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METHODS TO DETECT FECAL INDICATOR VIRUSES AND PROTOZOAN SURROGATES IN NC RECLAIMED WATER: OPTIMIZATION, PERFORMANCE EVALUATION, PROTOCOL DEVELOPMENT, VALIDATION, COLLABORATIVE TESTING, AND OUTREACH

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1 Abstract

1.1 Title: Methods to detect fecal indicator viruses and protozoan surrogates in NC reclaimed water: optimization, performance evaluation, protocol development, validation, collaborative testing, and outreach.**1.2 Objectives:** The objectives of this study were to better define, describe, improve, validate, collaboratively test, seek State certification for and disseminate the methods to detect fecal indicator viruses (coliphages) and protozoan parasite surrogates (*Clostridium perfringens*) in reclaimed water to support the NC Reclaimed Water Rule. **1.3 Methods**: For coliphage viruses, US EPA methods 1601, enrichment-spot plate, and 1602, single agar layer (SAL) were adapted and evaluated for use in reclaimed water and wastewaters. This methods comparison involved the use of multiple coliphage hosts including the somatic coliphage host (*E. coli* CN13), the male-specific/F+ coliphage host (*E. coli* Famp), and 3 total coliphage hosts (*E. coli* CB390, *E. coli* C3000, and *E. coli* C3322). For *C. perfringens,* three agar media (TSC, mCp, and CP ChromoSelect) were compared using a standard membrane filtration method to evaluate their ability to enumerate *C. perfringens* from reclaimed water samples. Presumptive positive and negative colonies were further subjected to two confirmation tests to better determine the ability of the media to correctly identify true *C. perfringens* colonies. A survey was also performed to determine the market potential and capacity to access testing for these indicators in reclaimed water.**1.4 Results:** Data for coliphage concentrations were subjected to statistical analyses, and no statistically signficant differences were found between the two coliphage methods, SAL and enrichment-spot plate, for any coliphage host. The total coliphage host, *E. coli* CB390, was not found to be statistically different from the sum of male-specific/F+ coliphages on *E. coli* Famp and somatic coliphages on *E. coli* CN13. For *C. perfringens* detection by membrane filtration*,* agar media comparisons indicated significant differences in detection in both pasteurized and unpasteurized pre-sand filter-treated reclaimed water samples. All agar media gave some false-positive and false negative colonies in addition to detecting *C. perfringens,* based on colony confirmation by acid phosphatase and stormy fermentation tests. The survey indicated that without significant demand increases for reclaimed water or changes in legislation, reclaimed water testing for coliphages and *C. perfringens* and expansion of type 2 reclaimed water systems may be slow to develop as many labs do not currently offer and are not currently interested in these microbial test methods.**1.5 Conclusions:** The two coliphage detection methods evaluated for reclaimed water and wastewater gave statistically equivalent results. However, the single agar layer method was preferred method based on cost, supplies used, time to obtain results and preferences of workshop participants. The total coliphage host *E. coli* CB390 is preferred over other *E. coli* hosts as it can detect simultaneously both somatic and male-specific/F+ coliphages. For the detection of *C. perfringens,* CP ChromoSelect agar medium is preferred, as it does not require a confirmation test or multiple and expensive medium additives and gives distinctive colonies. As the survey was conducted before new legislation regarding reclaimed water for potable reuse, a new survey is recommended to evaluate interest in type 2 reclaimed water and these microbial methods. **1.6 Recommendations:** For coliphage detection in reclaimed water or wastewater, the single agar layer method and *E. coli* total coliphage host *E.* CB390 are recommended for efficient and convenient detection of these fecal indicator viruses. For the enumeration of *C. perfringens* in reclaimed water or wastewater, membrane filtration using CP ChromoSelect agar medium is the recommended choice. With new legislation recently passed for type 2 reclaimed water to be used as source water for potable reuse in NC, it is recommended that new surveys be done to reevaluate interest in implementing both coliphage and *C. perfringens* methods for analysis of type 2 reclaimed water.

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3 Acknowledgements

WRRI (Water Resources Research Institute)

North Carolina Department of Environment and Natural Resources (NC DENR)

Participating Water Utilities
- OWASA (Orange Water and Sewer Authority) Wastewater Treatment Plant
- Raleigh Neuse River Wastewater Treatment Plant
- North Durham Water Reclamation Facility
- Holly Springs Wastewater Treatment Plant
- North Cary Water Reclamation Facility

Workshop and Hands-on Training Attendees (Employers noted below)
- Durham County Department of Public Health
- Eastern Band of Cherokee Indians
- Town of Holly Springs
- Johnston County Public Utilities
- North Carolina DENR Division of Water Resources Water Quality Programs Laboratory
- Onslow County
- City of Raleigh Neuse River Laboratory
- Secure Resources, PLLC
- Shellfish Sanitation and Recreational Water Quality NC Division of Marine Fisheries Office

Survey Participants

Labs: Environmental Testing Solutions, Inc. K&W Laboratories, Environment 1, Inc., Cub Creek WasteWater Lab, Leith Creek Wastewater Lab, OWASA Wastewater Lab, Durham County Triangle WWTP, Kinston Regional Water Reclamation Facility, Town of Clayton WWTP, Cherokee Waste Water Treatment Plant, Town of Holly Springs Environmental Compliance Lab, ONWASA Laboratory, MCAS Cherry Point Water QC Labs, City of Shelby First Broad WWTP Lab, Jimmy Smith WWTP, Tuckaseigee Water and Sewer Authority Lab, Mount Olive WWTP Lab, Rocky River Waste Water Treatment Plant Laboratory, City of Jacksonville WWTP, The Environmental Quality Institute, EM Johnson Water Plant Laboratory, Caper Fear EMD Laboratory, Brunswick County Utilities Wastewater Laboratory, Crowder's Laboratory, Norman H. Larkins POTW, Town of Carolina Beach Waste Water Treatment Plant Laboratory.

Bulk reclaimed water providers: OWASA, Raleigh, Cary, Durham, Pittsboro, Monroe

4 Body of Report

4.1 Introduction

N.C. is looking increasingly to reclaimed water as a water resource with many beneficial uses to better meet growing water needs, such as in agriculture and industry. In 2011 N.C. passed revised reclaimed water regulations (subchapter 02U – Reclaimed Water) (NC DENR, 2011) that expanded reclaimed water uses in part by establishing a higher quality reclaimed water, type 2 for a wider range of beneficial uses, including agricultural use for irrigation of food crops. To address risks from disease-causing pathogens in human wastewater, the rules for Type 2 reclaimed water specify effluent quality and treatment performance requirements not only for fecal bacteria, such as *E. coli*, but also for coliphages such as fecal indicator viruses and for the spore-forming bacterium *Clostridium perfringens* as a surrogate for protozoan parasite pathogens, such as *Giardia* and *Cryptosporidium*. Design criteria for wastewater treatment to produce type 2 reclaimed water must achieve a 5 $\log_{10}$ reduction of coliphages and a 4 $\log_{10}$ reduction of *Clostridium perfringens* and effluent quality for type 2 reclaimed water must meet a geometric mean of 5/100 mL and a daily maximum of 25/100 mL for both coliphages and *Clostridium perfringens*.

The key problem to be addressed by this project is the urgent need for specific, effective, optimized, validated, cost-effective, clearly written and eventually certified methods to analyze for fecal indicator viruses (coliphages) and protozoan parasite surrogates (*Clostridium perfringens* spores and possibly vegetative cells as well) in NC type 2 reclaimed water. Although the recent regulations for NC reclaimed water require type 2 reclaimed water to meet performance requirements for coliphages (indicator viruses) and *Clostridium perfringens* (protozoan parasite indicators), as specified in SUBCHAPTER 02U – RECLAIMED WATER SECTION .0100 – GENERAL REQUIREMENTS, 15A NCAC 02U (see at: http://reports.oah.state.nc.us/ncac/title%2015a%20-%20environment%20and%20natural%20resources/chapter%2002%20-%20environmental%20management/subchapter%20u/subchapter%20u%20rules.html), the methods to detect these additional fecal indicators were not specified. There is no specific information or guidance on which particular coliphages or what forms of *C. perfringens*, total cells or spores only, are to be detected and by what specific methods. One can assume that US EPA methods or methods from Standard Methods for the Examination of Water and Wastewater are to be used, although this is not explicitly stated in the regulation. Furthermore, those sources (US EPA and Standard Methods) do not specifically address the use of the coliphage or *Clostridium perfringens* methods for reclaimed water. Therefore, the methods to analyze for these virus and protozoan parasite indicators are not specified. As the methods to be used are ill defined, potential users encounter inconsistent and variable methods among reference method sources (US EPA and Standard Methods) that are unverified for performance in NC reclaimed water, unavailable in appropriate formats for specific use in NC type 2 reclaimed water and uncertified by NC DENR. For those wanting to analyze NC type 2 reclaimed water for coliphages and *C. perfringens*, they have no specific guidance from the State of NC or other authoritative sources and they face a morass of potentially confusing choices and options. Furthermore, it is unclear from the NC rule if the "coliphages" to measure are the somatic or male-specific/F+ groups of coliphages or both groups together and if *C. perfringens* are to be detected as spores only or both spores and vegetative cells. This lack of specificity adds further

uncertainly and confusion to the choice of methods to use for NC type 2 reclaimed water.

The new rule for NC type 2 reclaimed water now brings the urgent need for specified, effective, practical, accessible and affordable methods to detect and quantify coliphages and *Clostridium perfringens* in wastewater and type 2 reclaimed water effluent in order to determine if effluent microbial quality standards and specified $\log_{10}$ reduction requirements as treatment design criteria are being met. Methods for coliphages and *Clostridium perfringens* have been developed and are described by US EPA and/or in Standard Methods for the Examination of Water and Wastewater, both of which are sources of analytical methods accepted by the State of N.C. (APHA, 2005; US EPA 2001a; 2001), as well as by other sources (for example, the International Standards Organization). However, these available analytical methods are diverse, they were not specifically developed, performance-validated, written up in a specific form for NC type 2 reclaimed water analysis or approved by NC certification for application to NC type 2 reclaimed water. Therefore, their performance for this reclaimed water has not been adequately evaluated, adapted, performance-validated, documented and certified for this context and use. Furthermore, new and improved methods for analysis of coliphages and *Clostridium perfringens* in water and wastewater continue to be developed, sometimes claiming improved performance but sometimes not supported by method performance validation data at all or performance validation data that are independent. Currently, potential users of coliphage and *Clostridium perfringens* analysis methods who want to apply them to N.C. type 2 reclaimed water or wastewater are faced with a wide and confusing range of options, none of which are either clear or well-established and adequately documented in performance for this purpose. Additionally, users, regulators and managers face uncertainties about which groups of coliphages to measure and which form of *C. perfringens* to measure, spores only or spores plus vegetative cells, as such details were not specified in the revised reclaimed water rule for type 2 water.

Coliphages are viruses that infect *E. coli* bacteria and are considered reliable indicators of human enteric viruses in wastewater and fecally contaminated water (National Academy of Sciences, 2004). They are present in raw sewage at concentrations of 10,000 to 100,000 per 100 mL. There are two main groups of coliphages, somatic, which infect host cells by attaching directly to the host cell outer layer, and F+ male-specific, which attach to the pili (fimbrae) surface appendages of only those host cells that are F+ male-specific (Sobsey et al., 2004). Within each of these two broad coliphage groups, there are taxonomically different male-specific and somatic coliphages families, each with different morphologies, compositions and structural elements. Both somatic and male-specific coliphages are abundant in raw sewage, but because of their diverse properties, they may differ in survival and they may be reduced to different extents by wastewater treatment and disinfection processes. Little information is available on the presence of these two different groups of coliphages in type 2 reclaimed water or the abilities of type 2 reclaimed treatment processes to reduce their levels in wastewater.

Under the current rule for NC type 2 reclaimed water and because of the uncertainty about which coliphage groups may persist in treatment process for reclaimed water, it is probably prudent and most protective of public health to measure both coliphage groups, somatic and male-specific/F+ in NC type 2 reclaimed water. Currently, these two main groups of coliphages are analyzed separately on different *E. coli* host bacteria. However, with the right choice of *E. coli* bacteria host, it may be possible to measure both somatic and male-specific/F+ coliphages together on a single *E. coli* host, thereby reducing the analytical effort and workload to detect and quantify

"total" (F+ and male-specific) coliphages in NC type 2 reclaimed water and wastewater. Given the current lack of clarity of the NC reclaimed water rule on which coliphages to measure and the lack of guidance or regulatory requirements on what coliphage method(s) to use, it is probably advisable to measure at first all 3 categories of coliphages, (1) somatic coliphages, (2) male-specific/F+ coliphages and (3) "total" coliphages (by simultaneous analysis of both somatic and male-specific/F+ coliphages), before making a decision about which coliphages and which methods for them are most appropriate for use under the rule for type 2 reclaimed water.

Clostridium perfringens are spore-forming anaerobic bacteria present in raw sewage at about 10,000-100,000 per 100 mL. *C. perfringens* has been proposed as a possible fecal indicator for protozoan parasites such as Cryptosporidium and Giardia in wastewater and fecally contaminated water because it produces hardy spores that can survive for a long time in the environment and are relatively resistant to disinfection processes, as are the cysts and oocysts of protozoan parasites (National Academy of Sciences, 2004; Payment and Franco, 1993). *C. perfringens* in water and wastewater are typically detected and quantified by membrane filtration methods using specific plating media. In the USA, the standard medium has been mCp agar (or broth), which has been modified by reducing the concentration of the expensive ingredient indoxyl β-D-glucoside to 60 mg/L (1/10 the initial recommended concentration) to reduce cost without affecting method sensitivity (Bisson and Cabelli, 1979; Armon and Payment, 1988). However, using modified mCp medium, *C. perfringens* detection requires a confirmatory step in which the bacterial colonies on the plates are exposed to ammonium hydroxide fumes to determine if they produce a characteristic pink color that confirms their identity. This step is extra work and must be done under a fume hood, making it less practical for routine use in many labs. In addition, the mCp medium contains several heat-sensitive ingredients that must be made up sterile and then added aseptically after the medium base is autoclaved, which is another inconvenience that adds cost to performing the analysis. However, studies document mCp medium as inferior to other media for detection of *C. perfringens* in water by membrane filtration. Sartory et al. (1998) documented that TSC (Tryptose Sulphite Cycloserine) agar gave better recoveries of *C. perfringens* than did mCp medium, resulting in the selection of TSC medium for the ISO method for *C. perfringens* now widely used in many other countries. TSC is supposed to give distinctive black *C. perfringens* colonies on TSC medium and its superior detection to mCp medium has also been reported by others (Arujo et al., 2004). More recently another medium for *C. perfringens* detection in water by membrane filtration has been developed, CP ChromoSelect agar from Sigma Aldrich and Fluka that is easy to make, gives distinctive green colonies of *C. perfringens* and is reported to provide detection comparable to or better than other methods against which it has been compared (Manafi et al., 2013). Based on their reported effective performance and/or their acceptance as standard methods for official use, these three different media appear to be preferred candidates to evaluate for detection of *C. perfringens* in NC type 2 reclaimed water and wastewater.

Another issue to consider for detecting *C. perfringens* in NC type 2 water and wastewater is whether to detect spores only or spores plus vegetative cells. For the use of *C. perfringens* as a protozoan parasite indicator or surrogate, it is the *C. perfringens* spores that are most like the cysts and oocysts of protozoan parasites, in terms of environmental persistence and resistance to water and waste treatment processes. However, some studies of water and wastewater microbial reductions by treatment processes have analyzed for one or the other of *C. perfringens* spores only or spores plus vegetative cells or for both forms, spores only and spores plus vegetative

cells (Sartory et al., 1988; Payment et al., 1993; Wen et al, 2009). To detect the spores only, the water samples are first heated to a temperature between 60 to 80 degrees C for 15 to 30 minutes prior to analysis in order to kill off the vegetative cells. This heat treatment leaves only the spores to be detected by membrane filtration and culture. For the purpose of this study, the measurement of both spores and vegetative cells as well as spores only was advisable, given the current lack of information about the occurrence and treatment reductions of *C. perfringens* spores or vegetative cells in wastewater and NC type 2 reclaimed water.

Methods protocols or standard operating procedures (SOPs) specific for the detection of coliphages and *C. perfringens* in type 2 NC reclaimed water or wastewater are not available and none have been submitted to or formally accepted by the State certification program for such analysis, as far as we know. Hence, this project was intended to be a definitive study to urgently address the uncertainties, lack of clarity and lack of specificity that now exists in the choices of methods and microbial targets for detection of coliphages and *C. perfringens* in NC type 2 reclaimed water. A systematic and thorough study was designed to identify and screen the established, candidate and emerging methods for analysis of coliphages and *Clostridium perfringens* in N.C wastewater and reclaimed water, evaluate the best of these methods for their performance and other characteristics (complexity, time to results, costs, etc.), subject the best of these methods to a multi-laboratory performance validation study, write up the methods in readily accessible and end-user friendly forms as protocols or SOPs, submit them to the N.C. methods certification program, seek approval of them through the US EPA Alternative Test Procedure (ATP) Program and make them known and available to potential users in N.C. though workshops and other means of outreach, communication and dissemination. This report describes a directed study to screen, further develop, evaluate, verify, collaboratively test, write up in appropriate formats and distribute and communicate to stakeholders a set of practical, performance-proven, affordable, appropriate and consensus methods to detect and quantify coliphages and *C. perfringens* in NC type 2 reclaimed water and wastewater. The study of this report is intended to facilitate the use of the coliphage and *C. perfringens* methods in N.C. to support activities to improve type 2 reclaimed water development and access in N.C.

