8 hose clamps (Cole-Parmer Instrument Co order # 06832-08)
- One 4in length and one 6in length Tygon tubing 5/16" ID, 7/16" OD (Fisher 14-169-1M)
- Four #6 hose clamps (Cole-Parmer Instrument Co order # 06832-06)
- Two Luer lock Fresenius Filter connectors (Fresenius 04-9505-1)
- Pressure gauge up to 30 psi (Ashcroft order NC9551701)

- Fresenius Optiflux F200NR filter (Fresenius 0500320N or 0500320E) or Asahi REXEED 21S filter (Asahi 1623)
- Keck pinch clamp (Cole-Parmer Instrument Co order # 06835-07)
- Waste bucket or carboy (may need two if not adequate volume)

Peripherals

Ring stand

Clamp holders

Various sized open sided clamps

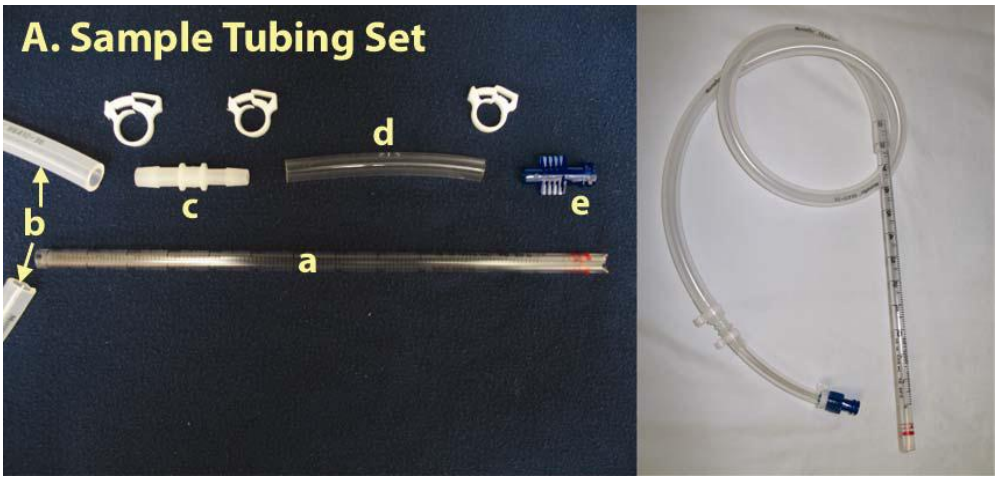
4" ring

Pliers

60cc sterile syringe

****Prepare the following hardware as aseptically as possible, wear gloves and wipe down with 70% ethanol****

Step 1.

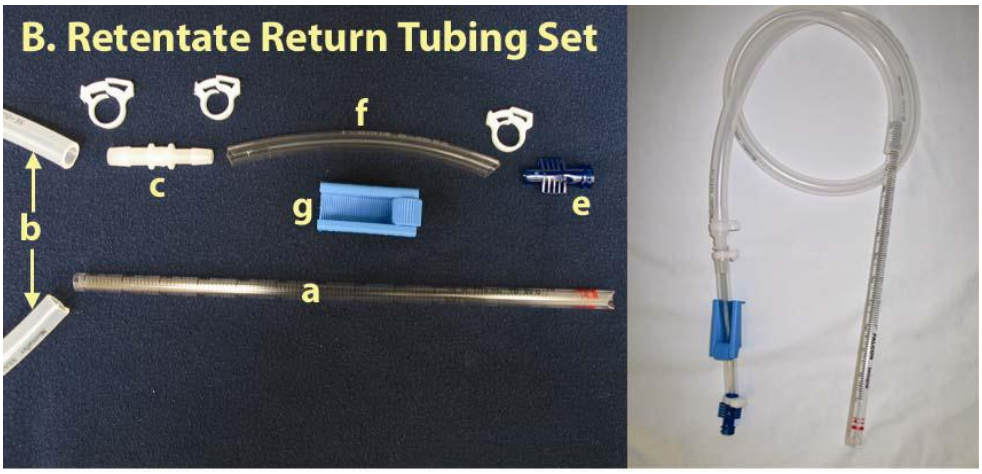


1. Remove (e.g. carefully break off) the tip of a 10 ml pipette (a)
2. Connect the 10-ml pipette (a) to 36 inches of #36 MF tubing (b), secure with a #8 hose clamp

3. Connect the #36 MF tubing (b) to a MF tubing connector (c); secure connection with a #8 hose clamp
4. Connect 4 inches of Tygon® tubing (d) to the MF tubing connector (c); secure connection with a #6 hose clamp
5. Connect the other end of the Tygon® tubing (d) to a Fresenius filter connector (e); secure connection with a #6 hose clamp
6. Store assembled tubing in large (gallon) zippered bag (label with contents, lot #'s, and expiration dates)

For Select Agents, every joint needs to be clamped in order to prevent leakage or connection failures during use

Step 2



1. Remove (e.g. carefully break off) the tip of a 10-ml pipette (a)
2. Connect the 10-ml pipette (a) to 36 inches of #36 MF tubing (b)
3. Connect the #36 MF tubing (b) to a MF tubing connector (c); secure connection with a #8 hose clamp
4. Connect 6 inches of Tygon® tubing (f) to the MF tubing connector (c); secure connection with a #6 hose clamp.
5. Connect the other end of the Tygon® tubing (f) to a Fresenius filter connector (e); secure connection with a #6 hose clamp.
6. Attach a flow regulator tubing clamp (g) to the 6-inch Tygon® tubing (f).
7. Store assembled tubing in large (gallon) zippered bag.

For Select Agents, every joint needs to be clamped in order to prevent leakage or connection failures during use

Step 3

1. Remove (e.g. carefully break off) the ends of a 10-ml pipette (a)
2. Connect the 10-ml pipette (a) to 24 inches of #36 MF tubing (h); secure with a #8 hose clamp
3. Store assembled tubing in a large (gallon) zippered bag.

***For Select Agents, every joint needs to be clamped in order to prevent leakage or connection failures during use*Finished System**

Sampling:

1. Put on gloves, disinfect with alcohol
2. When opening bottles to sample, be careful to handle caps aseptically
3. Add 0.5 mL/L (10.5 mL per 21L) 10% sodium thiosulfate to the chlorinated samples, mix.
4. Place carboy on ice

Upon return to the laboratory:

Aliquot 1L of each sample to be shipped to WPI in cooler

Place carboys at 4ºC or on ice until they can be concentrated

Filtration:

1. Put on gloves and disinfect with alcohol.
2. Wipe down work area with 70% ethanol.
3. Gather materials that needed (filters, calf serum, cell culture water, sterile syringes, sterile beaker or bottle, graduated cylinder(s), pipets, pipet-aids, etc).
4. Block the Asahi filters with 5% calf serum using 60cc sterile syringes (100mL for one filter). Shake at room temp for 30 min. Then keep the filters at 4°C.
5. Prepare pre-wash solution (labeled with different color from post-wash):
   1L cell culture water + 1mL 1000xP NaPP.
6. Prepare post-wash solution (labeled with different color from pre-wash):
   1L cell culture water + 1mL 1000xP NaPP + 100uL TWEEN + 10uL Antifoam Y-30.
7. Construct the complete set-up using blocked filter (Up-flow).
8. Add 1mL per L 1000x NaPP to the water sample and mix on stir plate at least 5 min before filtering.
9. Flush the blocked filter with 1L pre-wash solution. Place the sample tubing in pre-wash solution and retentate tubing to the waste bucket with the flow regulator clamp open. (Do not contact the retentate tubing with waste bucket), drain the tubing.
10. Drain the pre-wash bottle and weigh as the collection bottle (with cap on), record the empty bottle weight (Wt0).
11. Return the retentate return tubing to the sample carboy and start filtering. Use the flow regulator clamp to adjust the permeate rate equals to the return retentate, approximately.
12. Continue circulating until ~300-500 mL left in the sample carboy. Remove the retentate tubing to the collection bottle and filtrate all the sample to the collection bottle (drain the tubing, pipet the last few mL liquid in carboy to collection bottle), record the collection bottle weight with the concentrated sample (Wtf).
13. Place the sample and retentate tubing into 1L bottle of post-wash solution. With flow regulator open recirculate for 1-5 minutes until 300 mL liquid left in post-wash bottle.