4.2 Methods

4.2.1 Sample Collection

4.2.1.1Wastewater treatment plants

Treated wastewater effluents were collected at five wastewater treatment plants located in central North Carolina. These facilities were: (A) the Orange Water and Sewer Authority WWTP in Chapel Hill, (B) the Raleigh Neuse River WWTP, (C) the North Durham Water Reclamation Facility, (D) the Holly Springs WWTP and (E) the North Cary Water Reclamation Facility.

4.2.1.2 Selection of treated effluents

Sewage effluent samples were collected initially from two wastewater treatment plants, and analyzed for *Clostridium perfringens* and coliphages. It was found that both treatment plants

produced reclaimed water that consistently met the North Carolina Type 2 reclaimed water effluent standards of less than 5 *C. perfringens* colonies or coliphage plaque-forming units per 100 mL. Many of the final effluent samples had no detectable coliphages or *C. perfringens* in 100-mL sample volumes, and therefore were below the detection limits of the methods of analysis. Because the determination of the best methods for quantifying *C. perfringens* and coliphages requires reliable statistical analysis of data on quantifiable levels of the target microorganisms, samples containing sufficient numbers of these target microbes are required. Consequently, microbiological analysis was performed with samples collected from earlier stages in the water reclamation process. It was found that clarified secondary effluent collected prior to sand filtration and disinfection consistently yielded sufficient but not excessive concentrations of *Clostridium perfringens*, while post-sand filter effluent prior to disinfection provided adequate concentrations of coliphages. These same treatment plant sample locations yielded sufficient concentrations of both *Clostridium perfringens* and coliphages at all five wastewater treatment facilities where samples were collected when the appropriate partially treated samples indicated above were analyzed.

In the second stage of testing, in addition to the samples collected earlier in the treatment process, raw sewage and final reclaimed water samples were also collected for analysis and are included in this report. It is important to note that not all of the reclaimed water samples were treated by the identical physical, chemical and biological processes. These differences are summarized in Table 1 below. This table does not include information on steps prior to filtration and disinfection, as these steps were similar at each wastewater treatment plant and included primary clarification (sedimentation), anaerobic digestion of separated wastewater solids, aerobic biological treatment of primary effluent by some form of activated sludge treatment process and secondary clarification after aerobic biological treatment. Each plant, excepting plant C, uses free chlorine in the production of reclaimed water.

Table 1: Summary of Wastewater Treatment Sample Collection Scheme

Wastewater Treatment Plant	Reclaimed Water Treatment Steps after Primary and Secondary treatment	Samples Collected for Microbial Analysis
A	1. Filtration (Sand Filter) 2. UV Disinfection 3. Chlorine Disinfection	Coliphages: Pre UV Disinfection *Clostridium perfringens*: Pre Sand Filtration
B	1. Filtration (Sand Filter) 2. UV Disinfection 3. Chlorine Disinfection	Coliphages: Pre UV Disinfection *Clostridium perfringens*: Pre Sand Filtration
C	1. Filtration (Sand Filter) 2. UV Disinfection	Coliphages: Pre UV Disinfection *Clostridium perfringens*: Pre Sand Filtration

D	1. Filtration (Sand Filter) 2. UV Disinfection 3. Chlorine Disinfection	Coliphages: Pre UV Disinfection *Clostridium perfringens*: Pre Sand Filtration
E	1. Filtration (Sand Filter) 2. UV Disinfection 3. Chlorine Disinfection	Coliphages: Pre UV Disinfection *Clostridium perfringens*: Pre Sand Filtration

4.2.1.3 Sample handling

Treated and raw wastewater was collected from the appropriate WWTP sampling points in sterile polypropylene bottles, and kept chilled in coolers with ice during transport to Chapel Hill. The samples were stored at 4°C upon arrival at the laboratory. Coliphage and *Clostridium perfringens* assays were performed on the day of or the day following sample collection.

4.2.2 Coliphages

4.2.2.1 Coliphage Analysis Procedures

Procedures for coliphage detection and quantification were adapted from US EPA Method 1601 (two step enrichment spot-plate procedure for analysis of coliphages) and US EPA Method 1602 (Single Agar Layer method for analysis of coliphages). The methods as used in this study are attached to this report in the form of laboratory 'bench sheets,' which are intended to be easy-to-follow, step-by-step protocols that laboratory analysts may use to perform the analyses. These bench sheets were used by participants in the 1-day workshop and hands-on training held on February 21, 2014 at the Gillings School of Global Public Health, University of North Carolina, Chapel Hill.

This examination of methods for the detection of coliphages in reclaimed water was conducted in two phases. The initial phase of sample analysis in 2013 included the use of five *E. coli* coliphage hosts, specifically, total coliphage hosts CB390, C3322, C3000, the somatic coliphage host CN13, and the male-specific/F+ host Famp. The second phase of sample analysis in 2014 utilized the information from the initial phase to identify the best "total" coliphage host, *E. coli* CB390, based on 2013 results, with the goal of increasing the sample size while reducing workload and costs during the no-cost extension period. When the results from the secondary phase were analyzed, as presented below, the relevant host information, for *E.* coli CB390, from the initial phase was also included. The data analysis and explanation for the choice of *E. coli* CB390 as the total coliphage host will be presented below.

The coliphage hosts used in this study were obtained from a variety of sources. *E. coli* CN13 was obtained from the lab of Dr. Pierre Payment, Institut Armand-Frappier, Québec, Canada. *E. coli*

Famp was obtained from the lab of Dr. Victor Cabelli, University of Rhode Island, Kingston, Rhode Island. *E.coli* C3000 was obtained from the American Type Culture Collection (ATCC #15597), Manassas, Virginia. *E. coli* CB390 was obtained from the lab of Dr. Joan Jofre, Dept. of Microbiology, University of Barcelona, Barcelona, Spain. *E. coli* C3322 was obtained from the lab of Dr. GwangPyo Ko, Dept. of Environmental Health Sciences, Seoul National University, Seoul, South Korea. Stock *E. coli* suspensions for use in coliphage analysis were prepared by using a small scraping of previously prepared and frozen cell suspensions obtained from aforementioned sources, to inoculate a shaker flask partially filled with tryptic soy broth (TSB) containing appropriate antibiotics (see below), then placing the shaker flask into a 37°C incubator on a shaker tray for overnight incubation with continuous shaking at 100 RPM. To prepare new frozen stocks of each *E. coli* host, overnight cultures in TSB samples were supplemented with 20% sterile glycerol (V/V) and volumes were placed in sterile 2-mL screw capped tubes and frozen at -80°C. The antibiotics used for these coliphage hosts were Naladixic Acid for *E. coli* CN13 at 100 mg/L, Streptomycin and Ampicillin at 15 mg/L each for *E. coli* Famp, and Ampicillin at 100 mg/L for *E. coli* CB390, and *E. coli* C3322. *E. coli* C3000 did not require an antibiotic.

For each sampling week, a scraping from these prepared frozen stock suspensions was inoculated into TSB with appropriate antibiotics and allowed to shake at 100 RPM at 37°C on a shaker tray overnight (18-24 hours). The next morning, a 1 mL sample of this overnight culture was re-inoculated into a flask of 25 mL TSB and placed onto the shaker tray in a 37°C incubator with shaking at 100 RPM until reaching logarithmic phase (log phase). Log phase was based on each coliphage host achieving an absorbance between 0.2 and 0.8 absorbance units in a spectrophotometer, as compared to a TSB blank. The amount of time needed to achieve log phase for each coliphage host varied; therefore, a spectrophotometric analysis was conducted each time a log phase culture was prepared for each *E. coli* host.

Single Agar Layer Method

As described in US EPA Method 1602, the single agar layer method involves the combination of a 100 mL water sample with 100 mL of molten agar medium, *E. coli* coliphage host bacteria, appropriate antibiotics (as described above), and a divalent cation (as $MgCl_2$) at a final concentration of 0.05M. This method relies on the ability of coliphages to infect the provided *E. coli* host cells and create lysis zones or plaque forming units in the solidified agar medium. Double strength tryptic soy agar (TSA) is prepared and tempered in a waterbath first at 55°C and then at 45°C, while 100 mL volumes of the water sample is warmed in a waterbath first at 37°C and then at 45°C for a short time period. The time for sample to be tempered to 45°C is determined from a thermometer placed in 100 mL of Phosphate Buffered Saline (PBS) or deionized (DI) water to monitor the time required to reach 45°C. Careful temperature control is needed to prevent heat inactivation of coliphages from excessive exposure to 45°C and to prevent agar hardening if the water samples are combined with the molten agar that is not sufficiently high in temperature. *E. coli* coliphage host, appropriate antibiotic, and magnesium chloride ($MgCl_2$) are added to the 100mL water sample and this mixture is added to 100 mL of molten agar medium, mixed gently (not shaken) and poured into 5 150mm diameter sterile petri dishes. These plates are allowed to harden and dry for 10-15 minutes, then inverted and incubated overnight at 37°C. The next day the plates are read for lysis zones (plaque forming

units), and concentrations are reported as plaque forming (PFU) units per 100 mL.

Two Step Enrichment-Spot Plate Method

This method is a quantitative adaptation of US EPA Method 1601 consisting of two phases, with the first phase being overnight broth culture enrichment of the coliphages in water samples as multiple sub-volumes incubated at 37°C. First, 5 mL of 10X concentrated TSB, 10 mL of log phase *E. coli* coliphage host, appropriate antibiotics according to coliphage host, and $MgCl_2$ to a concentration of 0.05 M were added to a 100 mL water sample. The sample was then quickly subdivided into 3 volumes each of 30, 3.0, and 0.3 mL. These enrichment volumes were incubated overnight at 37°C. A TSA plate was prepared with *E. coli* coliphage host bacteria and appropriate antibiotics and stored in a cold room at 4°C until the second day of sample analysis. On~~e~~ the second day of the and after overnight incubation of the broth culture enrichments of sample volumes, enrichment volumes removed from the incubator and 0.32 mL volumes were transferred into 1mL conical bottom microcentrifuge tubes. Tubes were centrifuged at 10,000 RPM for 5 minutes, and after centrifugation, 10 µL of the supernatant was spotted onto the previously prepared agar medium plates with each *E. coli* coliphage host. Each coliphage host enrichment supernatant sample was spotted on the agar medium plate containing the same *E. coli* coliphage bacteria. A 100mL sample results in 9 spots on an agar medium culture plate containing *E. coli* coliphage host, 3 spots for the 30 mL volumes, 3 spots for the 3.0 mL volumes, and 3 spots for the 0.3 mL volumes. After the spots were allowed to dry for 15-20 minutes, plates were inverted and incubated at 37°C overnight. The next day, spots were scored for positive or negative growth of coliphages. Positive coliphage growth in a spotted, enriched sample volume is seen as a circular zone of lysis of the *E. coli* host bacteria in the spot. The positive results were then put into an MPN calculator (alternatively an MPN table could also be used instead), along with the appropriate sample volumes to return a MPN for the concentration of coliphages per 100 mL as MPN units per 100 mL. The 95% confidence interval was also reported for each of these MPN values, as is appropriate for any statistical maximum likelihood estimate (MLE) calculation from quantal data such as these.

4.2.2.2 Coliphage Data Analysis Procedures

The data collected on coliphage concentration was analyzed using the JMP Pro 9 statistical package (SAS Institute, Cary, NC). The data from phase one of this study were analyzed using nonparametric tests, including the Kruskal Wallis and Mann-Whitney Comparison Tests, because of the small sample size (n=11). As the sample size increased during the second phase of this study, hypotheses tests were conducted to examine the distribution of the data prior to performing any statistical tests. The null hypothesis (H_0) stated that the data are normally distributed, while the alternative hypothesis (H_a) stated that the data are not be normally distributed. For each comparison in the data analysis of the second phase of the study, H_0 did not describe these data; therefore, nonparametric tests were also performed on this data set, including the Kruskal Wallis and Mann-Whitney Comparison Tests. Additionally, a cumulative frequency distribution analysis was created to examine the distribution of the observed concentration values for each coliphage host in the second data set. Frequency distributions were generated in Microsoft Excel by sorting the coliphage concentrations in rank order and expressing them as percentages for frequencies of occurrence at or below various coliphage concentration values in

increments (bins) of coliphage concentrations per 100 mL.

4.2.3 *Clostridium perfringens*

4.2.3.1 *Clostridium perfringens* Analysis Procedures

Procedures for *C. perfringens* detection and enumeration were based on standard membrane filter (MF) methods. These methods were originally developed for US EPA by Cabelli and Bisson (1979). The methods used are attached to this report in the form of a laboratory 'bench sheet,' which is intended to be easy-to-follow, step-by-step protocols that laboratory analysts may use to perform the analyses. Following each assay, other confirmatory analyses were done to identify false-positive and false negative *C. perfringens* colonies obtained by initial membrane filter analysis. Individual presumptive positive and presumptive negative *C. perfringens* colonies from each of the three *C. perfringens* agar media tested were subjected to an alkaline-phosphatase (AP) test as described by Sartory et al. (2006). For the final 10 samples analyzed on this project, a further confirmatory test of stormy fermentation (SF) in tubes of iron milk medium was done to score for sulfite-reducing *Clostridium* species, the category to which *C. perfringens* belongs.

Prior to performing the membrane filtration (MF) method, the Acid-Phosphatase (AP) test and the Stormy Fermentation (SF) test as later described, the various media and reagents were prepared. The three agar media used for this investigation were Membrane *Clostridium perfringens* (mCp) agar, CP ChromoSelect Agar (CS), and Tryptose Sulfite Cycloserine agar (TSC). TSC agar medium was obtained from EMD Chemicals Inc. TSC agar was prepared by adding 3.9 grams of agar base /100 mL of deionized water in a 500 mL bottle and autoclaving for 15 minutes and cooling to keep molten. The mCp agar base (from Oxoid Microbiology Products was prepared by adding 7.11g of agar base /100 mL of deionized water, autoclaving for 15 minutes and cooling to keep molten. CP ChromoSelect agar base (from Fluka Analytical) was prepared by adding 6.28 grams/100 mL deionized water, bringing to a boil on a hot plate and then removing to cool and keep molten. After cooling, supplements were added to the various agar media as follows. CP Chromoselect and TSC agars got 0.04 grams of D-Cycloserine per 100 mL of molten agar medium base. The mCp agar got 0.2 mL of sterile 4.5% ferric chloride solution, 2 mL of sterile 0.5% phenolphthalein diphosphate solution, 0.8 mL of sterile 0.76% Indoxyl-β-glucoside, and 0.4 mL of mCp Selective Supplement, per 100 mL of molten agar base. Supplemented media were dispensed in 5-mL volumes in 50 mm diameter sterile, polystyrene Petri dishes and allowed to harden. Plates were stored at 4^{o}C until use.

In order to perform the various confirmation tests, the following reagents were required: 2% ferrous sulfate solution, canned evaporated milk, Columbia agar base, deionized water, glacial acetic acid, sodium acetate (anyhydrous), 1-naphtyl phosphate disodium salt, and Fast Blue B Salt. Columbia agar plates were prepared by adding 4.25 grams Columbia agar base per 100 mL deionized water in a 500 mL bottle, autoclaving for 15 minutes, dispensing into sterile polystyrene dishes and allowing agar medium to harden. Iron milk medium for the SF test was prepared by combining asepticall12 oz. of canned evaporated milk, 50 mL of 2% ferrous sulfate solution, and 938 mL of deionized water, mixing and then dispensing into glass culture tubes. Acid phosphatase reagent was prepared as described by Ueno et al. (1970) and adapted by Mead

et al. (1981) by combining 20 mL acetate buffer, 0.4g of 1-naphtyl phosphate disodium salt and 0.8g of Fast Blue B salt in a sterile, plastic 50 mL tube. The acetate buffer was made by combining 200 mL of deionized water, 0.067 mL of glacial acetic acid and 0.068 grams of sodium acetate. Upon combining the reagents for the acid-phosphatase solution, the solution was refrigerated for at least 1 hour. The solution was then centrifuged at 3,500 rpm for 15 minutes at 4°C, and the resulting supernatant can be recovered and stored for later use.

Membrane Filtration Method

C. perfringens spores and total *C. perfringens* (spores plus vegetative cells) were detected in reclaimed waters by standard membrane filter (MF) methods. These methods were originally developed for US EPA by Cabelli and Bisson (1979) and further modified (Armon and Payment, 1988) by changes in the composition of the bacteriological medium, mCp agar. Based on more recent evidence of the inferior performance of the MF method when using mCp medium, two alternative *C. perfringens* MF media, TSB and CP ChromoSelect agar, were evaluated in parallel with modified mCp (Sartory et al., 1998; Manafi and Siegrist, 2011; Manafi et al., 2013). All three agar media were applied simultaneously in MF analysis of samples of reclaimed water and other treated and untreated wastewaters, with a focus on samples having *C. perfringens* concentrations in the range of the treated effluent limits of 5 (as geometric mean) and 25 (as single sample maximum) per 100 mL as well as at higher concentrations for agar media comparisons by statistical analyses. In the MF method a volume of sample is vacuum-filtered through a standard 47 mm diameter, approximately 0.45 µM pore size cellulose ester membrane filter. The membrane filter is placed on the surface of an agar medium for *C. perfringens* (modified mCp, TSC or CP ChromoSelect) in a Petri dish and the dish is then incubated under anaerobic conditions at 44 °C. *C. perfringens* and related sulphite reducing clostridia produce characteristic colonies that are then counted. On TSC and CP ChromoSelect agars, *C. perfringens* colonies are black (although colonies of other colors are scored as positive as well) or green in color, respectively, and can be directly counted. On mCp agar *C. perfringens* colonies become pink after exposure to ammonium hydroxide fumes, which is an added step in the procedure when using this medium. Counted colonies of the distinctive color on their respective agar media are considered total presumptive *C. perfringens* per the volume of water sample analyzed. If the method is used to detect only *C. perfringens* spores, the sample is first heated at temperatures between 60 and 80 °C for 15 minutes prior to filtration in order to kill vegetative bacteria and provide colony counts of only spores. The numbers of *C. perfringens* colonies detected per unit volume of test water or wastewater on the three different agar media were then compared to evaluate their performance and to determine the absolute and relative abundance total *C. perfringens* (unheated samples) and *C. perfringens* spores (pre-heated samples) on each agar medium (mCp, TSC and CP ChromoSelect).

Acid-phosphatase confirmation method

Upon counting of plates for the three test agars, presumptive positive and negative isolated colonies were then used for performing a confirmation test with Acid-phosphatase reagent. The method used was that of Sartory et al. (2006), which was adapted from Ueno et al. (1970) and Mead et al. (1981). Presumptive *C. perfringens* colonies were streaked onto separate Columbia agar medium plates (as many as five presumptive positive and five presumptive negative

colonies per sample type, per agar) with a sterile wooden applicator stick. These plates were then grown overnight anaerobically in a 37°C incubator. On the following day, individual colonies from these plates were then inoculated onto a sterile cotton pad with a sterile wooden applicator stick and a 0.1 mL aliquot of Acid-phosphatase reagent was then pipetted onto the colony. If the mixture became a purple color after about a minute, it was scored confirmed positive by the acid-phosphatase test. If it did not become purple, it was scored confirmed negative by the AP test.

Stormy Fermentation method

During the final 10 sampling periods of the investigation, isolates obtained from the test agar media were further subjected to a secondary confirmation test for Stormy Fermentation in Iron-milk tubes. The method for this was adapted from Abeyta et al. (1985). After the acid-phosphatase tests a second colony from each of the Columbia agar medium plates was inoculated into a glass tube of ~9mL of iron-milk medium that was clearly marked to correspond with the water sample and agar medium from which it originally came as well as its result from the acid-phosphatase test. The inoculated tubes were then incubated for 24 hours in a 44°C incubator and checked for stormy fermentation of the media. Results were recorded accordingly.

4.2.3.2 *Clostridium perfringens* Data Analysis Procedures

The data collected on *C. perfringens* concentration was analyzed using the GraphPad Instat statistical package from GraphPad Software. All data was analyzed using nonparametric tests, including the Friedman one-way analysis of variance test and the Dunn Multiple Comparison Post-Test because the data were not normally distributed. The data from these tests is displayed in Table 16 below. In addition to these statistical tests used to determine if the agar media differed significantly in their ability to detect *C. perfringens* colonies, a cumulative frequency distribution analysis was conducted using Microsoft Excel. Figures were generated by sorting the frequency of occurrence of increments (bins) of *C. perfringens* concentrations per 100 mL on each agar medium as the number of repeated concentrations out of the total multiplied by 100%. Box and whisker plots of *C. perfringens* concentrations per 100 mL were also used to demonstrate the distributions of data and the differences between the three test agar media to detect *C. perfringens*. These graphs were created in JMP Pro 9 (SAS Institute, Cary NC).

For analysis of the confirmation test results, data were analyzed in both Microsoft Excel and GraphPad Instat. In Excel, the data were analyzed to determine the extent to which each C. perfringens confirmation test results agreed with what it was the isolate was considered presumptively (either *C. perfringens* positive of negative) when originally observed and the isolated from its respective agar medium. In GraphPad Instat, the data were further analyzed in a Chi-square test in which 12 different contingency tables were analyzed for Chi-square values. These values were intended to provide information on the extent to which an association is seen between the two *C. perfringens* confirmatory tests. The Chi-square tables listed in Appendix 6.3 display the occurrence of confirmed positives and confirmed negatives for both tests on colony isolates from the last 10 samples analyzed with pasteurization and no pasteurization for each agar medium.

4.2.4 Laboratory Survey

A survey of laboratory capacity and willingness to perform *C. perfringens* analysis as a service was sent to all microbiology laboratories registered with the North Carolina Department of Environment and Natural Resources (NCDENR). Of those laboratories, 44 responded to the survey. Questions asked to these labs were as follows:

Is your lab currently certified for bacterial analysis in drinking water, wastewater, or other waters?

If you are certified for bacterial analysis, what do you analyze and what methods do you use?

Do you or would you consider offering testing for *C. perfringens*? I

If you do, what is pricing structure for the tests be and to what capacity would you be able to perform such tests?

Have you ever used either TSC, mCP, or CP ChromoSelect agar media to test for *C. perfringens*?

Is your laboratory currently certified for coliphage analysis for drinking water, wastewater, or other waters?

If certified for coliphage analysis, what methods do you currently use?

Do you or would you consider offering coliphage analysis by use of the spot plate method or single agar layer method?

If so, what is or would your pricing structure be for such analysis and at what capacity would you be able to perform these tests?

If state certification for coliphage or *C. perfringens* testing in reclaimed water soon became available, would you be interested in obtaining certification?

Do you currently perform any tests for food safety microbiology and which tests do you perform if you do?

Do you currently test Type 1 reclaimed water and are you certified for those methods?

In contacting bulk reclaimed water providers, questions were asked to understand whether they produced or intended to produce type two reclaimed water, how much reclaimed water they currently produced, how they currently go about getting it tested, whether they had heard of other utilities planning to produce type two reclaimed water, who they would send type two reclaimed water to, and what their current pricing structure was.

4.3 Results

4.3.1 Coliphages

Water samples from the five wastewater treatment plants of the study were compared on the basis of coliphage concentrations for: male-specific/F+ coliphages on *E. coli* host Famp, somatic coliphages on *E. coli* host CN13 and 'total coliphages' on *E. coli* hosts CB390, C3322, C3000 and the sum of concentrations on *E. coli* hosts CN13 and Famp; and (2) by coliphage analysis method, either Single Agar Layer or Two-Step Enrichment-Spot plate. SAL analysis resulted in single point estimates of the concentration of coliphages in PFU per 100 milliliters. Data for the enrichment-spot plate method were MPN point estimates and their 95% confidence limits. If results from coliphage analysis of a sample were higher than the detection limit of the enrichment assay (i.e. >370 per 100 mL), then 371 was used as the point estimate concentration for the analysis. Similarly, any estimate of <1 per 100 mL was estimated as zero for this analysis.

Tables 2 and Table 3 present the single agar layer data for both the initial phase of experiments and the full data set, which included data from the initial data set. For the SAL method, results for analysis of reclaimed water samples were recorded as plaque forming units per 100 mL sample. Table 2 presents the coliphage data for "Pre UV" water samples, which are water samples subjected to primary and secondary treatment and sand filtration as a tertiary treatment (see Table 1). These samples provided coliphage concentrations suitable for comparison by statistical analysis. In the second phase of this study, reclaimed water samples additionally treated by disinfection processes and raw sewage samples were also analyzed for coliphages. In all following tables, "reclaimed" refers to a reclaimed water sample for which the type of treatment is shown in Table 1. "Raw Sewage" refers to an influent raw sample that received no treatment, and "Pre UV" again refers to samples subjected to primary, secondary treatment and sand filtration treatment.

Table 2: Coliphage Concentrations Detected by SAL (PFU per 100mL) in Pre UV Samples of Reclaimed Water on Different *E. coli* Hosts

Location	*E. coli* somatic host	*E. coli* male-specific (F+) host	Combined somatic and male-specific/ F+ hosts	"Total" coliphage *E. coli* hosts		
	CN13	Famp	CN13 + Famp	C3000	C3322	CB390
B1	230	61	291	44	13	129
B2	314	41	355	57	0	240
C1	5300	5	5305	11	14	4200
C2	1080	26	1106	27	53	1300
D1	90	8	98	3	5	60
D2	79	3	82	4	6	91
E1	52	2	54	1	5	77
E2	71	1	72	43	10	115
A1	637	0	637	0	2	607
A2	271	0	271	10	0	245
A3	112	0	112	0	17	93

As shown in Table 2, concentrations of somatic coliphages were consistently higher than concentrations of male-specific/F+ coliphages in all of the 11 samples tested. Based on the

concentrations of total coliphages as the sum of coliphage concentrations detected on the individual somatic (CN13) and male-specific/F+ *E. coli* hosts, the only *E. coli* host for simultaneous detection of total coliphages that gave similar concentrations was *E. coli* CB390. The total coliphage hosts *E. coli* C3000 and *E. coli* C3322 generally gave relatively low total coliphage concentrations compared to *E. coli* CB390. Statistical analysis comparing the concentrations of total coliphages as the sum of concentrations detected on *E. coli* CN13 and *E. coli* Famp to the concentrations detected on the total coliphage host *E. coli* CB390 indicated no significant difference by the Kruskal Wallis test ($p > 0.05$ Table 7, below).

Table 3: Coliphage Concentrations Detected by SAL (PFU per 100mL) in all Reclaimed Water and Raw Sewage Samples on Different *E. coli* Hosts

Location	Sample Type	*E. coli* somatic host	*E. coli* male-specific (F+) host	Combined somatic and male-specific/F+ *E. coli* hosts	"Total" coliphage host
		CN13	Famp	CN13 + Famp	CB390
A1	Pre UV	637	0	637	607
A2	Pre UV	271	0	271	245
A3	Pre UV	112	0	112	93
B1	Pre UV	230	61	291	129
B2	Pre UV	314	41	355	240
C1	Pre UV	5300	5	5305	4200
C2	Pre UV	1080	26	1106	1300
D1	Pre UV	90	8	98	60
D2	Pre UV	79	3	82	91
E1	Pre UV	52	2	54	77
E2	Pre UV	71	1	72	115
A1*	Pre UV	1220	5	1225	1270
	Reclaimed	2	7	9	0
A2*	Raw Sewage	9×10^4	8×10^4	1.7×10^5	4.3×10^5
	Pre UV	283	3	286	217
	Reclaimed	6	0	6	0
B1*	Pre UV	211	1	212	183
	Reclaimed	43	1	44	4
B2*	Pre UV	146	10	156	156
	Reclaimed	0	3	3	2
B3*	Raw Sewage	1.19×10^4	2.55×10^4	3.74×10^4	2.16×10^4
	Pre UV	83	17	100	118
	Reclaim	0	0	0	1

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C1*	Raw Sewage	2.6×10^5	6×10^4	3.4×10^5	3.5×10^5
	Pre UV	2	0	2	15
	Reclaimed	0	0	0	7
D1*	Pre UV	54	2	56	22
	Reclaimed	2	0	2	0
D2*	Pre UV	16	0	16	20
	Reclaimed	0	0	0	0
D3*	Raw Sewage	1.1×10^5	1.5×10^5	2.6×10^5	1.5×10^5
	Pre UV	123	7	130	92
	Reclaimed	2	0	2	4
E1*	Pre UV	38	3	40	36
	Reclaimed	2	2	4	13
E2*	Pre UV	258	2	260	263
	Reclaimed	6	2	8	3

* Indicates that sample was collected during second sampling period.

Table 3 presents the data for somatic, male-specific/F+ and total coliphage concentrations in the different wastewater samples analyzed by the SAL method on the different *E. coli* hosts selected from those used in the initial phase 1 studies. The trends observed in the phase 1 studies were observed again. Coliphage concentrations based on the sum of counts detected on *E. coli* hosts CN13 and *E. coli* Famp are similar to the concentration of coliphages detected by the total coliphage host *E. coli* CB390. Statistical analysis comparing the SAL concentrations of total coliphages as the sum of concentrations detected on *E. coli* CN13 and *E. coli* Famp compared to the concentrations detected on the total coliphage host *E. coli* CB390 indicated no significant difference by the Kruskal Wallis test ($p = 0.3126$, Table 9, below). As observed in the samples of phase 1, concentrations of somatic coliphages are generally greater than those of male-specific coliphages in reclaimed water samples. However, the concentrations of both somatic and male-specific/F+ coliphages are similar in raw sewage samples and much higher than their concentrations reclaimed water samples.

For the two step enrichment-spot plate method, 100 mL samples of water were analyzed as triplicate sub-volumes of 0.33 mL, 3 mL and 30 mL (100 mL total), and results were scored as Most Probable Number (MPN) per 100 mL sample, with upper and lower 95 percent confidence limits (c. l.) of coliphage concentrations. Table 4 presents coliphage concentrations in Pre-UV reclaimed water samples analyzed by the enrichment-spot plate method on the different hosts for somatic, male-specific/F+ and total coliphages.

Table 4: Coliphage Concentrations and Their 95% Confidence Limits as Detected by Enrichment-Spot Plate (MPN per 100mL) in Pre UV Samples of Reclaimed Water on Different *E. coli* Hosts

Location	Sample	CN-13	Famp	CN13 + Famp	C3000	C3322	CB390
B1	MPN/100 mL	370	370	740	12	3	370
	Lower 95% c. l.	86	86		3	0.8	86
	Upper 95% c. l.	1,600	1600		44	12	1600
B2	MPN/100 mL	> 370 (371)	150	521	1	6	31
	Lower 95% c. l.	> 86	35		0.2	2	8
	Upper 95% c. l.	> 1,600	680		8	22	130
C1	MPN/100 mL	> 370 (371)	370	741	9	9	80
	Lower 95% c. l.	> 86	86		3	3	22
	Upper 95% c. l.	> 1,600	1,600		33	33	290
C2	MPN/100 mL	> 370 (371)	25	396	6	1	310
	Lower 95% c. l.	> 86	6		2	0.2	77
	Upper 95% c. l.	> 1600	110		23	8	1,300
D1	MPN/100 mL	370	50	420	2	2	72
	Lower 95% c. l.	86	14		0.4	0.6	20
	Upper 95% c. l.	1,600	180		10	11	250
D2	MPN/100 mL	150	80	230	< 1 (0)	1	150
	Lower 95% c. l.	35	22		< 0.2	0.2	35
	Upper 95% c. l.	680	290		< 8.4	8.4	680
E1	MPN/100 mL	80	13	93	1	2	50
	Lower 95% c. l.	22	3		0.2	0.5	14
	Upper 95% c. l.	290	51		8	11	180
E2	MPN/100 mL	150	80	230	8	2	150
	Lower 95% c. l.	35	22		2	0.4	35
	Upper 95% c. l.	680	290		27	10	680
A1	MPN/100 mL	> 370 (371)	> 370 (371)	742	3	31	> 370 (371)
	Lower 95% c. l.	> 80	> 80		0.8	8	> 80
	Upper 95% c. l.	> 1,600	> 1,600		12	130	> 1,600
A2	MPN/100 mL	> 370 (371)	2	743	8	14	> 370 (371)
	Lower 95% c. l.	> 86	0.6		2	4	> 86
	Upper 95% c. l.	> 1,600	11		27	58	> 1,600
A3	MPN/100 mL	80	31	111	14	5	370
	Lower 95% c. l.	22	8		4	1	86
	Upper 95% c. l.	290	130		58	17	1,600

As shown in Table 3 for analysis of pre-UV samples by the enrichment spot plate method, concentrations of somatic coliphages are generally higher greater than concentration of male-

specific/F+ coliphages when detected on their respective *E. coli* hosts, CN13 and Famp, respectively. As previously observed for the SAL method, the enrichment-spot plate concentrations of total coliphages detected as the sum of coliphage concentrations on *E. coli* hosts CN13 for somatics and Famp for male-specifics/F+ were similar to their concentrations detected together on total coliphage host *E. coli* CB390. Total coliphage concentrations detected by enrichment-spot plate on *E. coli* hosts C3000 and *E. coli* C3322 were again much lower than those detected by total coliphage host *E. coli* CB390 or as the sum of their concentrations on their individual somatic and male-specific hosts, *E. coli* CN13 and *E. coli* Famp, respectively. Again, statistical analysis comparing the enrichment-spot plate concentrations of total coliphages as the sum of concentrations detected on *E. coli* CN13 and *E. coli* Famp to the enrichment-spot plate concentrations detected on the total coliphage host *E. coli* CB390 indicated no significant difference by the Kruskal Wallis test ($p > 0.05$, Table 7, below).