14. Remove the retentate tubing to collection bottle and continue collecting all the liquid into collection bottle. Drain the tubing and combine all the liquid left in post-wash bottle into collection bottle. Record the total weight (Wtt) of collection bottle.
15. Store the concentrated sample at 4°C (ship in cooler) for further analysis.

Post filtration:

1. Flush the tubing by circulating 1L 5% bleach for 3min.
2. Neutralize bleach by circulating a new bottle of 1L autoclaved lab water contain sodium thiosulfate for 3min.
3. Air dry.
4. Wipe out a Ziplock bag with ethanol.
5. UV the tubing for 5 minutes and turn over for another 5 minutes, aseptically place in bag for storage.

Appendix B.

Professor J. D. Plummer

Worcester Polytechnic Institute

Department of Civil and Environmental Engineering

Last Updated April 26, 2010

Standard Operating Procedure

Method 1602: Double Agar Layer (SAL) Procedure

Table of Contents

Part 1 – Double Layer Procedure

A. **Prepare Overnight *E. coli***

Check for refrigeration cultures of *E. coli* Famp and CN-13. If there are none (or if they are more than 1 month old) prepare fresh cultures from frozen stocks. Make new before one month is up. Transfer no more than 6 (8 max.) times.

1. Prepare *E. coli* - CN-13 and Famp
2. Make two of each
3. Make cultures in laminar flow hood, sprayed with 40% reagent alcohol.
4. Flame tube tops and caps and flame loop in between each use, flame loop very carefully between cultures

Overnight *E. coli* CN-13 (Somatic)

a. Add 50 mL TSB to a flask labeled "Somatic Overnight"
b. Autoclave
c. Add 0.5 mL 100X Naladixic Acid to Somatic Overnight flask
d. Add 0.5 mL of refrigeration *E.coli* CN-13 to Somatic Overnight flask

Overnight *E. coli* Famp (F+)

*more sensitive to time

a. Add 50 mL TSB to a labeled flask: Famp Overnight
b. Autoclave
c. Add 0.5 mL 100X Strep/Amp to Famp Overnight Flask
d. Add 0.5 mL of refrigeration *E. coli* Famp to Famp Overnight flask

5. Cap overnight flasks and shake/incubate at 36°C at 100-150 rpm for 16-18 hrs
6. Use after inoculation (overnight *E. coli*) or save refrigeration flasks in culture fridge (1 month)

B. Prepare Log Phase *E. coli*

1. Start **4 hr** log phase *E. coli* F_{amp}/ *E. coli* CN-13 hosts from overnight *E. coli*
2. Make Log phase *E. coli*

Log-phase *E. coli* CN-13 (somatic)

a. Add 100 mL 1X TSB per log-phase flask
b. Autoclave
c. Add 1 mL of 100X Naladixic Acid to Somatic log-phase flask
d. Add 1 mL overnight *E. coli* CN-13 to Somatic log-phase flask

Log-phase *E. coli* Famp

a. Add 100 mL TSB per log-phase flask
b. Autoclave
c. Add 1 mL of 100X Strep/Amp to Famp log-phase flask
d. Add 1 mL overnight *E. coli* Famp to Famp log-phase flask

3. Incubate at 36°C, shaking at 100-150 rpm for 4 ± 1 hours or until visibly turbid
4. Immediately chill on ice or at 4°C until ready for use
5. Must be used within 2 hours of placing on ice
6. For larger time window, a second set of log-phase cultures can be started an hour after the first

C. Make TSA

1. Prepare 2X TSA for Large Plates – See Recipes
2. Autoclave
3. Set in 48°C waterbath

D. Samples

1. Complete the following steps for each sample twice; once for F+ (Famp) Enumeration and then repeat the steps for Somatic (CN 13) Enumeration.
2. Prepare bottles of samples, and concentrated samples
    a. Prepare samples and a duplicate of each by dispensing 100mL of each sample into separate sterile 250mL screw cap bottles.
    b. Prepare concentrated samples and a duplicate of each.
        i. For groundwater, and treated drinking water (25X only)
            1. Prepare a 25X concentrate
                a. Add 50 mL of each HFUF sample into separate sterile 250mL screw cap bottles.
                b. Add 50 mL PBS
        ii. For surface source water (5X and 25X)
            1. Prepare a 5X concentrate
                a. Add 10 mL of each HFUF sample into separate sterile 250mL screw cap bottles.
                b. Add 90 mL PBS
            2. Prepare a 25X concentrate
                a. Add 50 mL of each HFUF sample into separate sterile 250mL screw cap bottles.

b. Add 50 mL PBS

3. Prepare Positive Controls (Matrix spike and OPR) for one of the samples.
    a. Aseptically prepare two sterile 250mL screw cap bottles.
        i. Dispense 100mL of one of your samples separate sterile 250mL screw cap bottle.
        ii. Dispense 100 mL of PBS into separate sterile 250mL screw cap bottle
4. Add Phage
    a. Add Phage to Matrix Spike and OPR
    b. For F+ enumeration add a known amount (31.3 µL ~ 80PFU) of MS2 to the positive controls (Sample MS/OPR).
    c. For Somatic enumeration add a known amount (59 µL ~ 80PFU) of ΦX to the positive controls (Sample MS/OPR).
5. Prepare Temperature Control
    a. Prepare a temperature control by dispensing 100mL PBS into a separate sterile 250mL screw cap bottle.
6. Aseptically add 0.5mL of 4M MgCl2 to all of the 250 ml sample bottles, including temperature control.
7. Prepare Centrifuge Tubes for Negative Controls
    a. Add 15mL PBS into two separate sterile 50mL centrifuge tubes. Label one as "Host" and one as "Agar"
    b. Add 0.075mL of 4M MgCl2 to each of the two centrifuge tubes.
8. Uncap the temperature control and insert a thermometer.
9. Place the sample bottles (including controls and centrifuge tubes) into a 48°C water bath and shake for 5min or until the temperature control reaches 36°C.
10. Remove bottles/tubes from water bath
11. Add E. coli (Should be plated within 20 minutes)
    a. For F+:
        i. Add 10mL log-phase *E. coli* F_{amp} to each sample bottle (including temperature/positive control).
        ii. Add 1.5mL log-phase host to F+ "host" centrifuge tube.
    b. For Somatic:
        i. Add 10mL log-phase *E. coli* CN13 to each sample bottle (including temperature/positive control).
        ii. Add 1.5mL of log-phase host to the Somatic "host" centrifuge tube.
12. Place bottles and centrifuge tubes back into the 48°C water bath and shake until temperature reaches 43°C +/- 1C. Once temperature is reached, transfer to 43°C water bath.
13. Prepare Agar
    a. Antibiotic Quantities
        i. F+: Add 2.0mL of 100X Strep/Amp per 100mL 2X TSA.
        ii. Somatic: Add 2.0mL of 100X naladixic acid per 100mL 2X TSA.
        iii. Note:
            1. 6.0 mL per 300 mL 2X TSA
            2. 7.0 mL per 350 mL 2X TSA
            3. 12 mL per 600 mL 2X TSA
            4. 17 mL per 850 mL 2X TSA
    b. Add the antibiotic along the inside of the container to reduce the formation of bubbles,
    c. Gently rock the container slowly to mix.
14. Once antibiotics are added, you have 10min to add agar to sample before antibiotics degrade.
15. Add the Agar with antibiotics to the Sample Bottles
    a. Pour the agar until the contents of the bottle are approximately doubled (thumb check).
    b. Tilt and turn gently to mix – avoid introducing bubbles.

c. For "host" and "agar" tubes, add approximately 15-17mL agar/antibiotic.

16. Pour the contents of the sample bottle into a series of five – 150mm Petri dishes. Use the entire solution. For "host" and "agar" tubes pour entire contents into one Petri plate each.
17. Repeat as needed for each of the samples and controls.
18. Leave the tops of the Petri plates askew until agar has hardened (about 5min). Cover, stack, invert, and bag. Incubate at 37C for 16-24hours.
19. Count all plaque forming units and note any contamination. Plaques can be isolated in 300ul 20% glycerol/TSB in cryotubes for further serotyping or genotyping.