Table 5: Coliphage Concentrations Detected by Two Step Enrichment-Spot Plate (PFU per 100 mL) in all Reclaimed Water and Raw Sewage Samples on Different *E. coli* Hosts

Location	Sample Type	Somatic *E.coli* Host: CN-13	Lower 95% c. l. – Upper 95% c.l.	F+ *E. coli* Host: Famp	Lower 95% c. l. – Upper 95% c.l.	CN13 + Famp	"Total" Coliphage Host: CB390	Lower 95% c. l. – Upper 95% c.l.
A1	Pre UV	>370 (371)	86-1,600	>370 (371)	86-1,600	742	>370(371)	86-1,600
A2	Pre UV	>370 (371)	86-1,600	2	0.6-11	743	>370(371)	86-1,600
A3	Pre UV	80	22-290	31	8-130	111	370	86-1,600
B1	Pre UV	370	86-1,600	370	86-1,600	740	370	86-1,600
B2	Pre UV	>370 (371)	86-1,600	150	35-680	521	31	8-130
C1	Pre UV	>370 (371)	86-1,600	370	86-1,600	741	80	22-290
C2	Pre UV	>370 (371)	86-1,600	25	6-110	396	310	77-1,300
D1	Pre UV	370	86-1,600	50	14-180	420	72	20-250
D2	Pre UV	150	35-680	80	22-290	230	0	0-8.4
E1	Pre UV	80	22-290	13	3-51	93	50	14-180
E2	Pre UV	150	35-680	80	22-290	230	150	35-680
A1*	Pre UV	1,500	350-6,800	370	86-1,600	1,870	3,701	860-16,000
	Reclaimed	1	0.12-8.2	1.2	0.17 – 8.4	2.2	3.1	0.76-12
A2*	Raw Sewage	2.3×10^5	7.5×10^4-7.2×10^5	2.3×10^5	7.5×10^4–7.2×10^5	4.6×10^5	5.0×10^5	1.4×10^5-1.80×10^6
	Pre UV	23	7.5-72	23	7.5-72	46	150	35-680
	Reclaimed	0	0-8.4	0	0-8.4	0	0	0-8.4
B1*	Pre UV	150	35-680	0	0-8.4	150	>370(371)	86-1,600

	Reclaimed	0	0-8.4	0	0-8.4	0	0	0-8.4
B2*	Pre UV	80	2-290	0	0-8.4	80	80	22-290
	Reclaimed	0	0-8.4	0	0-8.4	0	0	0-8.4
B3*	Raw Sewage	1.5×10^4	3.5×10^3–6.8×10^4	3.71×10^4	8.6×10^3–1.6×10^5	5.21×10^4	3.71×10^4	8.6×10^3 – 1.6×10^5
	Pre UV	370	86-1,600	>370 (371)	86 – 1,600	741	6.8	2 – 24
	Reclaimed	0	0-8.4	0	0-8.4	0	14	3.5 – 58
C1*	Raw Sewage	1.5×10^5	4.6×10^4-4.7×10^6	2.3×10^5	7.5×10^4 - 7.2×10^6	3.8×10^5	1.5×10^5	4.6×10^4 – 4.7×10^6
	Pre UV	2.7	0.69-11	6	1.9-19	8.7	4.3	
	Reclaimed	0	0-8.4]	1.1	0.16 – 7.6	1.1	2.7	0.69-11
D1*	Pre UV	50	14 – 180	0	0-8.4	50	50	14 – 180
	Reclaimed	0	0-8.4	0	0-8.4	0	0	0-8.4
D2*	Pre UV	50	14 – 180	3.1	0.76 – 12	53.1	150	35 – 680
	Reclaimed	0	0-8.4	0	0-8.4	0	0	0-8.4
D3*	Raw Sewage	7.7×10^4	2.2×10^4–2.7×10^5	3.7×10^6	8.6×10^5–1.6×10^7	3.777×10^6	1.3×10^5	3.5×10^4 – 5.8×10^5
	Pre UV	150	35 – 680	150	35 – 680	300	72	20 -250
	Reclaimed	0	0-8.4	0	0-8.4	0	0	0-8.4
E1*	Pre UV	50	14 – 180	150	35 – 680	200	80	22 – 290
	Reclaimed	1.2	0.17 – 8.4	7.4	2.2 – 27	8.9	7.7	2.2 – 27
E2*	Pre UV	>370(371)	86 – 1,600	80	22 – 290	451	370	86 – 1,600
	Reclaimed	0	0-8.4	0	0-8.4	0	0	0-8.4

*Indicates that sample was collected during second sampling period.

Table 5 presents the full data set for enrichment-spot plate coliphage concentrations per 100 mL in all samples collected and analyzed, including Pre-UV samples, reclaimed water and raw sewage, when assayed on *E. coli* somatic host CN13, male-specific/F+ host Famp, total coliphage host CB390, as well as the sum of the concentrations on somatic host CN13 and male-specific/F+ host Famp. The trends in coliphage concentrations observed in the phase 1 studies were observed again for the full data set that includes raw sewage and reclaimed water samples. Coliphage concentrations based on the sum of counts detected on *E. coli* hosts CN13 and *E. coli* Famp were similar to the concentration of coliphages detected by the total coliphage host *E. coli*

CB390. Again, statistical analysis comparing the enrichment-spot plate concentrations of total coliphages as the sum of concentrations detected on *E. coli* CN13 and *E. coli* Famp to the enrichment-spot plate concentrations detected on the total coliphage host *E. coli* CB390 indicated no significant difference by the Kruskal Wallis test ($p = 0.1865$, Table 9, below). Enrichment-spot plate concentrations of somatic coliphages were often similar those of male-specific coliphages in reclaimed water samples. The concentrations of both somatic and male-specific/F+ coliphages also were similar in raw sewage samples and were much higher than their concentrations reclaimed water samples.

In Figures 1-6 below are geometric mean concentrations and 95% confidence intervals of coliphages detected on the different *E. coli* hosts in the different sample types and by the different method of coliphage analysis, either SAL or two-step enrichment-spot plate.

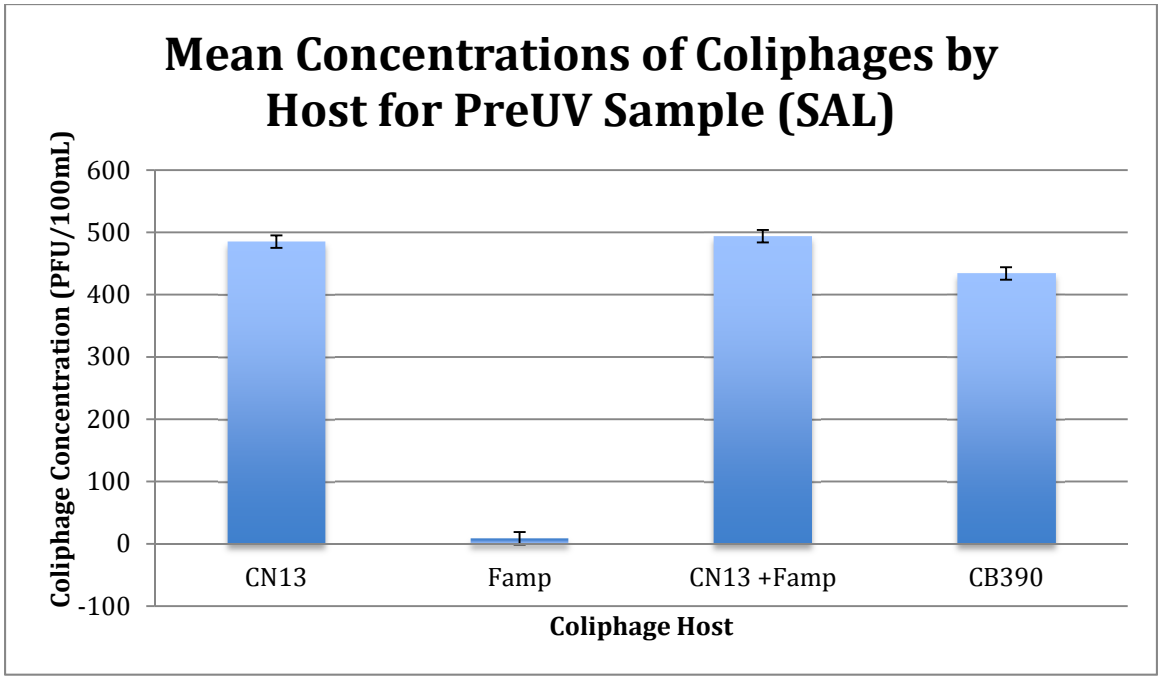

Figure 1: Geometric Mean Concentrations of Coliphages in All 22 Pre UV Reclaimed Water Samples Analyzed by the Single Agar Layer Method on Different *E. coli* Hosts (CN13 = Somatic, Famp = Male-Specific/F+ and CB390 = Total Coliphages).

Figure 1 presents geometric mean SAL concentrations of coliphages per 100 mL for all 22 pre UV samples subjected to primary, secondary and filter treatments, based on data presented in Table 3. It is apparent that somatic coliphages are present at much higher concentrations than male-specific/F+ coliphages. The geometric mean SAL concentration of total coliphages in pre UV reclaimed water samples based on the sum of the concentrations on somatic *E. coli* host CN13 and the male-specific/F+ host *E. coli* Famp is similar to the total coliphage SAL concentration detected on *E. coli* host CB390. However, the geometric mean concentrations are somewhat different, with the CB390 concentration being lower than the summed somatic and male-specific/F+ concentration and their 95% confidence intervals not overlap.

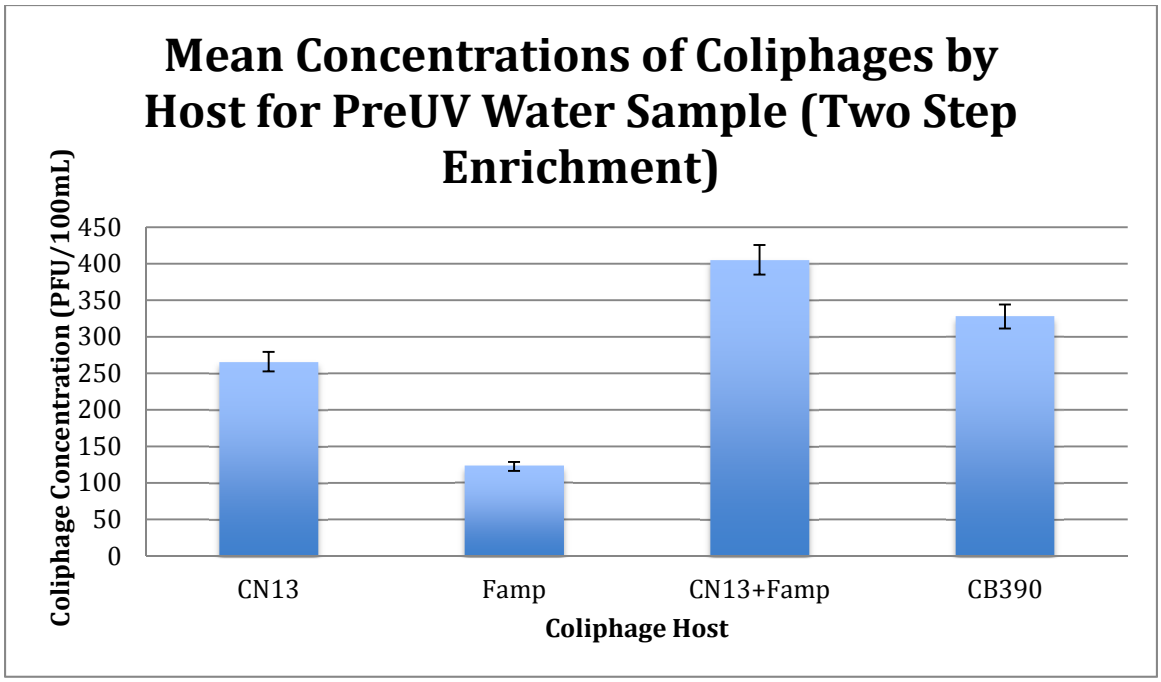

Figure 2: Geometric Mean Concentrations of Coliphages in All 22 Pre UV Reclaimed Water Samples Analyzed by the Two Step Enrichment-Spot Plate Method on Different *E. coli* Hosts (CN13 = Somatic, Famp = Male-specific/F+ and CB390 = Total Coliphages).

Figure 2 presents geometric mean enrichment-spot plate concentrations of coliphages per 100 mL for all 22 pre UV samples subjected to primary, secondary and filter treatments, based on data presented in Table 5. It is apparent that somatic coliphages are present at higher concentrations than male-specific/F+ coliphages. The geometric mean enrichment-spot plate concentration of total coliphages in pre UV reclaimed water samples based on the sum of the concentrations on somatic *E. coli* host CN13 and the male-specific/F+ host *E. coli* Famp is similar to the total coliphage enrichment-spot plate concentration detected on *E. coli* host CB390. However, the geometric mean concentrations are somewhat different, with the CB390 concentration being lower than the summed somatic and male-specific/F+ concentrations and their 95% confidence intervals do not overlap.

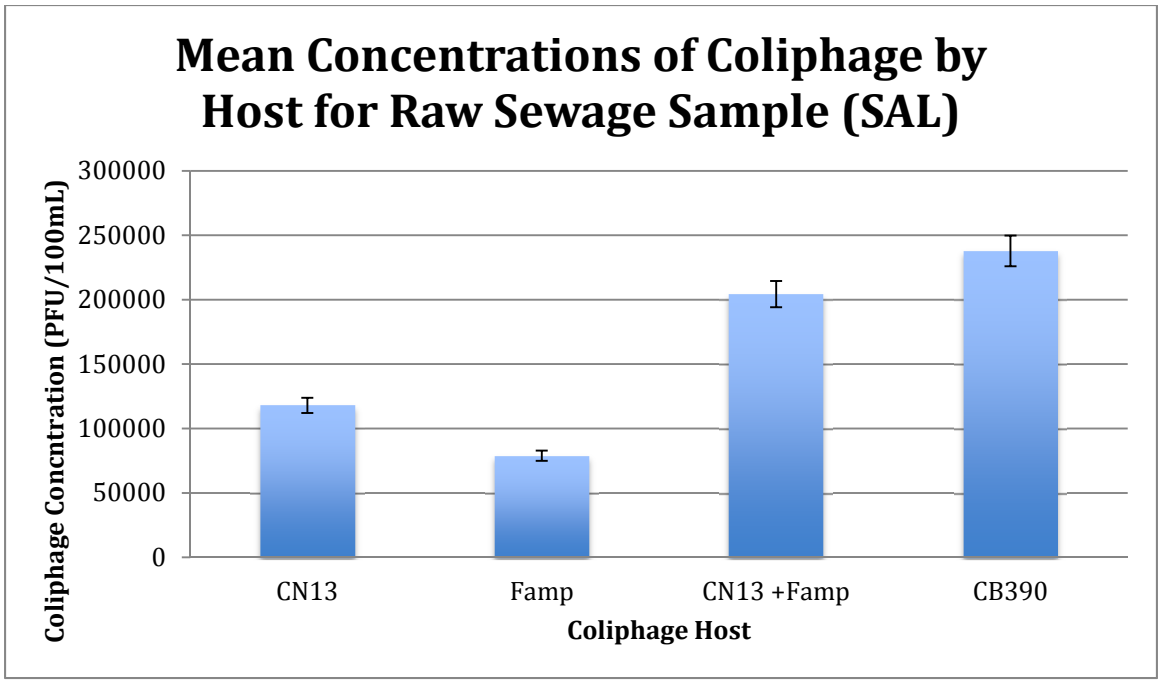

Figure 3: Geometric Mean Concentrations of Coliphages in All 4 Raw Sewage Samples Analyzed by the Single Agar Layer Method on Different *E. coli* Hosts (CN13 = Somatic, Famp = Male-Specific/F+ and CB390 = Total Coliphages).

Figure 3 presents geometric mean SAL concentrations of coliphages per 100 mL for all 4 raw sewage samples analyzed, based on data presented in Table 3. Somatic coliphages are present at somewhat higher concentrations than male-specific/F+ coliphages in raw sewage. The concentrations of somatic and male-specific/F+ coliphages are within the same order of magnitude in concentration, but their 95% confidence intervals do not overlap. The geometric mean SAL concentration of total coliphages in raw sewage samples based on the sums of the concentrations on somatic *E. coli* host CN13 and the male-specific/F+ host *E. coli* Famp is similar to the total coliphage SAL concentration detected on *E. coli* host CB390. However, the geometric mean concentrations are somewhat different, with the CB390 concentration being higher than the summed somatic and male-specific/F+ concentration and their 95% confidence intervals do not overlap.