Part 2 - Schedule

Prior to Sample Day:

Make Overnight *E. coli*

Label plates and tubes

Get ice

Autoclave pipette tips – blue box (1 mL), green box (0.1 mL), 10 mL

Make Coliphage PBS 250 mL Bottles

Autoclave 250 mL bottle with appropriate amounts of PBS

Prepare and Autoclave

- TSA (Agar)
- $MgCl_2$
- PBS – Phage Only
- TSB
    - 4 – 50 mL flasks
    - 6 – 100 mL flasks

Move Antibiotics to Refrigerator

Turn on Incubator 36 ^{o}C

Prepare PBS temp control bottle and place in fridge

Prepare smaller centrifuge tubes of $MgCl_2$

Sample Day:

Prepare Log Phase Cultures

Turn on Waterbath 48^{O}C

Second Water bath 36 ^{O}C

Autoclave TSA (agar)

Prepare dilutions

Add antibiotic to TSA (agar)

Prepare plates with sample, *E. coli* Culture, and TSA with antibiotics

Prepare controls

Day After Sample Day:

Count Plates

Reorder any needed supplies

Make any necessary cultures

Part 3 - Check List – MaterialsPlates (320-420)

- Agar (10)
- Agar + Famp (5)
- Agar + CN13 (5)
- Famp + MS2, 5 per sample (25)
- Famp + MS2 + Sample, 5 per sample (25)
- CN13 + ΦX, 5 per sample (25)
- CN13 + ΦX + Sample, 5 per sample (25)
- Famp DW1 through Famp DW5, A and B for each, 5x2 per sample (50)
- CN13 DW1 through CN13 DW5, A and B for each, 5x2 per sample (50)
- Famp DW1 5X through Famp DW5 5X, A and B for each, 5x2 per sample (50)
- CN13 DW1 5Xthrough CN13 DW5 5X, A and B for each, 5x2 per sample (50)

For Surface Water Source Only

- Famp DW1 25X through Famp DW5 25X, A and B for each, 5x2 per sample (50)
- CN13 DW1 25Xthrough CN13 DW5 25X, A and B for each, 5x2 per sample (50)

- Antibiotics
  - Naladixic Acid
  - 100X Strep Amp
- Famp and CN-13 Cultures
- TSB
- Phage Only PBS - Autoclaved
- 4M – 80X MgCl2
  - 10mL vials
- Enumerated MS2/ΦX174
- Large Petri Plates
  - GW – 45 plates X 2 = 90 plates per sample
  - SW – 55 plates X 2 = 100 plates per sample
- Pipette Tips
- Auto pipette tips
- Ice
- Autoclave all 500mL glass bottles
- Mark 250mL bottle with lines for 100mL and 200 mL

- Autoclave 250mL bottles with 50mL PBS
    - GW – at least 20
- Autoclave 250mL bottles with 90mL PBS
    - For surface water only
- Autoclave 250 mL bottle for temperature control – 100mL PBS
- 2X Agar
    - GW
        - At least 2 bottles of 650mL – per sample
        - At least 1 bottle of 650mL, 1 bottle of 300mL, and 1 bottle of 350mL – per sample
    - SW
        - At least 850 mL - per sample

Part 4 - Coliphage Enumeration Recipes

Tryptic Soy Broth (TSB) 1X

- Add 30 g tryptic soy broth to a sterile 2000 mL bottle
- Add 1000 mL Epure water
- Mix and warm to dissolve, autoclave, store refrigerated for 1 month
- Prior to sampling, autoclave 50 mL and 100 mL quantities as necessary

100X Naladixic Acid

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Add 1.0g Naladixic Acid Sodium Salt to a sterile bottle
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

100X Streptomycin/Ampicillin

- Materials: Sterile beaker, (2) sterile bottles, sterile serological pipet, sterilization filter apparatus, pump
- Collect at least 100 mL of Epure water in a sterile beaker
- Add 0.15 g ampicillin to a sterile bottle
- Add 0.15 g streptomycin
- Add 100 mL Epure water using a sterile serological pipet and swirl to dissolve
- Filter sterilize into a sterile bottle
- Freeze 5 mL aliquots at -20°C

TSA - 2X TSB + 0.85% Agar (100 mL) – TSA for large plates

- Add 6 g tryptic soy broth to a sterile bottle (60g for 1L)
- Add 1.8 g Bacto Agar (18 g for 1L)
- Add 100 mL Epure water (1L)
- While stirring, heat to dissolve
- Autoclave
- Place in 48°C waterbath until use

4M (80X) $MgCl_2$

- Add about 1/3 Epure water to 100 mL Volumetric Flask
- Add 81.4 g $MgCl_2 \cdot 6H_2O$

- Bring final volume to 100 mL (Total Volume)
- Stir to dissolve
- Autoclave, store refrigerated

Phage Phosphate Buffered Saline (PBS) – Label Phage Only

- Add 8.0 g NaCl to a sterile 1000 mL bottle
- Add 0.2 g KH_2PO_4
- Add 0.12 g KCl
- Add 0.91 g anhydrous Na_2HPO_4 (or 2.9 g $Na_2HPO_4 \cdot 12H_20$)
- Bring up to 1L with Epure water
- Adjust pH to 7.2-7.4 (with 1N HCl or NaOH)
- Autoclave, store refrigerated for 1 year

Appendix C. Spearman's rho-value table

α(1): / n	0.25	0.10	0.05	0.025	0.01	0.005	0.0025	0.001	0.0005
4	0.600	1.000	1.000						
5	0.500	0.800	0.900	1.000	1.000				
6	0.371	0.657	0.829	0.886	0.943	1.000	1.000		
7	0.321	0.571	0.714	0.786	0.893	0.929	0.964	1.000	1.000
8	0.310	0.524	0.643	0.738	0.833	0.881	0.905	0.952	0.976
9	0.267	0.483	0.600	0.700	0.783	0.833	0.867	0.917	0.933
10	0.248	0.455	0.564	0.648	0.745	0.794	0.830	0.879	0.903
11	0.236	0.427	0.536	0.618	0.709	0.755	0.800	0.845	0.873
12	0.217	0.406	0.503	0.587	0.678	0.727	0.769	0.818	0.846
13	0.209	0.385	0.484	0.560	0.648	0.703	0.747	0.791	0.824
14	0.200	0.367	0.464	0.538	0.626	0.679	0.723	0.771	0.802
15	0.189	0.354	0.446	0.521	0.604	0.654	0.700	0.750	0.779
16	0.182	0.341	0.429	0.503	0.582	0.635	0.679	0.729	0.762
17	0.176	0.328	0.414	0.485	0.566	0.615	0.662	0.713	0.748
18	0.170	0.317	0.401	0.472	0.550	0.600	0.643	0.695	0.728
19	0.165	0.309	0.391	0.460	0.535	0.584	0.628	0.677	0.712
20	0.161	0.299	0.380	0.447	0.520	0.570	0.612	0.662	0.696
21	0.156	0.292	0.370	0.435	0.508	0.556	0.599	0.648	0.681
22	0.152	0.284	0.361	0.425	0.496	0.544	0.586	0.634	0.667
23	0.148	0.278	0.353	0.415	0.486	0.532	0.573	0.622	0.654
24	0.144	0.271	0.344	0.406	0.476	0.521	0.562	0.610	0.642
25	0.142	0.265	0.337	0.398	0.466	0.511	0.551	0.598	0.630
26	0.138	0.259	0.331	0.390	0.457	0.501	0.541	0.587	0.619
27	0.136	0.255	0.324	0.382	0.448	0.491	0.531	0.577	0.608
28	0.133	0.250	0.317	0.375	0.440	0.483	0.522	0.567	0.598
29	0.130	0.245	0.312	0.368	0.433	0.475	0.513	0.558	0.589
30	0.128	0.240	0.306	0.362	0.425	0.467	0.504	0.549	0.580
31	0.126	0.236	0.301	0.356	0.418	0.459	0.496	0.541	0.571
32	0.124	0.232	0.296	0.350	0.412	0.452	0.489	0.533	0.563
33	0.121	0.229	0.291	0.345	0.405	0.446	0.482	0.525	0.554
34	0.120	0.225	0.287	0.340	0.399	0.439	0.475	0.517	0.547
35	0.118	0.222	0.283	0.335	0.394	0.433	0.468	0.510	0.539
36	0.116	0.219	0.279	0.330	0.388	0.427	0.462	0.504	0.533
37	0.114	0.216	0.275	0.325	0.383	0.421	0.456	0.497	0.526
38	0.113	0.212	0.271	0.321	0.378	0.415	0.450	0.491	0.519
39	0.111	0.210	0.267	0.317	0.373	0.410	0.444	0.485	0.513
40	0.110	0.207	0.264	0.313	0.368	0.405	0.439	0.479	0.507
41	0.108	0.204	0.261	0.309	0.364	0.400	0.433	0.473	0.501
42	0.107	0.202	0.257	0.305	0.359	0.395	0.428	0.468	0.495
43	0.105	0.199	0.254	0.301	0.355	0.391	0.423	0.463	0.490
44	0.104	0.197	0.251	0.298	0.351	0.386	0.419	0.458	0.484
45	0.103	0.194	0.248	0.294	0.347	0.382	0.414	0.453	0.479
46	0.102	0.192	0.246	0.291	0.343	0.378	0.410	0.448	0.474
47	0.101	0.190	0.243	0.288	0.340	0.374	0.405	0.443	0.469
48	0.100	0.188	0.240	0.285	0.336	0.370	0.401	0.439	0.465
49	0.098	0.186	0.238	0.282	0.333	0.366	0.397	0.434	0.460
50	0.097	0.184	0.235	0.279	0.329	0.363	0.393	0.430	0.456

n \ α(1):	0.25	0.10	0.05	0.025	0.01	0.005	0.0025	0.001	0.0005
51	0.096	0.182	0.233	0.276	0.326	0.359	0.390	0.426	0.451
52	0.095	0.180	0.231	0.274	0.323	0.356	0.386	0.422	0.447
53	0.095	0.179	0.228	0.271	0.320	0.352	0.382	0.418	0.443
54	0.094	0.177	0.226	0.268	0.317	0.349	0.379	0.414	0.439
55	0.093	0.175	0.224	0.266	0.314	0.346	0.375	0.411	0.435
56	0.092	0.174	0.222	0.264	0.311	0.343	0.372	0.407	0.432
57	0.091	0.172	0.220	0.261	0.308	0.340	0.369	0.404	0.428
58	0.090	0.171	0.218	0.259	0.306	0.337	0.366	0.400	0.424
59	0.089	0.169	0.216	0.257	0.303	0.334	0.363	0.397	0.421
60	0.089	0.168	0.214	0.255	0.300	0.331	0.360	0.394	0.418
61	0.088	0.166	0.213	0.252	0.298	0.329	0.357	0.391	0.414
62	0.087	0.165	0.211	0.250	0.296	0.326	0.354	0.388	0.411
63	0.086	0.163	0.209	0.248	0.293	0.323	0.351	0.385	0.408
64	0.086	0.162	0.207	0.246	0.291	0.321	0.348	0.382	0.405
65	0.085	0.161	0.206	0.244	0.289	0.318	0.346	0.379	0.402
66	0.084	0.160	0.204	0.243	0.287	0.316	0.343	0.376	0.399
67	0.084	0.158	0.203	0.241	0.284	0.314	0.341	0.373	0.396
68	0.083	0.157	0.201	0.239	0.282	0.311	0.338	0.370	0.393
69	0.082	0.156	0.200	0.237	0.280	0.309	0.336	0.368	0.390
70	0.082	0.155	0.198	0.235	0.278	0.307	0.333	0.365	0.388
71	0.081	0.154	0.197	0.234	0.276	0.305	0.331	0.363	0.385
72	0.081	0.153	0.195	0.232	0.274	0.303	0.329	0.360	0.382
73	0.080	0.152	0.194	0.230	0.272	0.301	0.327	0.358	0.380
74	0.080	0.151	0.193	0.229	0.271	0.299	0.324	0.355	0.377
75	0.079	0.150	0.191	0.227	0.269	0.297	0.322	0.353	0.375
76	0.078	0.149	0.190	0.226	0.267	0.295	0.320	0.351	0.372
77	0.078	0.148	0.189	0.224	0.265	0.293	0.318	0.349	0.370
78	0.077	0.147	0.188	0.223	0.264	0.291	0.316	0.346	0.368
79	0.077	0.146	0.186	0.221	0.262	0.289	0.314	0.344	0.365
80	0.076	0.145	0.185	0.220	0.260	0.287	0.312	0.342	0.363
81	0.076	0.144	0.184	0.219	0.259	0.285	0.310	0.340	0.361
82	0.075	0.143	0.183	0.217	0.257	0.284	0.308	0.338	0.359
83	0.075	0.142	0.182	0.216	0.255	0.282	0.306	0.336	0.357
84	0.074	0.141	0.181	0.215	0.254	0.280	0.305	0.334	0.355
85	0.074	0.140	0.180	0.213	0.252	0.279	0.303	0.332	0.353
86	0.074	0.139	0.179	0.212	0.251	0.277	0.301	0.330	0.351
87	0.073	0.139	0.177	0.211	0.250	0.276	0.299	0.328	0.349
88	0.073	0.138	0.176	0.210	0.248	0.274	0.298	0.327	0.347
89	0.072	0.137	0.175	0.209	0.247	0.272	0.296	0.325	0.345
90	0.072	0.136	0.174	0.207	0.245	0.271	0.294	0.323	0.343
91	0.072	0.135	0.173	0.206	0.244	0.269	0.293	0.321	0.341
92	0.071	0.135	0.173	0.205	0.243	0.268	0.291	0.319	0.339
93	0.071	0.134	0.172	0.204	0.241	0.267	0.290	0.318	0.338
94	0.070	0.133	0.171	0.203	0.240	0.265	0.288	0.316	0.336
95	0.070	0.133	0.170	0.202	0.239	0.264	0.287	0.314	0.334
96	0.070	0.132	0.169	0.201	0.238	0.262	0.285	0.313	0.332
97	0.069	0.131	0.168	0.200	0.236	0.261	0.284	0.311	0.331
98	0.069	0.130	0.167	0.199	0.235	0.260	0.282	0.310	0.329
99	0.068	0.130	0.166	0.198	0.234	0.258	0.281	0.308	0.327
100	0.068	0.129	0.165	0.197	0.233	0.257	0.279	0.307	0.326