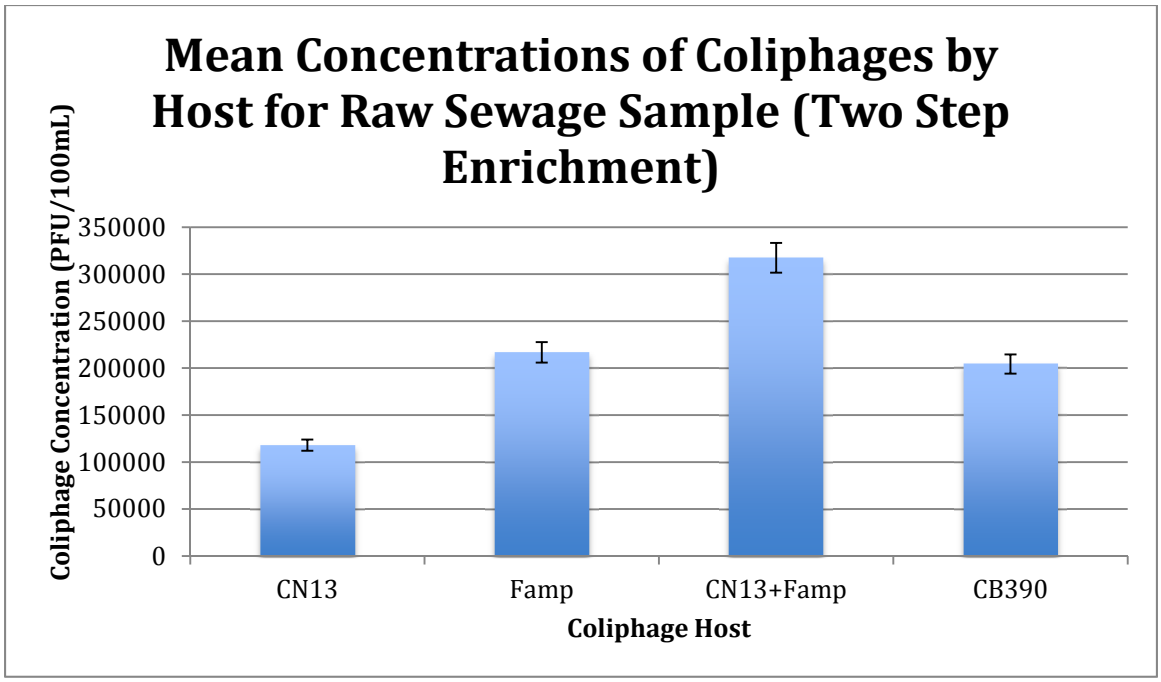

Figure 4: Geometric Mean Concentrations of Coliphages in All 4 Raw Sewage Samples Analyzed by the Enrichment-Spot Plate Method on Different *E. coli* Hosts (CN13 = Somatic, Famp = Male-Specific/F+ and CB390 = Total Coliphages).

Figure 4 presents geometric mean SAL concentrations of coliphages per 100 mL for all 4 raw sewage samples analyzed, based on data presented in Table 5. Somatic coliphages are present at somewhat lower concentrations than male-specific/F+ coliphages in raw sewage. The concentrations of somatic and male-specific/F+ coliphages are within the same order of magnitude in concentration, but their 95% confidence intervals do not overlap. The geometric mean enrichment-spot plate concentration of total coliphages in raw sewage samples based on the sums of the concentrations on somatic *E. coli* host CN13 and the male-specific/F+ host *E. coli* Famp is similar to the total coliphage SAL concentration detected on *E. coli* host CB390. However, the geometric mean concentrations are somewhat different, with the CB390 concentration being lower than the summed somatic and male-specific/F+ concentrations and their 95% confidence intervals do not overlap.

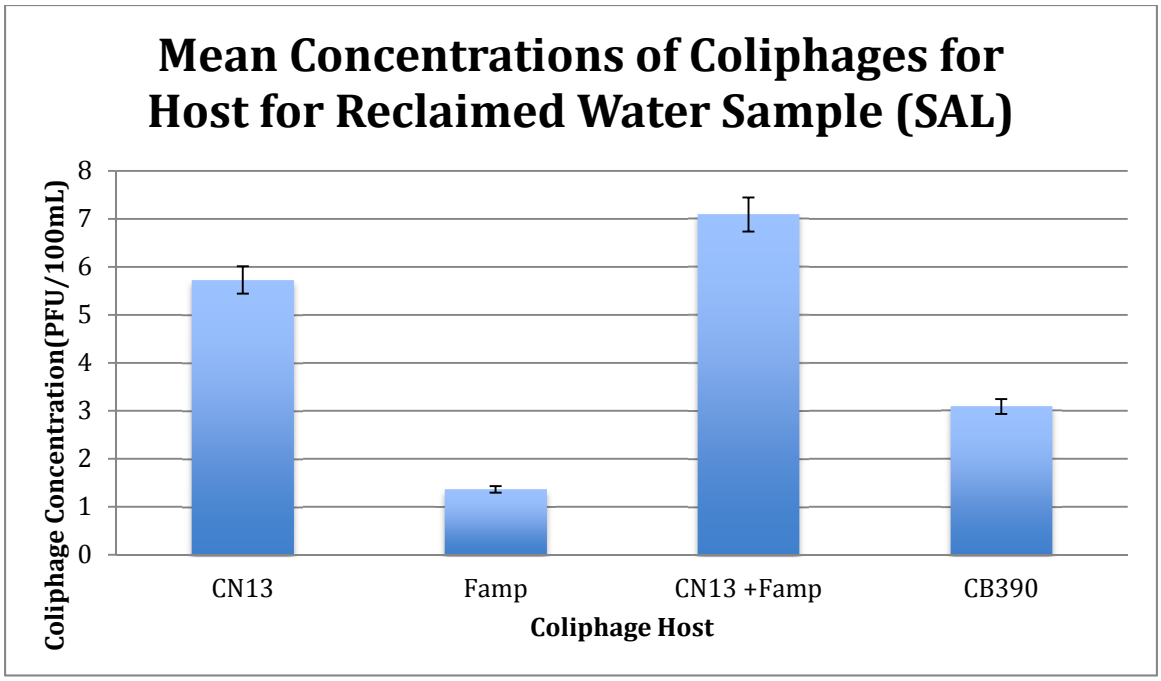

Figure 5: Geometric Mean Concentrations of Coliphages in All 11 Reclaimed Water Samples Analyzed by the Single Agar Layer Method on Different *E. coli* Hosts (CN13 = Somatic, Famp = Male-Specific/F+ and CB390 = Total Coliphages).

Figure 5 presents geometric mean SAL concentrations of coliphages per 100 mL for all 11 reclaimed water samples analyzed, based on data presented in Table 3. Somatic coliphages are present at somewhat higher concentrations than male-specific/F+ coliphages in raw sewage. The concentrations of somatic and male-specific/F+ coliphages are within the same order of magnitude in concentration, but their 95% confidence intervals do not overlap. The geometric mean SAL concentration of total coliphages in reclaimed water samples based on the sums of the concentrations on somatic host *E. coli* CN13 and the male-specific/F+ host *E. coli* Famp is similar to the total coliphage SAL concentration detected on *E. coli* host CB390. However, the geometric mean concentrations of total coliphages are somewhat different, with the CB390 concentration being lower than the summed somatic and male-specific/F+ concentrations and their 95% confidence intervals do not overlap.

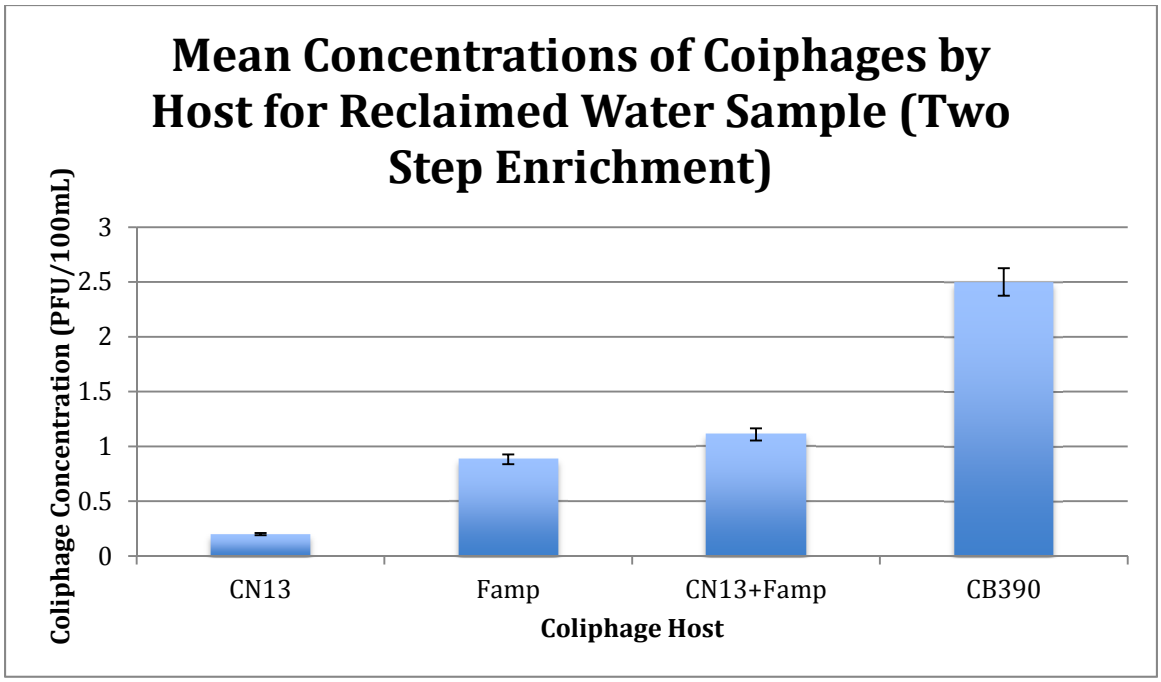

Figure 6: Geometric Mean Concentrations of Coliphages in All 11 Reclaimed Water Samples Analyzed by the Enrichment-Spot Plate Method on Different *E. coli* Hosts (CN13 = Somatic, Famp = Male-Specific/F+ and CB390 = Total Coliphages).

Figure 6 presents geometric mean SAL concentrations of coliphages per 100 mL for all 11 reclaimed water samples analyzed, based on data presented in Table 5. Somatic coliphages are present at somewhat lower concentrations than male-specific/F+ coliphages in reclaimed water. The concentrations of somatic and male-specific/F+ coliphages are within the same order of magnitude in concentration, but their 95% confidence intervals do not overlap. The geometric mean enrichment-spot plate concentration of total coliphages in reclaimed water samples based on the sums of the concentrations on somatic *E. coli* host CN13 and the male-specific/F+ host *E. coli* Famp is similar to the total coliphage SAL concentration detected on *E. coli* host CB390. However, the geometric mean concentrations are somewhat different, with the CB390 concentration being somewhat higher than the summed somatic and male-specific/F+ concentrations and their 95% confidence intervals do not overlap.

In order to further examine these data for coliphage concentrations in raw and treated wastewater samples by the two assay methods, SAL and two step enrichment-spot plate, each data set, the initial one from phase 1 in 2013 and then all of the data combined for phase 1 in 2013 plus phase 2 in 2014 were subjected to a Kruskal-Wallis (nonparametric) ANOVA Test. A nonparametric test was used for the initial phase 1 data set because the sample size was small (n=11), and the data did not follow a normal distribution. For detection of total coliphages by both the single agar layer (SAL) method and the enrichment-spot plate method, there were no significant differences between the sum of CN13 and Famp versus CB390, or between C3000 versus C33220. However, there were significant differences between CN13 + Famp versus C3000, CN13 + Famp versus C3322, CB390 versus C3000, and CB390 versus C3322. This information is summarized in Table 6. These results indicate that *E. coli* hosts C3000 and 3322 detect significantly fewer total coliphages than does *E. coli* host CB390 or than detected as the sum of

coliphages detected by somatic host *E. coli* CN13 and male-specific/F+ *E. coli* host Famp.

Table 6: Kruskal Wallis Comparisons of *E. coli* Hosts and Assay Methods for Detection of Total Coliphages in 11 Pre-UV Reclaimed Water Samples

E. coli Host 1	*E. coli* Host 2	Laboratory method	K-W p-value
CN13+Famp	CB390	Single Agar Layer	>0.05
CN13+Famp	CB390	Two-Step Enrichment	>0.05
CN13+Famp	C3000	Single Agar Layer	<0.001
CN13+Famp	C3000	Two-Step Enrichment	<0.001
CN13+Famp	C3322	Single Agar Layer	<0.001
CN13+Famp	C3322	Two-Step Enrichment	<0.001
CB390	C3322	Single Agar Layer	<0.001
CB390	C3322	Two-Step Enrichment	<0.01
CB390	C3000	Single Agar Layer	<0.001
CB390	C3000	Two-Step Enrichment	<0.01
C3322	C3000	Single Agar Layer	>0.05
C3322	C3000	Two-Step Enrichment	>0.05

In addition, the concentrations of coliphages detected in 11 Pre-UV reclaimed water samples by SAL and enrichment-spot pate methods were compared for each *E. coli* host by the non-parametric Mann-Whitney test. For CN13, CB390, C3000, C3322, and CN13+Famp, there were no significant differences between the median coliphage concentrations detected by SAL versus enrichment-spot plate; these p-values are summarized in Table 7. However, there was a significant difference between the medians of the SAL and enrichment-spot plate concentrations of male-specific/F+ coliphages detected on *E. coli* Famp, with a p-value of 0.0035. This significant p-value may be a result of the significant number of zero values or it may be associated with the small sample size, n=11, or both. With the increased sample size in the second phase, this analysis will be performed a second time.

Table 7: Mann-Whitney Comparison of SAL to Enrichment-Spot Plate for Coliphage Detection in Pre-UV Reclaimed Water Samples by each *E. coli* Host

E. coli Host	M-W p-value between SAL and enrichment methods
CN13	0.4779
Famp	0.0035
CN13 + Famp	0.3000
CB390	0.7477
C3322	0.4496
C3000	0.3750

Based on the p-values of Table 7 there were no significant differences in coliphage concentrations detected by either the SAL or enrichment spot plate methods as detected on *E. coli* host CN13 for somatic coliphages, *E. coli* host CB390 for total coliphages, *E. coli* C3322 for total coliphages, *E. coli* C3000 for total coliphages, or for the sum of coliphage concentrations detected on *E. coli* CN13 for somatic coliphages and *E. coli* Famp for male-specific/F+ coliphages. However, were differences in male-specific/F+ coliphage detection between SAL

and enrichment-spot plate methods on *E. coli* Famp host. With the exception of the *E. coli* Famp host for male-specific/F+ coliphage detection, there were no significant differences in coliphage detection in Pre-UV reclaimed water between the results of the SAL and enrichment-spot plate methods for the *E. coli* hosts that detect somatic and total coliphages.

With the additional data collected in 2014, as noted by the asterisks in Table 3 and Table 5, additional tests were carried out on the complete data set. Based on the results of the phase 1 study with pre-UV reclaimed water only the total coliphage host *E. coli* CB390, which gave similar total coliphage concentrations to the sum of the male-specific/F+ and somatic coliphages detected on their individual *E. coli* hosts, continued to be evaluated by both the SAL and two-step enrichment-spot plate methods in 2014. The other total coliphage hosts, *E. coli* C3322 and *E. coli* C3000, were excluded as a result of poor performance compared to the combined somatic and male- specific/F+ data. The continued testing with a greater variety of samples provided a bigger data set to evaluate the performance of the total coliphage host *E. coli* CB390, compared to the sum of the coliphages detected by the individual somatic and male-specific/F+ hosts.

The full data set for coliphage analysis on the different *E. coli* hosts was subjected to initial cumulative frequency distribution analyses, as presented in the Figures 7 and 8 for the SAL and enrichment-spot plate methods, respectively. This analysis graphically presents the cumulative frequency distributions for a visual comparison of the detection of coliphage concentrations on the different *E. coli* hosts, including the sum of total coliphage concentrations as detected on hosts *E. coli* CN13 for somatic coliphages and *E. coli* Famp for male-specific/F+ coliphages, compared to the simultaneous detection of total coliphages on *E. coli* CB390. As evident in Figures 13 and 14 below, the general trends in coliphage detection frequency are very similar among the different *E. coli* hosts. Cumulative frequency distributions for coliphages detected on *E. coli* hosts CN13 for somatic coliphages, Famp for male-specific/F+ coliphages, CB390 for total coliphages and the sum of total coliphages detected on CN13 for somatic and Famp for male-specific/F+ coliphages are described reasonably well by linear relationships, with R^2 values of 0.85-0.89. The only exception is the detection of male-specific/F+ coliphages on *E. coli* Famp by the SAL method, for which the R^2 value is only 0.54, due to a rapid initial increase in coliphage concentration followed little further increase in coliphage concentrations.