Appendix D. Fecal Data

0	0	Sample	Sample	Turbidity	pH	TOC	DOC	Coliform	E. coli	Male-Specific Colipage	Somatic Coliphage
Region	State	Type	Name	(ntu)	0	(mg/L)	(mg/L)	(cfu/100 mL)	(cfu/100 mL)	(pfu/mL; pfu/g)	(pfu/mL; pfu/g)
Midwest	WI	Cow	Little Wig	nd	7.47	nd	nd	5.2435E+04	5.0810E+04	<5	8.8667E+02
Midwest	WI	Cow	187	nd	7.3	nd	nd	2.5600E+07	1.0890E+07	6.6667E+00	6.6154E+02
Midwest	WI	Cow	211	nd	7.125	nd	nd	0.0000E+00	0.0000E+00	<5	<3
Midwest	WI	Dog	Leidener	nd	6.9	nd	nd	6.4348E+06	6.4348E+06	<3	5.5000E+01
Midwest	WI	Dog	Tasha	nd	7.23	nd	nd	4.3363E+06	4.0650E+06	<3	<3
Midwest	WI	Dog	Sadie	nd	6.995	nd	nd	1.0163E+08	1.0163E+08	<3	5.7500E+02
Midwest	WI	Dog	Phoebe	nd	7.31	nd	nd	2.0850E+05	2.0850E+05	1.0000E+01	<3
Midwest	WI	WW	Primary	55	7.08	85.825	58.305	3.5900E+07	4.5873E+06	6.6667E+03	9.6667E+04
Midwest	WI	WW	Final	2.23	7.375	8.929	7.7975	8.0913E+04	9.6500E+03	<33	5.1000E+05
Midwest	WI	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Northeast	MA	Cow	48	nd	6.67	nd	nd	9.0328E+06	8.0080E+06	1.9249E+03	8.3667E+04
Northeast	MA	Horse	Misty	nd	6.41	nd	nd	4.9985E+04	7.6350E+03	7.0571E+02	1.0033E+05
Northeast	MA	Horse	Possum	nd	6.565	nd	nd	3.2650E+03	4.7000E+02	7.5758E+01	2.3739E+03
Northeast	MA	Dog	Lucky	nd	7.005	nd	nd	2.9021E+07	2.6821E+07	9.0909E+00	1.7879E+02
Northeast	MA	Chicken	Chicken	nd	6.75	nd	nd	5.3233E+06	3.9443E+06	7.1667E+03	>2.5E+7
Northeast	MA	Horse	Pie	nd	6.75	nd	nd	1.0350E+03	0.0000E+00	<3	6.6667E+00
Northeast	MA	nd	nd	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Northeast	MA	WW	Raw	54.3	6.32	65.06	45.515	1.6116E+07	2.8280E+06	1.0303E+05	3.0000E+04
Northeast	MA	WW	Primary	37.5	6.22	52.485	43.75	2.3355E+07	2.0748E+06	9.0909E+03	1.2121E+04
Northeast	MA	WW	Final	1.43	6.145	6.7155	6.0425	7.4580E+04	5.4450E+03	6.6667E+01	9.6970E+02
South	FL	Cassowary	Rare bird	nd	4.49	nd	nd	3.1450E+07	4.6750E+05	<3	<3
South	FL	Chicken	Australorp	nd	7.185	nd	nd	5.0195E+08	2.7765E+08	2.0303E+06	>3.4E+04
South	FL	Cow	Brama bull	nd	7.3	nd	nd	3.6000E+02	2.5500E+02	<3	<3
South	FL	Goat	Angora	nd	7.45	nd	nd	1.1060E+06	1.1060E+06	<3	<3
South	FL	Horse	Paso fino	nd	6.725	nd	nd	2.3514E+05	9.3773E+04	2.4909E+04	5.8667E+03

South	FL	nd	nd	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
South	FL	nd	nd	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
South	FL	WW	Raw	82.75	6.895	94.97	54.355	1.7624E+07	4.1455E+06	2.1021E+04	1.5758E+05
South	FL	WW	Final	0.795	7.085	8.3855	8.0595	1.8285E+04	2.0000E+03	6.6667E+01	1.8485E+03
South	FL	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
West	CO	Rabbit	White	nd	8.465	nd	nd	0.0000E+00	0.0000E+00	3.6877E+02	<3
West	CO	Rabbit	Black	nd	7.075	nd	nd	3.7450E+03	1.5500E+02	4.8500E+04	>3.0e+05
West	CO	Horse	Mini white 1	nd	6.67	nd	nd	3.9700E+07	1.9696E+05	<3	2.6456E+03
West	CO	Horse	Mini white 2	nd	6.485	nd	nd	4.5700E+07	9.0500E+04	5.0964E+00	2.6426E+02
West	CO	Donkey	Donkey	nd	6.59	nd	nd	3.6000E+05	1.8028E+04	2.9333E+04	2.4024E+02
West	CO	Sheep	Sheep	nd	8.35	nd	nd	1.9320E+06	1.6770E+06	<3	<3
West	CO	Llama	Llama	nd	8.115	nd	nd	1.0686E+07	1.1300E+07	5.2000E+04	1.8788E+02
West	WA	WW	Raw	115.5	6.8	136.72	97.275	8.7300E+07	6.9428E+06	6.6667E+04	1.0606E+05
West	WA	WW	Final	5.155	7.015	17.895	15.555	9.7650E+05	8.9428E+04	7.5758E+02	1.2633E+04
West	WA	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Midwest	WI	Cow	Little Wig	nd	8.005	nd	nd	1.6140E+06	1.6140E+06	<3	1.0000E+01
Midwest	WI	Cow	237	nd	7.74	nd	nd	1.3550E+06	1.3550E+06	3.3333E+00	6.9697E+01
Midwest	WI	Cow	187	nd	7.86	nd	nd	7.5250E+05	8.0500E+05	<3	3.2000E+02
Midwest	WI	Dog	Tasha	nd	6.84	nd	nd	1.9045E+06	1.9045E+06	<3	2.7027E+01
Midwest	WI	Dog	Phoebe	nd	6.925	nd	nd	1.0200E+05	8.5580E+04	3.3333E+00	6.0606E+00
Midwest	WI	Dog	Quincy	nd	6.675	nd	nd	5.3500E+04	6.6120E+04	<3	<3
Midwest	WI	Dog	Sadie	nd	6.9	nd	nd	3.0288E+06	3.0288E+06	<3	<3
Midwest	WI	WW	Raw	71.7	6.795	193.8	68.845	6.6350E+05	5.7548E+04	3.9039E+04	1.6364E+05
Midwest	WI	WW	Final	1.56	6.2415	82.06	6.914	8.9763E+02	1.8420E+02	1.6667E+02	3.1667E+03
Midwest	WI	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Northeast	MA	Horse	Possum	nd	6.845	nd	nd	1.2660E+04	6.9925E+03	<3	<3
Northeast	MA	Horse	Benny	nd	6.755	nd	nd	5.8150E+03	2.8450E+03	1.3333E+01	1.5152E+02
Northeast	MA	Horse	Daisy	nd	7.19	nd	nd	4.1650E+05	1.4660E+04	2.6667E+01	2.6667E+01

Northeast	MA	Cow	186	nd	5.23	nd	nd	2.9650E+05	2.7100E+05	1.4600E+04	2.2913E+03
Northeast	MA	Rabbit	Rabbit	nd	8.935	nd	nd	2.5582E+05	2.5582E+05	<3	<3
Northeast	MA	Chicken	Buff	nd	5.175	nd	nd	1.1883E+07	9.2680E+06	<3	2.3030E+02
Northeast	MA	nd	nd	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Northeast	MA	WW	Raw	54.15	7.17	60.47	34.94	7.1400E+05	3.5225E+04	2.2121E+03	7.3333E+02
Northeast	MA	WW	Final	1.095	6.875	8.002	7.2985	1.5290E+03	1.1765E+02	6.6667E+01	6.6667E+02
Northeast	MA	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Northeast	MA	Chicken	Chicken 1	nd	8.565	nd	nd	>241960	0.0000E+00	1.6665E+02	3.3333E+03
Northeast	MA	Cow	168	nd	6.55	nd	nd	7.4200E+05	2.1340E+05	8.5000E+03	1.5067E+04
Northeast	MA	Horse	Red	nd	7.185	nd	nd	3.0500E+02	3.0500E+02	9.3909E+02	<3
Northeast	MA	Horse	Possum	nd	7.13	nd	nd	2.2050E+03	1.7200E+03	1.5422E+03	<3
Northeast	MA	Horse	Black	nd	7.325	nd	nd	2.2850E+03	1.7750E+03	1.4341E+03	<3
Northeast	MA	Horse	Daisy	nd	7.33	nd	nd	1.8665E+04	1.8520E+04	4.8485E+03	<3
Northeast	MA	nd	nd	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Northeast	MA	WW	Raw	44.95	7.02	54.545	47.16	1.1425E+07	9.0400E+05	2.0167E+05	3.2333E+04
Northeast	MA	WW	Final	1.32	7.075	8.686	9.3245	1.6648E+05	4.5700E+03	3.6304E+02	1.7576E+03
Northeast	MA	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
South	FL	Cow	Brama Bull	nd	6.85	nd	nd	4.5550E+05	4.5550E+05	<3	4.4333E+02
South	FL	Chicken	Australorp	nd	7.015	nd	nd	2.7233E+08	7.6650E+07	2.8022E+03	3.7333E+06
South	FL	Dog	Australian Shepherd	nd	6.42	nd	nd	3.7450E+07	3.5150E+07	1.7102E+02	2.9667E+02
South	FL	Horse	Paso Fino	nd	7.38	nd	nd	4.8550E+07	4.7000E+07	1.4701E+02	5.3667E+03
South	FL	Goat	Angora	nd	7.45	nd	nd	1.1845E+07	1.1111E+07	2.7003E+01	1.7718E+02
South	FL	Sheep	Suffolk	nd	7.28	nd	nd	4.1388E+06	3.3830E+06	4.8005E+01	1.0933E+04
South	FL	nd	nd	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
South	FL	WW	Raw	899.5	6.885	73.58	71.16	1.0310E+08	7.3080E+06	1.4033E+05	1.5100E+05
South	FL	WW	Final	1.36	7.025	8.311	7.931	3.5373E+04	3.5200E+03	<33	8.0000E+02
South	FL	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
West	CO	Horse	Horse1	nd	7.125	nd	nd	5.0000E+01	5.0000E+01	<3	<3