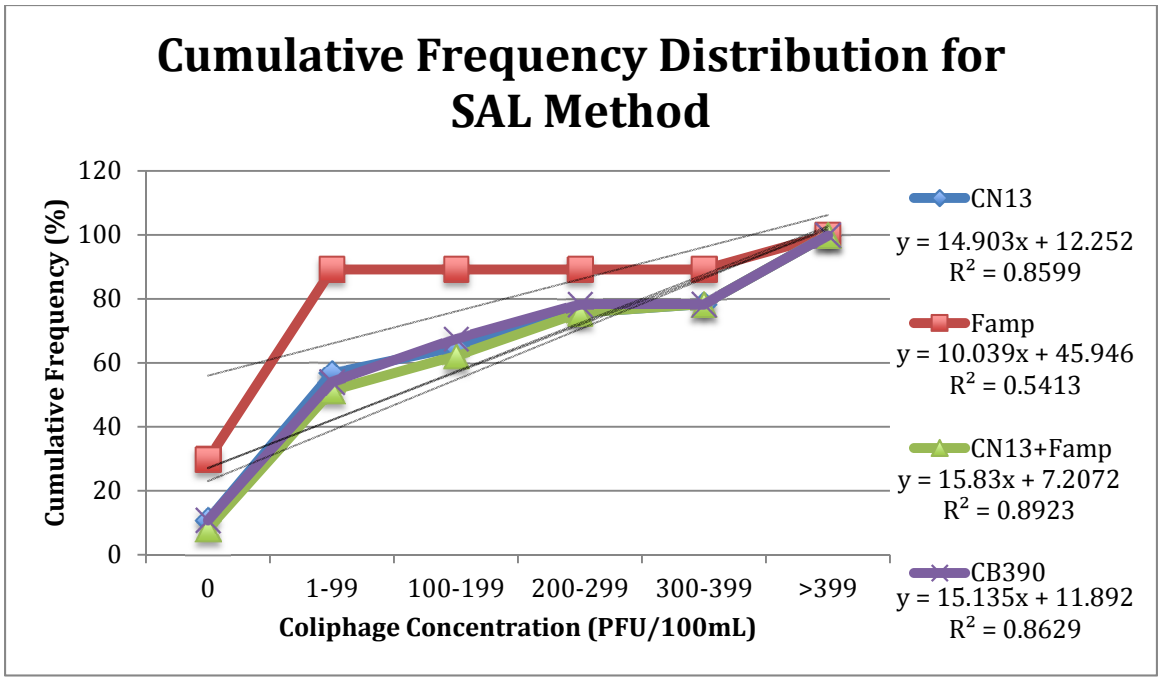

Figure 7: Cumulative Frequency Distributions of Coliphage Concentrations on Different *E. coli* Hosts by the Single Agar Layer Method.

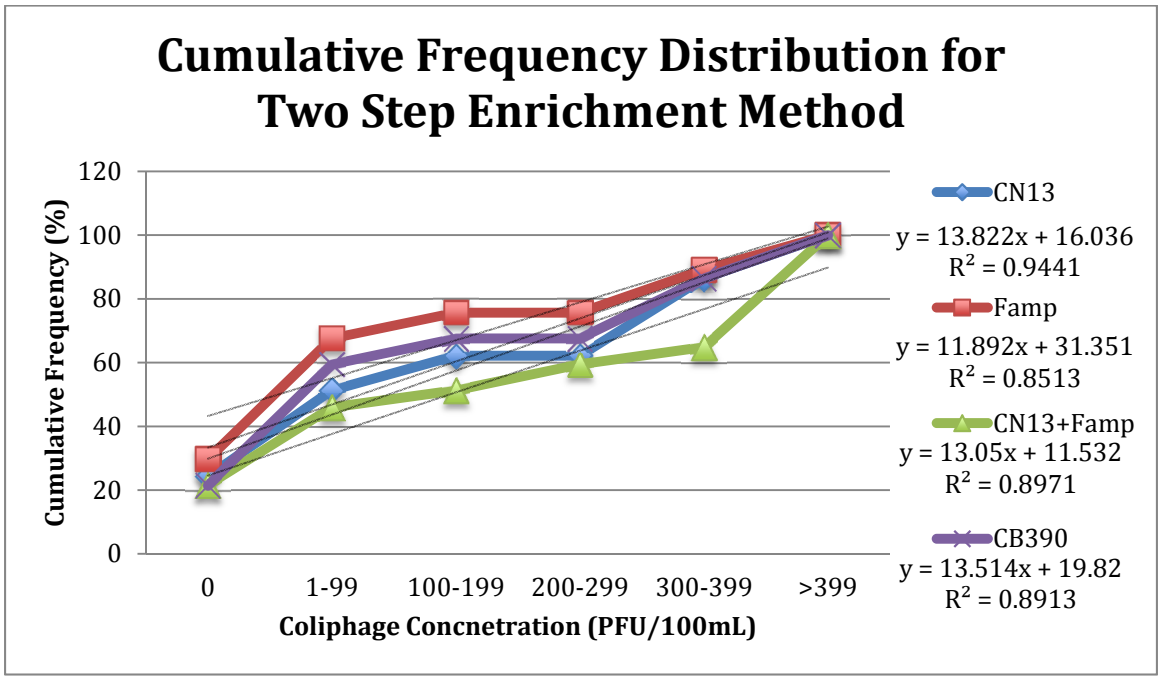

Figure 8: Cumulative Frequency Distributions of Coliphage Concentrations on Different *E. coli* Hosts for the Two Step Enrichment Method.

The full data set for all 37 wastewater and reclaimed water samples was analyzed to determine if the simultaneous detection of total coliphages on the single *E. coli* host CB390 was comparable to the detection of total coliphages as the sum of their detection on the individual hosts, *E. coli*

CN13 for somatic coliphages and *E. coli* Famp for male-specific/F+ coliphages. As shown in Table 8 there was no difference in total coliphage detection by these two approaches for either the SAL methods or the enrichment-spot plate method, with Kruskal-Wallis P-values of 0.3126 and 0.1865, respectively.

Table 8: Comparison of Total Coliphage Concentrations Detected on *E. coli* Host CB390 compared to Sum of Coliphages Detected on Somatic Host *E. coli* CN13 Plus Male-Specific/F+ Host *E. coli* Famp by the Kruskal Wallis Test

E. coli Host 1	*E. coli* Host 2	Laboratory Method	K-W P-value
CN13 + Famp	CB390	Single Agar Layer	0.3126
CN13 + Famp	CB390	Two-Step Enrichment	0.1865

The detection of coliphages by the SAL test compared to the enrichment-spot plate test on each separate *E. coli* host and for total coliphages detected as the sum of coliphage concentrations on *E. coli* hosts CN13 and Famp was analyzed for paired data on coliphage concentrations in all 37 samples. As shown by the results summarized in Table 9 for Mann-Whitney tests, there were no significant differences in coliphage detection by SAL and enrichment-spot plate on any *E. coli* hosts, with P-values of >0.5 for all comparisons.

Table 9: Comparison of Coliphage Concentrations Detected by SAL compared to Enrichment-Spot Plate on Different *E. coli* Hosts by Mann Whitney Tests

E. coli Host	M-W p-value
CN13	0.1529
Famp	0.0934
CN13 + Famp	0.3010
CB390	0.2944

The results presented in Table 9 indicate that for each host, *E. coli* CN13, *E. coli* Famp, *E. coli* CN13+ *E. coli* Famp, and *E. coli* CB390, there is no statistically significant difference between the values obtained in the SAL or the two-step enrichment-spot plate method. This finding is consistent with what was found in the initial data analysis for a smaller number of Pre-UV reclaimed water samples (Table 7), with the exception of the *E. coli* Famp host. In Table 7 the *E. coli* Famp did show a statistically significant difference between the two methods, but in the full data set this same trend is not evident. This is likely a result of the increased sample size and the variety of samples collected, as both of these factors allowed for fewer '0' values for this host, providing a more robust analysis.

From the data analysis presented above, it appears that there is no significant difference in coliphage detection between the two methods examined, SAL and enrichment-spot plate, or in total coliphage detection between total coliphage host *E. coli* CB390 and the sum of somatic host *E. coli* CN13 + male-specific/F+ host *E. coli* Famp. As there was no significant difference between the sum of *E. coli* CN13 and *E. coli* Famp when compared to *E. coli* CB390, for either the SAL or enrichment-spot plate method, a Kruskal Wallis *E. coli* host comparison was performed on data from both the SAL and two-step enrichment analysis to compare the *E. coli* CN13+ *E. coli* Famp (for SAL and two-step enrichment methods) to *E. coli* CB390 (for SAL and two-step enrichment methods) with a larger sample size of n=74. This analysis resulted in a p-

value of 0.1657, indicating no significant difference in coliphage detection between *E. coli* CB390 for total coliphages and the sum of *E. coli* CN13 for somatic coliphages and *E. coli* Famp for male-specific/F+ coliphages.

4.3.2 *Clostridium perfringens*

Reclaimed water and wastewater samples from the five wastewater treatment plants indicated previously were also compared on the basis of *C. perfringens* detected as spores only or spores plus vegetative cells. Using aliquots of 1 mL, 5 mL, or 10 mL per sample, depending on the expected contamination level, the various samples were vacuum filtered through a 0.45 μm pore size, 47 mm diameter cellulose membrane filters. Each sample volume was plated in triplicate. Reclaimed water and raw sewage samples were filter for only one volume, while pre-sand filtered samples were filtered for either two or three different volumes. If colony count results from a given membrane filter plate were above the detectable limit, they were deemed too numerous to count and a value of 225 colonies was used in its place. This colony count value was derived by multiplying two by the average of the upper detectable counting limit from plates in which one hundred or more colonies were counted. If a set of triplicate membrane plates for a raw sewage sample plated on an individual agar medium experienced no growth, a value of 0.5 colonies was assigned to one of the plates. Because raw sewage samples had to be diluted to display countable colonies, a plate detecting no *C. perfringens* would have underrepresented the ability of an agar medium to culture *C. perfringens* in a sewage sample, where it is expected that bacteria will always be present. Thus, the value of 0.5 was assigned to provide a conservative estimate of how many *C. perfringens* colonies would be detected by the agar medium in an undiluted sample.

Tables 10 and 11 below present the data for total *C. perfringens* detected from each of the agar media for all samples, both unpasteurized and pasteurized. The data were converted from their original aggregate colony counts to CFU/100 mL. This was done to provide uniformity in the results and allow for interpretation in the context of various standards or other targets as limits for *C. perfringens* per CFU/100mL. In all following tables, “reclaimed” refers to a reclaimed water sample, with the amount of treatment at a wastewater treatment plant shown in Table 1. “Raw Sewage” refers to an influent sample that has received no treatment at the wastewater treatment plant, and “Pre-sand filter” continues to refer to samples that have not yet gone through the sand filtration treatment step.

Table 10: *C. perfringens* Concentrations (CFU/100mL) on All Agar Media Tested for Unpasteurized Waste Water Samples

Sample Site	Sample Type	TSC	CS	mCp
A	Pre-sand filter	264	367	67
A	Pre-sand filter	615	458	67
A	Pre-sand filter	221	339	21
A	Pre-sand filter	264	367	67
A	Pre-sand filter	615	458	67
B	Pre-sand filter	521	824	439
B	Pre-sand filter	182	864	273
C	Pre-sand filter	285	900	855
C	Pre-sand filter	418	>2203*	561
D	Pre-sand filter	221	348	100
D	Pre-sand filter	473	>1961*	691
E	Pre-sand filter	261	636	39
E	Pre-sand filter	358	730	288
A	Pre-sand filter	539	579	185
A	Pre-sand filter	6	836	400
B	Pre-sand filter	279	>2264*	842
D	Pre-sand filter	144	161	11
C	Pre-sand filter	367	>1988*	375
A	Pre-sand filter	215	458	148
B	Pre-sand filter	360	673	258
D	Pre-sand filter	156	227	52
E	Pre-sand filter	198	598	115
C	Pre-sand filter	303	>2285*	718
B	Pre-sand filter	427	1033	500
D	Pre-sand filter	321	333	94
D	Raw Sewage	100000	133333	<16667**
E	Raw Sewage	133333	166667	<16667**
C	Raw Sewage	143333	173333	30000
B	Raw Sewage	53333	80000	26667
D	Raw Sewage	56667	60000	10000
A	Reclaimed	0	0	0
B	Reclaimed	0	0	0
D	Reclaimed	0	0	0
C	Reclaimed	40	20	3
A	Reclaimed	0	0	0
B	Reclaimed	0	0	0
D	Reclaimed	0	0	0
E	Reclaimed	0	0	0
C	Reclaimed	70	40	10
B	Reclaimed	0	0	0
D	Reclaimed	0	0	0

*denotes that one or more plates was TNTC and a value of 225 was assigned to calculate CFU/100mL. A value of 225 is two times the highest countable limit for any of the agar media

** denotes that no colonies grew on a sewage sample. Because sewage samples were diluted several 10-fold, a value of 0.5 was used as a lower detection limit to calculate CFU/100 mL of any agar medium that detected 0 colonies for a sewage sample.

Table 11: *C. perfringens* Concentrations (CFU/100mL) on All Agar Media Tested for Pasteurized Waste Water Samples

Sample Site	Sample Type	TSCΔ	CSΔ	mCpΔ
A	Pre-sand	121	221	15
A	Pre-sand	312	267	9
A	Pre-sand	124	106	3
A	Pre-sand	121	221	15
A	Pre-sand	312	267	9
B	Pre-sand	452	521	48
B	Pre-sand	197	515	24
C	Pre-sand	391	515	85
C	Pre-sand	406	>1464*	79
D	Pre-sand	61	155	21
D	Pre-sand	406	467	206
E	Pre-sand	233	188	21
E	Pre-sand	418	497	27
A	Pre-sand	288	158	9
A	Pre-sand	2	994	24
B	Pre-sand	179	>1182*	24
D	Pre-sand	117	133	3
C	Pre-sand	313	>1535*	56
A	Pre-sand	165	198	4
B	Pre-sand	288	413	150
D	Pre-sand	50	42	1
E	Pre-sand	77	125	2
C	Pre-sand	391	>1318*	33
B	Pre-sand	418	688	27
D	Pre-sand	200	148	2
D	Raw Sewage	<16667**	66667	<16667**
E	Raw Sewage	66667	<16667**	<16667**
C	Raw Sewage	103333	100000	<1667**
B	Raw Sewage	66667	43333	<1667**
D	Raw Sewage	36667	<1667**	<1667**
A	Reclaimed	0	3	0
B	Reclaimed	0	0	0
D	Reclaimed	0	0	0
C	Reclaimed	70	17	3
A	Reclaimed	0	0	0

B	Reclaimed	0	0	0
D	Reclaimed	0	0	0
E	Reclaimed	0	0	0
C	Reclaimed	83	10	3
B	Reclaimed	0	0	0
D	Reclaimed	0	0	0

*denotes one or more plates was TNTC and a value of 225 was used to calculate CFU/100mL. The value 225 is two times the highest detectable limit for any of the agar media

** denotes that no colonies grew on a sewage sample. Because sewage samples were diluted several 10-fold, a value of 0.5 was used as a lower detection limit to calculate CFU/100mL of any agar medium that detected 0 colonies for a sewage sample.

Presumptive positive and negative *C. perfringens* colony isolates for pre-sand filtered samples were collected from each of the test agar media for both pasteurized and unpasteurized samples. The data collected from these tests is presented in Tables 12-14 below. An acid-phosphatase (AP) test was performed on colony isolates from every pre-sand filtered sample and the Stormy Fermentation (SF) test was performed on colony isolates from the last 10 pre-sand filtered samples. The tables present how many total isolated colonies, presumed positive, tested positive for each test and how many total isolated colonies, presumed negative, tested negative for each test. The decimal values of presumptive colonies that were positive by confirmatory test results are also presented in these tables. The tables show the separate data from both pasteurized and unpasteurized samples, and then followed by each one individually.