West	CO	Horse	Horse2	nd	7.19	nd	nd	2.7150E+03	2.5550E+03	<3	<3
West	CO	Chicken	Chicken1	nd	7.425	nd	nd	5.7500E+04	3.0500E+04	<3	5.6667E+03
West	CO	Chicken	Chicken2	nd	7.635	nd	nd	6.0950E+05	6.0950E+05	<3	1.1567E+05
West	CO	Cow	Cow1	nd	7.87	nd	nd	5.0000E+01	5.0000E+01	3.3333E+00	<3
West	CO	Cow	Cow2	nd	8.24	nd	nd	>241960	>241960	<3	<3
West	CO	Donkey	Donkey	nd	7.685	nd	nd	3.6000E+02	3.6000E+02	<3	<3
West	WA	WW	Raw	95.9	6.81	42.325	30.925	3.8308E+06	1.9288E+06	9.0000E+04	2.2830E+04
West	WA	WW	Final	7.815	7.05	15.45	10.585	9.1825E+04	5.1593E+04	6.9697E+02	6.8333E+03
West	WA	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
Midwest	WI	Cow	190	nd	7.675	nd	nd	3.8445E+06	3.8445E+06	5.1333E+02	9.6970E+02
Midwest	WI	Cow	290	nd	7.685	nd	nd	5.9605E+06	5.9605E+06	<3	7.1667E+02
Midwest	WI	Cow	234	nd	7.96	nd	nd	1.5230E+04	6.6000E+03	4.9667E+02	3.6667E+02
Midwest	WI	Dog	Tasha	nd	7.735	nd	nd	5.9875E+05	6.9750E+05	<3	<3
Midwest	WI	Dog	Phoebe	nd	7.955	nd	nd	>241960	>241960	<3	<3
Midwest	WI	Dog	Quincy	nd	7.495	nd	nd	2.0482E+07	2.0482E+07	<3	3.3333E+02
Midwest	WI	Dog	Sadie	nd	7.28	nd	nd	>241960	>241960	<3	<3
Midwest	WI	WW	Raw	109	7.475	90.555	40.84	2.9455E+06	1.7000E+06	1.5533E+05	4.0333E+04
Midwest	WI	WW	Final	1.525	7.62	19.675	10.4575	1.6910E+04	5.1900E+03	<33	3.0000E+02
Midwest	WI	nd	nd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00
South	FL	Dog	Australian Shepherd	nd	6.98	nd	nd	85000000	82575000	16.6666667	17566.66667
South	FL	Sheep	Angora	nd	8.04	nd	nd	147000	135000	<3	<3
South	FL	Cow	Angus	nd	7.49	nd	nd	102232.5	83492.5	<3	<3
South	FL	Chicken	Australorp	nd	7.175	nd	nd	605150000	339200000	<3	2606666.667
South	FL	Horse	Paso Fino	nd	7.155	nd	nd	150565	85070	<3	<3
South	FL	0	0	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0	0
South	FL	0	0	nd	#DIV/0!	nd	nd	#DIV/0!	#DIV/0!	0	0
South	FL	WW	Raw	88.9	7.06	91.445	40.56	28300000	3138250	303333.333	81333.33333
South	FL	WW	Final	1.13	7.275	10.85	9.8195	16122.5	1860	272.727273	454.5454545

South	FL	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0	0
West	CO	Horse	1	nd	7.04	nd	nd	6925	6925	<3	<3
West	CO	Horse	2	nd	6.63	nd	nd	>24196000	>24196000	<3	<3
West	CO	Chicken	1	nd	8.385	nd	nd	100	100	<3	<3
West	CO	Chicken	2	nd	7.685	nd	nd	0	0	<3	<3
West	CO	Cow	1	nd	7.2	nd	nd	5400	845	<3	<3
West	CO	Cow	2	nd	6.36	nd	nd	1185	565	<3	133.3333333
West	CO	Donkey	Donkey 1	nd	7.71	nd	nd	305	305	<3	<3
West	WA	WW	Raw	255	7.07	75.325	29.355	2332000	1315250	93333.3333	39666.66667
West	WA	WW	Final	5.365	7.27	24.085	10.29	109830	34732.5	166.666667	166.6666667
West	WA	0	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0	0

Appendix E. Wastewater Data

ID	Notes	Date	Season	Region	State	Type	Name	Turb	pH	TOC	DOC	Coliform	E. coli	F+	Somatic
WW2	0	06/10/10	Spring	Midwest	WI	WW	Final	2.23	7.375	8.929	7.7975	8.091E+04	9.650E+03	0.000E+00	5.100E+05
WW1	0	06/10/10	Spring	Midwest	WI	WW	Primary	55	7.08	85.825	58.305	3.590E+07	4.587E+06	6.667E+03	9.667E+04
WW2	0	08/10/10	Summer	Midwest	WI	WW	Final	1.56	6.2415	82.06	6.914	8.976E+02	1.842E+02	1.667E+02	3.167E+03
WW1	0	08/10/10	Summer	Midwest	WI	WW	Raw	71.7	6.795	193.8	68.845	6.635E+05	5.755E+04	3.904E+04	1.636E+05
WW2	0	02/28/11	Winter	Midwest	WI	WW	Final	1.525	7.62	19.675	10.458	1.691E+04	5.190E+03	0.000E+00	3.000E+02
WW1	0	02/28/11	Winter	Midwest	WI	WW	Raw	109	7.475	90.555	40.84	2.946E+06	1.700E+06	1.553E+05	4.033E+04
WW3	0	06/15/10	Spring	Northeast	MA	WW	Final	1.43	6.145	6.7155	6.0425	7.458E+04	5.445E+03	6.667E+01	9.697E+02
WW2	0	06/15/10	Spring	Northeast	MA	WW	Primary	37.5	6.22	52.485	43.75	2.336E+07	2.075E+06	9.091E+03	1.212E+04
WW1	0	06/15/10	Spring	Northeast	MA	WW	Raw	54.3	6.32	65.06	45.515	1.612E+07	2.828E+06	1.030E+05	3.000E+04
WW2	0	09/14/10	Summer	Northeast	MA	WW	Final	1.095	6.875	8.002	7.2985	1.529E+03	1.177E+02	6.667E+01	6.667E+02
WW1	0	09/14/10	Summer	Northeast	MA	WW	Raw	54.15	7.17	60.47	34.94	7.140E+05	3.523E+04	2.212E+03	7.333E+02
WW2	0	01/11/11	Winter	Northeast	MA	WW	Final	1.32	7.075	8.686	9.3245	1.665E+05	4.570E+03	3.630E+02	1.758E+03
WW1	0	01/11/11	Winter	Northeast	MA	WW	Raw	44.95	7.02	54.545	47.16	1.143E+07	9.040E+05	2.017E+05	3.233E+04
WW2	0	07/13/10	Summer	South	FL	WW	Final	0.795	7.085	8.3855	8.0595	1.829E+04	2.000E+03	6.667E+01	1.848E+03
WW1	0	07/13/10	Summer	South	FL	WW	Raw	82.75	6.895	94.97	54.355	1.762E+07	4.146E+06	2.102E+04	1.576E+05
WW2	0	01/25/11	Winter	South	FL	WW	Final	1.36	7.025	8.311	7.931	3.537E+04	3.520E+03	0.000E+00	8.000E+02
WW1	0	01/25/11	Winter	South	FL	WW	Raw	899.5	6.885	73.58	71.16	1.031E+08	7.308E+06	1.403E+05	1.510E+05
WW2	0	03/29/11	Spring	South	FL	WW	Final	1.13	7.275	10.85	9.8195	1.612E+04	1.860E+03	2.727E+02	4.545E+02
WW1	0	03/29/11	Spring	South	FL	WW	Raw	88.9	7.06	91.445	40.56	2.830E+07	3.138E+06	3.033E+05	8.133E+04
WW2	0	08/04/10	Summer	West	WA	WW	Final	5.155	7.015	17.895	15.555	9.765E+05	8.943E+04	7.576E+02	1.263E+04
WW1	0	08/04/10	Summer	West	WA	WW	Raw	115.5	6.8	136.72	97.275	8.730E+07	6.943E+06	6.667E+04	1.061E+05
WW2	0	02/07/11	Winter	West	WA	WW	Final	7.815	7.05	15.45	10.585	9.183E+04	5.159E+04	6.970E+02	6.833E+03
WW1	0	02/07/11	Winter	West	WA	WW	Raw	95.9	6.81	42.325	30.925	3.831E+06	1.929E+06	9.000E+04	2.283E+04
WW2	0	04/12/11	Spring	West	WA	WW	Final	5.365	7.27	24.085	10.29	1.098E+05	3.473E+04	1.667E+02	1.667E+02
WW1	0	04/12/11	Spring	West	WA	WW	Raw	255	7.07	75.325	29.355	2.332E+06	1.315E+06	9.333E+04	3.967E+04