Table 12: Combined Data for Acid-phosphatase (AP) and Stormy Fermentation (SF) Confirmatory Tests of Presumptive Positive and Negative *C. perfringens* Colonies from Unpasteurized and Pasteurized Pre-sand Filtered Water Samples

Agar (presumptive positive or negative colony)	# presump-tive colonies (AP test)	# Confirmed colonies (AP test)	Agree-ment (AP test)	# presumptive colonies (SF Test	# Confirm-ed colonies (SF test)	Agree-ment (AP test)
TSC (positive)	242	175	0.72	100	91	0.91
TSC (negative)	249	107	0.43	100	35	0.35
ChromoSelect (positive)	248	205	0.82	100	94	0.94
ChromoSelect (negative)	219	200	0.91	89	69	0.78
mCp (positive)	215	166	0.77	75	62	0.83
mCp (negative)	207	204	0.99	83	82	0.99

Table 13: Combined Data for Acid-phosphatase (AP) and Stormy Fermentation (SF) Confirmatory Tests of Presumptive Positive and Negative *C. perfringens* Colonies from Unpasteurized Pre-sand Filtered Water Samples

Agar (presumptive positive or negative)	# presumptive colonies (AP test)	# Confirmed colonies (AP test)	% Agreement (AP test)	# presumptive colonies (SF Test	# Confirmed colonies (SF test)	% Agreement (AP test)
TSC (positive)	122	90	.74	50	46	.92
TSC (negative)	125	55	.44	50	16	.32
ChromoSelect (positive)	125	109	.87	50	46	.91
ChromoSelect (negative)	108	100	.93	47	38	.81
mCp (positive)	122	101	.83	47	41	.87
mCp (negative)	111	109	.98	48	47	.98

Table 14: Combined Data for Acid-phosphatase (AP) and Stormy Fermentation (SF) Confirmatory Tests of Presumptive Positive and Negative *C. perfringens* Colonies for Pasteurized Pre-sand Filtered Water Samples

Agar (presumptive positive or negative)	# presumptive colonies (AP test)	# Confirm-ed colonies (AP test)	% Agreement (AP test)	# presumptive colonies (SF Test)	# Confirm-ed colonies (SF test)	% Agree-ment (AP test)
TSC (positive)	120	85	0.71	50	45	0.90
TSC (negative)	124	52	0.42	50	19	0.38
ChromoSelect (positive)	123	96	0.78	50	48	0.96
ChromoSelect (negative)	111	100	0.90	42	31	0.74
mCp (positive)	93	65	0.70	28	21	0.75
mCp (negative)	96	95	0.99	35	35	1.0

Figures 9 through 12 below display box and whisker plots of the distributions of *C. perfringens* concentrations as corrected and uncorrected values for both pasteurized and unpasteurized pre-sand filtered samples. Each box plot per figure is derived from the data collected from 25 pre-

sand filtered samples and the total detection of *C. perfringens* on a test agar medium for a sampling period. Features of the data represented in the box and whisker plots include an upper 95% confidence interval, an upper quartile, a median value of the data, a lower quartile, a lower 95% confidence interval, and outlier points; these values are represented by the top line, second line from the top, middle line, second line from the bottom, bottom line, and other points removed from the box plot, respectively. For each of these graphs, the upper end of the 95% confidence interval for the CP ChromoSelect agar medium lies outside of the confidence intervals for the other two agar media and its median value is higher than the other two agar media. The top line and bottom line of the body of each box represents the value of the upper and lower quartiles of all samples included. The upper and lower quartiles for the CP Chromoselect agar are highest of the three agar media. These box-and-whisker figures suggest that the CP ChromoSelect agar medium may be capable of detecting higher numbers of *C. perfringens* colonies than the other two agar media that were evaluated and compared.

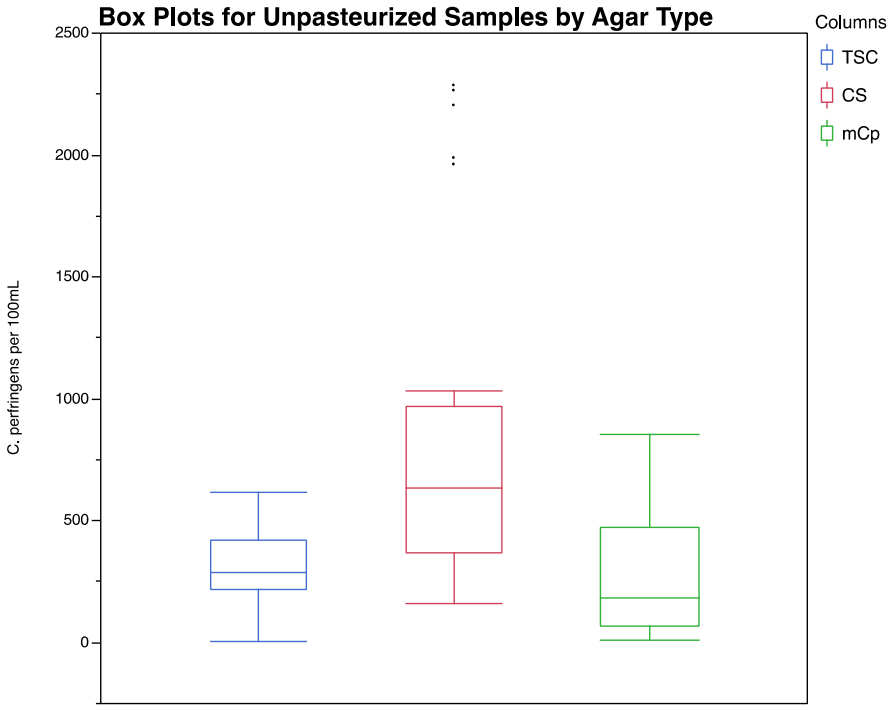

Figure 9: Box and Whisker Plot for Distribution of *C. perfringens* Detection in Unpasteurized Pre-sand Filtered Water Samples.

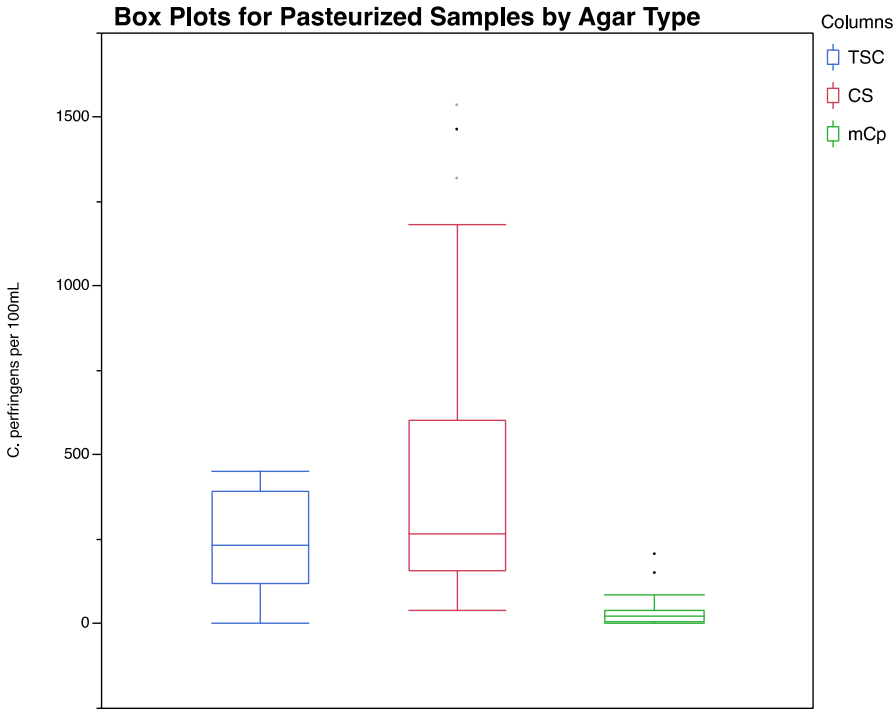

Figure 10: Box and Whisker Plot for Distribution of *C. perfringens* Detection in Pasteurized Pre-sand Filtered Water Samples.

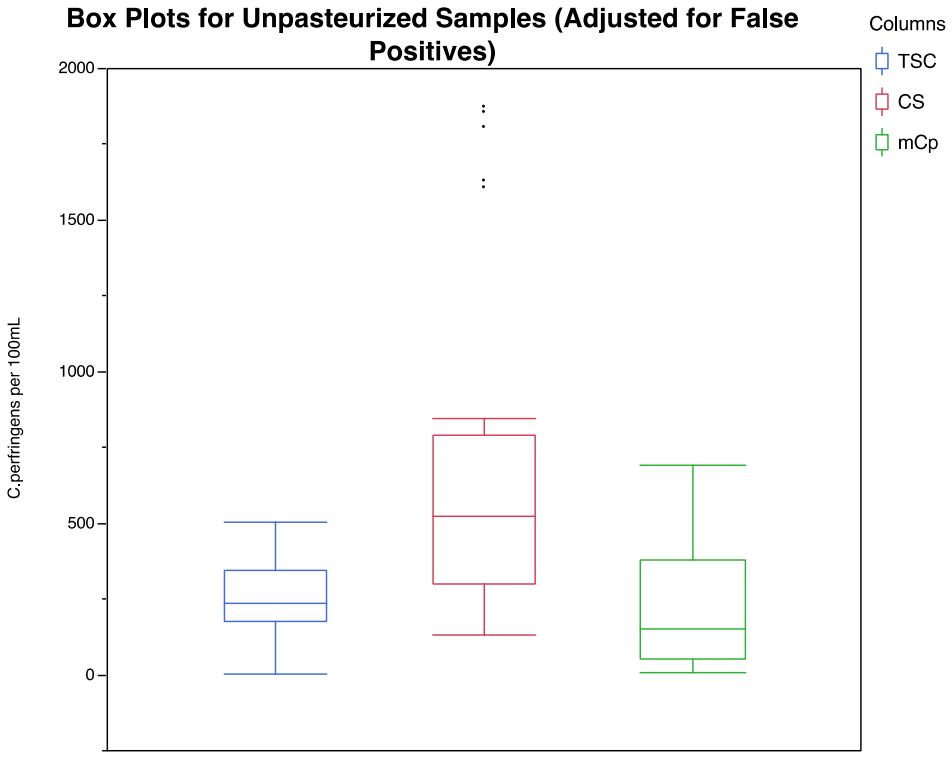

Figure 11: Box and Whisker Plot for Distribution of *C. perfringens* Detection in Unpasteurized

Pre-sand Filtered Water Samples Adjusted for False-positivity.

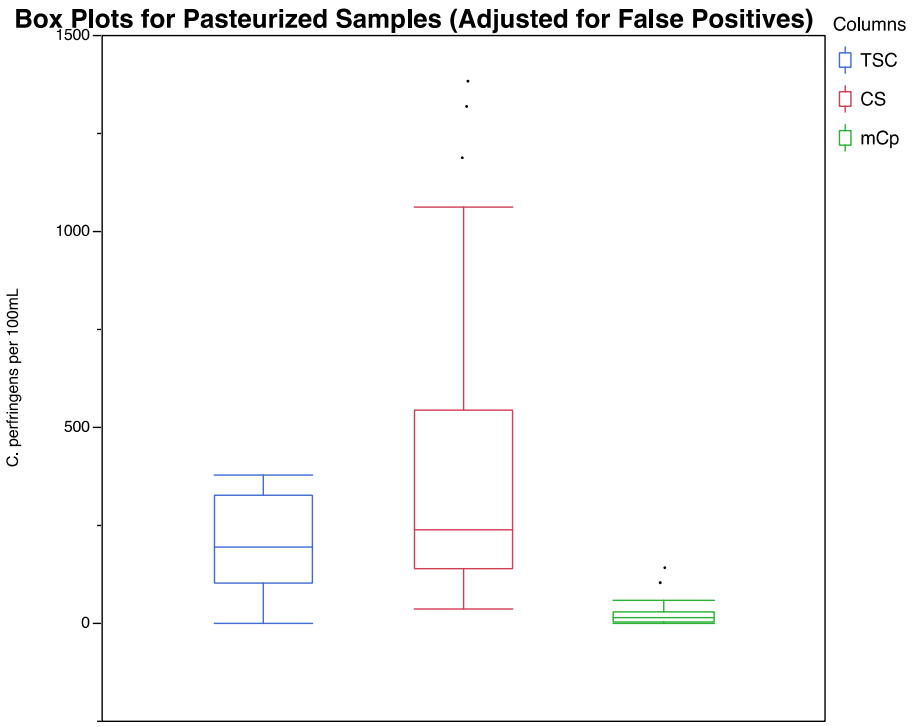

Figure 12: Box and Whisker Plot for Distribution of *C. perfringens* Detection in Pasteurized Pre-sand Filtered Water Samples Adjusted for False-Positivity.

Forty-one water samples from 5 wastewater treatment plants were compared for *C. perfringens* concentrations in unpasteurized and pasteurized reclaimed water samples analyzed by membrane filtration using 3 different agar media. A total of 82 water samples were analyzed, with an equal number pasteurized and unpasteurized. Of these 82 samples, 50 were pre-sand filter treated water, 10 were raw sewage, and 22 were final reclaimed water. Analysis of the data resulted in single point estimates of concentration of *C. perfringens* as CFU per 100 mL. If results from a sample were higher than the detection limit of the analysis or too numerous to count (TNTC), then a value of 2x the upper detection limit was used in the analysis. In this case, the upper detection limit was 112.5, so 225 was used as the TNTC concentration for samples that met this TNTC criterion. Sewage samples were diluted either 1000-fold or 10,000-fold for enumeration. If an agar medium detected no colonies in a sewage sample, a value of 0.5 was used to calculate CFU per 100 milliliters. This was done in order to ensure that an agar medium's performance was not underestimated due to the fact that in an undiluted sewage sample, it is expected that *C. perfringens* will always be present and be detected by all three of the agar media tested.

In order to examine the *C. perfringens* concentration data collected for the two sample preparation methods, pasteurized and unpasteurized, the data sets for unpasteurized samples and pasteurized samples were individually subjected to a Friedman one-way analysis of variance (nonparametric ANOVA) Test in order to compare results for the three agar media tested. This test determined if there was a significant difference in performance in *C. perfringens* detection among the three agar media tested. As shown in Table 15, the Friedman test found significant

differences in *C. perfringens* detection among the three agar media for many of the wastewater and reclaimed water samples types tested in groups of sample types or in individual sample types. Only in reclaimed water samples, where *C. perfringens* concentrations were very low, were there no differences in *C. perfringens* concentrations detected on the three agar media tested. Therefore, after the Friedman tests, Dunn Multiple Comparison Post-tests were performed to compare how the agar media detection of *C. perfringens* compared pairwise with one another in the different samples, pasteurized and unpasteurized (no treatment). The results of these analyses by the Dunn Multiple Comparison Post Tests are shown in Table 16.

By Dunn Multiple Comparison analysis, there was no significant difference between the mCp and TSC agar media for *C. perfringens* concentrations in unpasteurized pre-sand filtered samples. However, there were significant differences between the ChromoSelect and mCp agar media and the ChromoSelect and TSC agar media for *C. perfringens* concentrations in unpasteurized pre-sand filtered samples. In pasteurized pre-sand filtered samples there was no significant difference between the TSC and the ChromoSelect agar media. However, in pasteurized pre-sand filtered samples there were significant differences between the ChromoSelect and mCp agar media, and the TSC and mCp agar media. . In summary, the p-values of the Dunn Multiple Comparison tests indicate significant differences in detection of *C. perfringens* between TSC and mCp agar media and ChromoSelect and mCp agar media. However, no significant differences were found in *C. perfringens* detection in pasteurized and unpasteurized samples between the TSC and the ChromoSelect agar media, by these analyses.

In raw sewage and reclaimed waters as both pasteurized and non-pasteurized samples, there was no significant difference between the agar media in detection of *C. perfringens*, except between ChromoSelect and mCp in unpasteurized sewage samples. Thus, there is a significant difference in *C. perfringens* detection in unpasteurized raw sewage between ChromoSelect and mCp agar media. No significant differences were observed between the different agar media pairs for other raw sewage and reclaimed water samples. However, these findings are limited by a small data-set. Also, when adjusting for false-positivity, all of these associations remain the same.

Table 15: Friedman One-way Analysis of Variance Test of *C. perfringens* Results from Water Samples on Different Agar Media

Sample type	Friedman test p-value	Friedman test p-value adjusted for False-positivity
Pre-sand filter, no treatment	<0.0001	<0.0001
Pre-sand filter, pasteurized	<0.0001	<0.0001
Raw sewage, no treatment	.0008	0.0008
Raw sewage, pasteurized	0.056	0.0239
Reclaimed	0.1353	0.1353
Reclaimed, pasteurized	0.1778	0.1178
Pre-sand, raw sewage, and reclaimed	<0.0001	<0.0001
Pre-sand, raw sewage, and reclaimed; pasteurized	<.00001	<0.0001

Table 16: Dunn Multiple Comparison Post-test Comparisons of Different Agar Media for *C. perfringens* Results in Water Samples Adjusted for False-positivity

Agar 1	Agar 2	Sample type	Dunn Multiple Compari-son p-value (raw data)	Rank Sum Differenc e (raw data)	Dunn Multiple Comparison p-value (adjusted for false-positivity)	Rank Sum Difference (adjusted for false-positivity)
TSC	ChromoSelec t	Pre-sand	<0.001	-30	<0.001	-30
TSC	mCp	Pre-sand	>0.05	9	>0.05	9
CS	mCp	Pre-sand	<0.001	39	<0.001	39
TSC	ChromoSelec t	Pre-sand Δ	>0.05	-12	>0.05	-12
TSC	mCp	Pre-sand Δ	<0.001	30	<0.001	30
CS	mCp	Pre-sand Δ	<0.001	42	<0.001	42
TSC	ChromoSelec t	Raw sewage	>0.05	-5	>0.05	-5
TSC	mCp	Raw sewage	>0.05	5	>0.05	5
CS	mCp	Raw sewage	<0.01	10	<0.01	10
TSC	ChromoSelec t	Raw sewageΔ	>0.05	3.5	>0.05	1
TSC	mCp	Raw sewageΔ	>0.05	7	<0.05	8
CS	mCp	Raw sewageΔ	>0.05	3.5	>0.05	7
TSC	ChromoSelec t	Reclaime d	>0.05	2	>0.05	2
TSC	mCp	Reclaime d	>0.05	4	>0.05	4
CS	mCp	Reclaime d	>0.05	2	>0.05	2
TSC	ChromoSelec t	Reclaime dΔ	>0.05	.5	>0.05	.5
TSC	mCp	Reclaime dΔ	>0.05	4	>0.05	4
CS	mCp	Reclaime dΔ	>0.05	3	>0.05	3.5

In Figures 13 and 14 below are cumulative frequency distributions of the number *C. perfringens* colonies per 100 mL detected by each agar medium for pre-sand filtered wastewater samples. If all agar media exhibited the same or similar performance in *C. perfringens* detection, then the

regression lines of these agars would be coincident or nearly so. The differences among the cumulative frequency distributions demonstrate differences among the performance of the agar media for *C. perfringens* detection in pre-sand samples, based on data that are summarized in the tables above.