Appendix F. Drinking Water Data

ID	Sampling Notes	Date	Season	Region	State	Surface/ Ground	Treated/ Untreated	Sample Description	Turbidity (ntu)	pH	TOC (mg/L)	DOC (mg/L)	Coliform (cfu/100 mL)	E. coli (cfu/100 mL)	Male-Specific Colipage (pfu/100 mL)	Somatic Coliphage (pfu/100mL)
1		5/3/2011	Spring	Midwest	WI	ground	Untreated	Deep Well	2.81	7.61	2.51	1.7830E+00	0.0000E+00	0.0000E+00	9.9558E-01	0.341672
2		5/3/2011	Spring	Midwest	WI	Ground	treated	Chlorinated	0.975	7.58	7.77	1.4640E+00	0.0000E+00	0.0000E+00	9.5355E-01	0.44351
3		5/3/2011	Spring	Midwest	WI	Ground	treated	Dist System	0.986	7.62	2.405	1.6925E+00	0.0000E+00	0.0000E+00	2.3250E-01	0
1		5/16/2011	Spring	Northeast	MA	Surface	Untreated	Raw	5.34	6.68	#DIV/0!	5.3795E+00	1.3895E+02	2.1755E+00	0.0000E+00	0
2		5/16/2011	Spring	Northeast	MA	Surface	Treated	Ozone	2.175	6.45	#DIV/0!	3.8265E+00	2.3281E+00	0.0000E+00	1.0199E+03	0
3		5/16/2011	Spring	Northeast	MA	Surface	Treated	Filtration	0.8195	6.03	#DIV/0!	3.1955E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
4		5/16/2011	Spring	Northeast	MA	Surface	Treated	Chlorination	3.45	6.19	#DIV/0!	3.2570E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
5		5/16/2011	Spring	Northeast	MA	Surface	Treated	Distribution Syst.	2.27	6.54	#DIV/0!	3.5590E+00	0.0000E+00	0.0000E+00	1.9300E+02	0
1		6/21/2011	Summer	West	NV	surface	Untreated	Raw	0.49	8.03	6.56	#DIV/0!	5.42E+00	0.00E+00	0.00E+00	1.25E+00
2		6/21/2011	Summer	West	NV	surface	Treated	Filtered Effluent	0.25	7.99	6.39	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
3		6/21/2011	Summer	West	NV	surface	Treated	Finished / clear	0.12	7.85	5.89	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4		6/21/2011	Summer	West	NV	Ground	Treated	Distribution-Ground	0.21	7.81	5.89	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
5		6/21/2011	Summer	West	NV	surface	Treated	Distribution-Lake	0.30	7.73	0.81	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1		7/12/2011	SUMMER	MIDWEST	WI	GROUND	UNTREATED	DEEP WELL WATER #7	2.61	7.55	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
2		7/12/2011	SUMMER	MIDWEST	WI	GROUND	TREATED	CHLORINATION WELL WATER	0.35	7.63	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
3		7/12/2011	SUMMER	MIDWEST	WI	GROUND	TREATED	DISTRIBUTION SYSTEM WELL WATER	0.36	7.60	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4	0	7/12/2011	SUMMER	MIDWEST	WI	GROUND	LAB	WI LAB WATER	0.10	7.30	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1		7/25/2011	SUMMER	NORTHEAST	MA	SURFACE	Untreated	RAW	0.7845	6.23	#DIV/0!	#DIV/0!	4.6479E+02	3.4910E-01	0.0000E+00	4.19E-01
2		7/25/2011	SUMMER	NORTHEAST	MA	SURFACE	TREATED	AFTER OZINATION	0.172	6.89	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00	4.7660E+00	5.02E-01
3		7/25/2011	SUMMER	NORTHEAST	MA	SURFACE	TREATED	AFTER FILTRATION	0.0805	6.45	#DIV/0!	#DIV/0!	1.2483E+01	0.0000E+00	0.0000E+00	0.00E+00
4		7/25/2011	SUMMER	NORTHEAST	MA	SURFACE	TREATED	AFTER CHLORINATION	0.3755	7.52	#DIV/0!	#DIV/0!	2.5879E+02	1.0000E+00	0.0000E+00	0.00E+00
5		7/25/2011	SUMMER	NORTHEAST	MA	SURFACE	TREATED	DISTRIBUTION SYSTEM	0.3085	6.94	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00	2.0979E-01	4.90E-01
6		7/25/2011	SUMMER	NORTHEAST	MA	LAB	TREATED	WPI LAB WATER	0.0995	5.25	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00	#DIV/0!	#DIV/0!
1		8/10/2011	SUMMER	SOUTH	NC	GROUND	UNTREATED	NC University LAKE	5.815	7.19	3.8005	4.0385E+00	6.5844E+02	5.9288E-01	0.0000E+00	0.00E+00

2		8/10/2011	SUMMER	SOUTH	NC	GROUND	UNTREATED	NC CANE CREEK	1.555	7.32	4.3165	3.0415E+00	5.0017E+02	6.6523E-01	0.0000E+00	0.00E+00
3		8/10/2011	SUMMER	SOUTH	NC	GROUND	TREATED	FILTERED	0.319	7.49	3.268	3.1525E+00	0.0000E+00	0.0000E+00	0.0000E+00	0.00E+00
4		8/10/2011	SUMMER	SOUTH	NC	GROUND	TREATED	FINISHED	0.1955	8.47	3.123	3.5070E+00	0.0000E+00	0.0000E+00	2.4633E-01	4.22E-01
5		8/10/2011	SUMMER	SOUTH	NC	GROUND	TREATED	DBN	0.261	8.43	3.428	4.1790E+00	6.3000E+00	0.0000E+00	8.4810E-01	2.31E-01
1		8/23/2011	SUMMER	WEST	NV	SURFACE	UNTREATED	RAW WATER	0.356	7.70	#DIV/0!	#DIV/0!	1.6119E+02	0.0000E+00	0.0000E+00	4.80E-01
2		8/23/2011	SUMMER	WEST	NV	SURFACE	TREATED	FILTER EFFLUENT	0.417	7.79	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00	0.0000E+00	2.80E-01
3		8/23/2011	SUMMER	WEST	NV	SURFACE	TREATED	FINISHED WATER	0.288	7.80	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00	0.0000E+00	7.80E-01
4		8/23/2011	SUMMER	WEST	NV	GROUND	TREATED	DISTRIBUTION -GW	0.204	7.81	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00	0.0000E+00	0.00E+00
5		8/23/2011	SUMMER	WEST	NV	SURFACE	TREATED	DISTRIBUTION-LAKE MEAD	0.2525	7.79	#DIV/0!	#DIV/0!	0.0000E+00	0.0000E+00	0.0000E+00	0.26
1		10/25/2011	FALL	MIDWEST	WI	GROUND	UNTREATED	DEEP WELL	2.93	7.785	0.959	#DIV/0!	0	0	0	0
2		10/25/2011	FALL	MIDWEST	WI	GROUND	TREATED	CHLORINATED	1.06	7.725	1.5805	#DIV/0!	0	0	0	0
3		10/25/2011	FALL	MIDWEST	WI	GROUND	TREATED	DISTRIBUTION SYSTEM	1.015	7.73	0.6575	#DIV/0!	0	0	0	0
4		10/25/2011	FALL	MIDWEST	WI	LAB	TREATED	LAB WATER	0.3	8.175	0.12	#DIV/0!	0	0	#DIV/0!	#DIV/0!
1		11/08/11	FALL	SOUTH	NC	SURFACE	UNTREATED	RAW, CANE CREEK	3.83	8.26	7.42	7.03	1.41E+02	1.69E+00	8.00E-02	1.03E+00
2		11/08/11	FALL	SOUTH	NC	SURFACE	UNTREATED	RAW, UNIVERSITY LAKE	3.01	7.52	7.36	6.87	1.45E+03	9.18E+00	0.00E+00	4.88E+00
3		11/08/11	FALL	SOUTH	NC		TREATED	FILTER EFFLUENT	0.13	8.10	2.82	2.88	0.00E+00	0.00E+00	4.00E-02	8.00E-02
4		11/08/11	FALL	SOUTH	NC	GROUND	TREATED	FINAL CLEAR WELL	0.24	8.88	2.82	2.66	0.00E+00	0.00E+00	6.00E-01	0.00E+00
5		11/08/11	FALL	SOUTH	NC			DISTRIBUTION SYSTEM	0.15	8.89	2.47	2.37	0.00E+00	0.00E+00	0.00E+00	0.00E+00
6		11/08/11	FALL	SOUTH	NC			RECYCLED WATER	3.88	7.77	4.94	4.21	0.00E+00	0.00E+00	1.00E+00	1.00E+00
7		11/08/11	FALL	SOUTH	NC			LAB WATER	0.17	6.78	0.05	0.13	2.42E+03	2.42E+03	0.00E+00	0.00E+00
1		11/29/11	FALL	NORTHEAST	MA	SURFFACE	UNTREATED	RAW	1.35	7.16	3.28	3.04	1.39E+02	8.00E-01	0.00E+00	0.00E+00
2		11/29/11	FALL	NORTHEAST	MA	SURFFACE	TREATED	OZONATED	1.47	6.28	3.12	2.15	6.07E-01	0.00E+00	0.00E+00	0.00E+00
3		11/29/11	FALL	NORTHEAST	MA	SURFFACE	TREATED	AFTER FILTRATION	0.16	6.28	2.15	2.02	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4		11/29/11	FALL	NORTHEAST	MA	SURFFACE	TREATED	POST CHLORINATED	0.39	6.78	2.08	2.00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
5		11/29/11	FALL	NORTHEAST	MA	SURFFACE	TREATED	DISTRIBUTION SYSTEM	0.87	6.78	2.07	1.93	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1		12/07/11	FALL	west	NV	Surface	Untreated	RAW WATER	0.30	8.07	#DIV/0!	#DIV/0!	1.26E+01	1.00E+00	6.00E-01	6.60E-01
2		12/07/11	FALL	west	NV	SUFACE	TREATED	FILTER EFFLUENT	0.36	7.85	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	8.80E-01	0.00E+00
3		12/07/11	FALL	west	NV	SURFACE	TREATED	FINISHED / CLEAN WELL	0.25	8.06	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00

4		12/07/11	FALL	west	NV	SURFACE	TREATED	DISTRIBUTION-6621 BRANDYWINE WAY	0.60	7.84	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	4.00E-02
5		12/07/11	FALL	west	NV	SURFACE	TREATED	DISTRIBUTION-113 ROSEMEADE ST	0.38	7.82	#DIV/0!	#DIV/0!	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	TOC Run 1 week later, not refridgerated	01/05/12	WINTER	SOUTH	NC	Surface	Untreated	UNIVERSITY LAKE RAW	9.69	6.76	8.50	6.52	1.34E+02	1.50E-01	0.00E+00	0.00E+00
2	TOC Run 1 week later, not refridgerated	01/05/12	WINTER	SOUTH	NC	Surface	Untreated	CANE CREEK RAW	3.32	6.97	11.58	5.62	6.52E+00	6.00E-01	0.00E+00	1.33E+00
3	TOC Run 1 week later, not refridgerated	01/05/12	WINTER	SOUTH	NC	Ground	Treated	FILTERED EFFLUENT	0.22	8.61	6.63	2.10	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4	TOC Run 1 week later, not refridgerated	01/05/12	WINTER	SOUTH	NC	Ground	Treated	FINAL CLEAR WELL	0.13	7.05	9.57	2.08	0.00E+00	0.00E+00	0.00E+00	0.00E+00
5	TOC Run 1 week later, not refridgerated	01/05/12	WINTER	SOUTH	NC	Ground	Treated	DISTRIBUTION SYSTEM	0.19	8.71	10.82	2.30	0.00E+00	0.00E+00	0.00E+00	0.00E+00
6	TOC Run 1 week later, not refridgerated	01/05/12	WINTER	SOUTH	NC	Ground	Treated	Lab Water	2.25	6.71	0.61	0.20	0.00E+00	0.00E+00	0.00E+00	0.00E+00
7	TOC Run 1 week later, not refridgerated	01/05/12	WINTER	SOUTH	NC	Ground	Treated	RECYCLED WATER	3.45	7.66	6.31	3.29	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1		1/19/2012	winter	southwest	nv	Surface	Untreated	Raw Water	0.6745	8.11	2.775	2.7735E+00	1.9547E+02	1.0000E+00	1.3400E+00	0.2
2		1/19/2012	winter	southwest	nv	surface	treated	Filtered Water	0.44	8.08	2.694	2.6215E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
3		1/19/2012	winter	southwest	nv	surface	treated	Finished Water	0.2485	7.98	2.6535	2.7015E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
4		1/19/2012	winter	southwest	nv	surface	treated	Lake Mead Dist. (1)	0.3605	8.01	2.638	2.6670E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
5		1/19/2012	winter	southwest	nv	surface	treated	Lake Mead Dist. (2)	0.4755	7.86	2.6635	2.6265E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
1		2/7/2012	Winter	Northeast	MA	surface	Untreated	Raw	0.864	6.55	3.1505	3.1885E+00	2.2792E+02	2.0000E-01	0.0000E+00	0
2		2/7/2012	Winter	Northeast	MA	surface	treated	After Ozone	0.912	6.29	2.8035	2.1855E+00	4.6667E-01	0.0000E+00	0.0000E+00	0
3		2/7/2012	Winter	Northeast	MA	surface	treated	After Filtration	0.2545	7.41	2.1995	2.1870E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
4		2/7/2012	Winter	Northeast	MA	surface	treated	After Chlorination	0.2125	7.02	2.062	2.0475E+00	0.0000E+00	0.0000E+00	0.0000E+00	0.2
5		2/7/2012	Winter	Northeast	MA	surface	treated	Distribution System	0.9805	6.87	1.9665	1.9210E+00	0.0000E+00	0.0000E+00	0.0000E+00	0
6		2/7/2012	Winter	Northeast	MA	surface	treated	Lab Water	0.1985	6.88	0.0415	1.1300E-01	0.0000E+00	0.0000E+00	0.0000E+00	0
1		2/22/2012	Winter	Midwest	WI	Ground	Untreated	Deep Well	3.155	7.72	5.3015	7.5950E-01	0.0000E+00	0.0000E+00	0.0000E+00	0
2		2/22/2012	Winter	Midwest	WI	Ground	Untreated	Clear Well 1	1.79	7.65	5.206	6.7800E-01	0.0000E+00	0.0000E+00	0.0000E+00	0
3		2/22/2012	Winter	Midwest	WI	Ground	Untreated	Clear Well 2	0.645	7.00	0.2865	7.1800E-01	0.0000E+00	0.0000E+00	0.0000E+00	0
4		2/22/2012	Winter	Midwest	WI	Ground	Untreated	Distribution	1.12	7.18	0.264	7.4750E-01	0.0000E+00	0.0000E+00	0.0000E+00	0
5		2/22/2012	Winter	Midwest	WI	Ground	treated	Lab Water	0.31	7.55	0.407	1.6750E-01	0.0000E+00	0.0000E+00	0.0000E+00	0