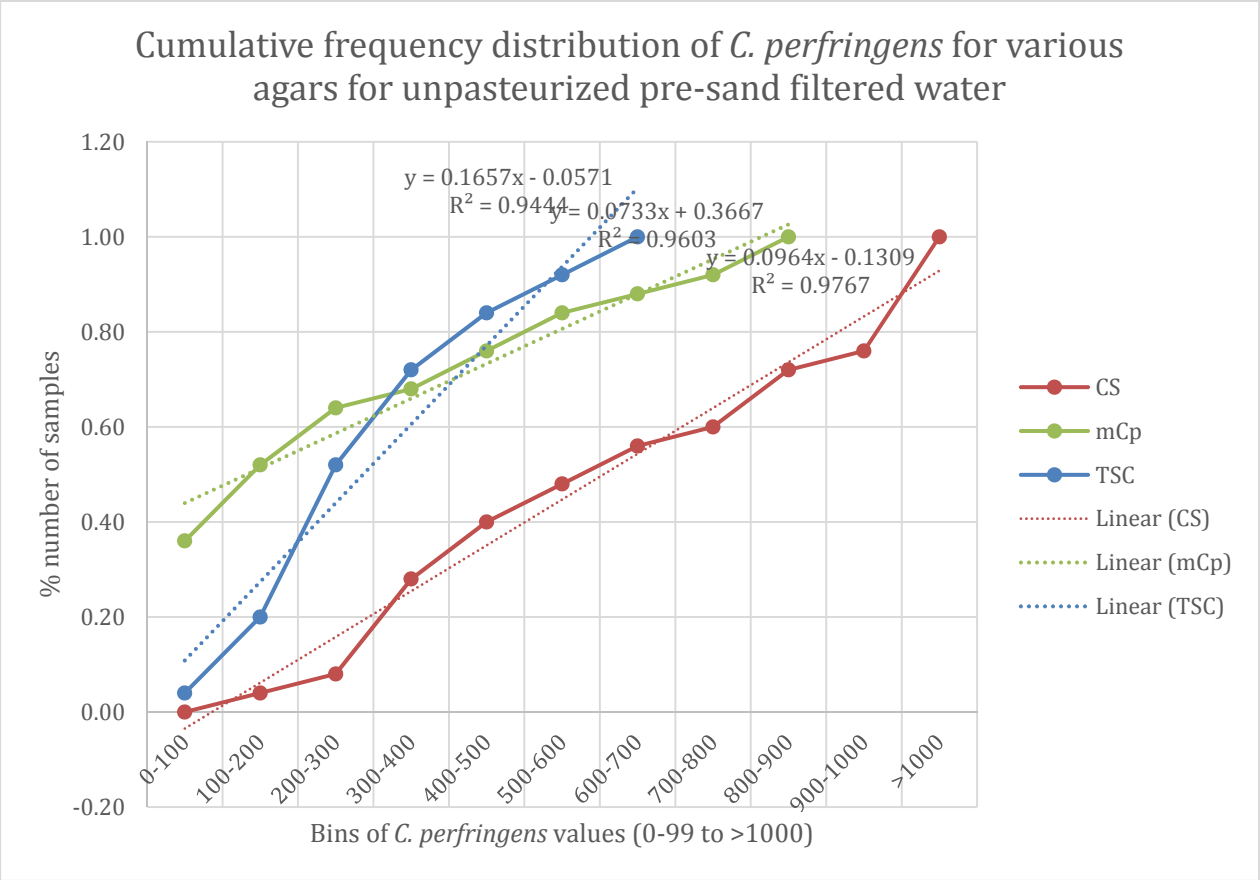

Figure 13: Cumulative Frequency Distribution of *C. perfringens* for Unpasteurized Pre-sand Filtered Water Samples.

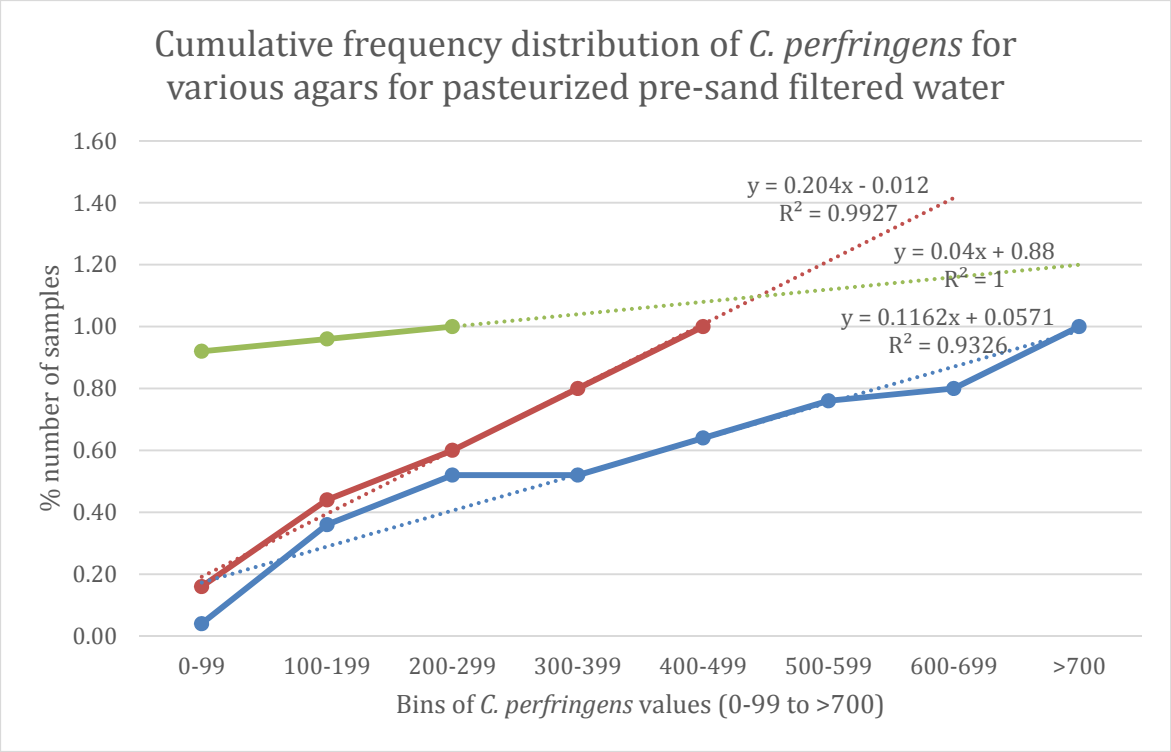

Figure 14: Cumulative Frequency Distribution of *C. perfringens* for Pasteurized Pre-sand Filtered Water Samples.

An additional step for analysis of the performance of these agar media involved further confirmation and characterization of the properties of presumptive positive and negative colony isolates taken from the membrane filter plates of the pre-sand filter reclaimed water samples. Up to five presumptive positive and five presumptive negative colonies were inoculated with a sterile wooden applicator stick from the original agar medium onto a non-selective Columbia agar medium plate and grown overnight at 37.5ºC. Material from a colony isolate from each of these plates was then exposed to acid-phosphatase (AP) reagent to score for the appearance of a purple color signifying a positive test and confirming that the colony was *C. perfringens*. For the final 10 samples analyzed in this study, a second confirmatory test was performed for stormy fermentation (SF) in iron-milk medium. Presumptive colonies were inoculated into separate tubes of iron milk medium and then grown overnight at 44ºC. Tubes positive for growth of *C. perfringens* or other sulphite-reducing clostridia species would show characteristic stormy fermentation, with gas pockets and clotting in the medium. If a colony tested positive for both the AP and SF tests, then the colony was considered to be *C. perfringens*. If the colony tested negative for both tests, then the colony was scored as not *C. perfringens*. Colonies testing positive for one test and not the other were considered inconclusive and not counted as confirmed positives for *C. perfringens*. The results from both tests are presented in contingency tables for both selected presumptive negative and positive colonies from pasteurized and non-pasteurized samples analyzed on each type of agar medium. A total of 12 different contingency tables displayed the rates of confirmation for these two additional tests. The contingency tables are found in Appendix section 6.3. In order to calculate an adjusted value for false-positivity for the overall colony detection in each agar medium, the total of isolates positive for both

confirmation tests was divided by the total number of isolates analyzed for each condition to give the false-positivity rate. When these adjusted positivity values were then applied to the raw data values, there were no changes in the results of previous statistical significance tests. The results of the confirmation tests used to obtain rates of false-positivity and false-negativity for each agar medium are presented in Tables 17 and 18. The results for the two confirmation tests were also tested for their agreement or association using Chi-square tests. This was done to demonstrate whether a significant association was observed between colonies confirmed positive or colonies confirmed negative between the two confirmatory tests for each of the colonies isolated from different agar media and reclaimed water samples. The results of the Chi-square tests are also displayed in Tables 17 and 18. A significant association was found between the results for the two confirmatory tests for presumptive positive isolates taken from mCp agar medium in unpasteurized samples, TSC agar medium of pasteurized samples, and ChromoSelect agar medium of pasteurized samples. A significant association was found between the results for the two confirmatory tests for presumptive negative isolates from unpasteurized samples on TSC and ChromoSelect agar media, and from all agar media in pasteurized samples. Of the 12 conditions analyzed for presumptive positive and negative colony isolates from pasteurized and unpasteurized samples plated on the three different agar media, significant associations were found between the tests for 8 (67%) of these conditions.

Table 17: Summary of false-positivity Results from Confirmatory AP and SF Tests on Presumptive *C. perfringens* Colony Isolates of Pasteurized and Unpasteurized Samples Analyzed on TSC, CS and mCp Agar Media by Chi-square Analysis

Presumptive positive	Rate of false-positivity	Chi-square value	p-value
TSC	0.18	1.994	0.1579
CP ChromoSelect	0.18	1.994	0.1579
mCp	0.19	8.252	0.0041
TSCΔ	0.16	28.187	<0.0001
CP ChromoSelectΔ	0.10	9.78	0.0018
mCpΔ	0.32	2.029	0.1543

Table 18: Summary of False-negativity Results from Confirmatory AP and SF Tests on Presumptive *C. perfringens* Colony Isolates of Pasteurized and Unpasteurized Samples Analyzed on TSC, CS and mCp Agar Media by Chi-square Analysis

Presumptive negative	Rate of false-negativity	Chi-square value	p-value
TSC	0.74	20.415	0.0001
CP ChromoSelect	0.21	5.307	0.0212
mCp	0.02	.02127	0.8841
TSCΔ	0.76	11.46	0.0007
CP ChromoSelectΔ	0.35	3.359	0.0668
mCpΔ	0.03	8.493	0.0036

From the results summarized in Tables 17 and 18, TSC agar medium appears to have the greatest false-negative rate of the three agar media tested. Pasteurized samples analyzed on mCp plates

have the false-positive highest rates according to this analysis. Overall, rates of false-positivity among the different agar media for pasteurized and unpasteurized samples ranged from 0.1 to 0.32, with 4 of the 6 conditions tested having rates between 0.16 and 0.19. Rates for false-negativity were greater and more variable than false-positivity among the sample conditions and agar media tested, ranging from very low (0.02 and 0.03) for mCp agar medium, to moderate (0.21 and 0.35) for CS agar medium) to high (0.74 and 0.76) for TSC agar medium. Rates of false-positivity and false-negativity are higher than would be desired for highly selective agar media, with the exception of the false-negative rate of mCp agar medium. Further studies are recommended to better determine the rates of false-positivity and false-negativity from use of these agar media by further biochemical and genetic analysis of colony isolates from each agar medium and sample type, when applied to a larger number of representative wastewater and reclaimed water samples.

4.3.3 Laboratory Survey

A survey was conducted to determine if laboratories in NC were able and willing to perform coliphage and *C. perfringens* analysis of NC type reclaimed water or other water and wastewater samples. This survey was done because attempts to recruit laboratories able and willing to participate in a performance validation study of the candidate coliphage and *C. perfringens* methods developed and evaluated in this study were unsuccessful. Therefore, it was not possible to meet this intended objective of the study. The survey was an attempt to better quantify and understand the status of capacity and willingness to perform coliphage and *C. perfringens* analysis by microbial water quality analysis labs operating in NC.

Of the 44 labs that elected to answer the survey about willingness and ability to do *C. perfringens* and coliphage analysis and certification status for microbial analysis in various water types, 16 of the labs currently are certified to test drinking water and 34 are certified to test wastewater for bacteriological quality. There were also 5 labs that analyzed other sources such as lakes, streams and other surface waters and 3 that analyzed reclaimed water of some type. Methods for bacterial analysis included membrane filtration, Colilert, Heterotrophic Plate Count, and Enterolert. The most common bacteria detected by use of these methods were *E. coli*, total coliforms, Fecal Coliforms and enterococci. While none of the labs specifically mentioned *C. perfringens* as a bacterium that is tested for, the fact that almost all private labs use membrane filtration suggests that transition to include *C. perfringens* testing would be quite possible in principle, by using an appropriate *C. perfringens* medium and implementing anaerobic incubation. In reality, however, the response to the question on whether the lab would consider offering *C. perfringens* testing, 33 responded “no” and only 2 responded “yes”. The two responding “yes” were both labs operated by public utilities. The one lab that answered about its pricing structure for offering new microbial tests claimed that they would only offer new tests if the annual revenue from providing such a test exceeded $10,000 dollars. Because none of the labs answering this survey currently tests for *C. perfringens*, none had experience using any of the three agar media of this study that are selective for *C. perfringens* colony growth by membrane filtration methods.

Only 1 lab responded that they perform coliphage testing of surface waters and they did not elaborate on the kinds of coliphage tests that they do. Furthermore, this was the only lab that

expressed any interest in using and implementing the enrichment-spot plate and single agar layer methods used for coliphage detection. The pricing structure for microbial testing was either not known yet or was done on contract for a public utility for which the pricing information was not available. Food safety microbiological testing was even less common and only one lab of the forty-four surveyed currently offered such tests. The survey data summarized are presented in greater detail in Tables 10-20 below.

Table 19: Number of NC Labs Certified for Bacterial Analyses of Certain Water Sources

Water Source	Number certified	Percentage
Drinking water	16	46
Wastewater	34	97
Others (Mentioned: reclaimed, lakes, streams, recycled discharge, stormwater)	5	14

Table 20: Number of NC Labs Certified for Specific Bacterial Analysis and Methods

Indicator organism	Detection Method used	Number of labs	% out of total labs offering
Total Coliform	Colilert	12	27.3
Total coliform	Membrane filtration	5	11.4
Fecal coliform	Membrane filtration	28	63.6
Fecal coliform	Colilert	8	18.2
Heterotrophic Plate Count bacteria	Heterotrophic plate count methods (either spread plate or membrane filtration)	8	18.2
E. coli	Coliliert	8	18.2
E. coli	Membrane Filtration	3	6.8
Enterococci	Enterolert	6	13.6

Enterococci	Membrane Filtration	1	2.3

Table 21: NC Labs That Would Presently Consider Offering *C. perfringens* Testing

Offer or would offer testing	Number of labs	Percentage
Yes	2	6
No	33	94

Table 22: Responses to What Pricing Structure Would be for Offering *C. perfringens* Testing

Response 1	"We don't get requests for this procedure. We only add new tests if we can generate approximately 10K or more in revenue per year
Response 2	"Not known at this time"
Response 3 (OWASA)	"We are a municipal lab and cannot contract out and charge for services. If we did the testing, it would be for internal purposes."
Response 4 (Clinton, NC)	"These would be internal tests only conducted by us for our own reclaimed water

Table 23: Response to Question: Have You Ever Used Any of the Selective Agars for *C. perfringens* Detection?

Agar type	Number of labs
TSC agar	0
MCP	0
ChromoSelect	0
None	27

Table 24: NC Labs Currently Certified for Coliphage Analysis

Certified	Number of labs	Percentage
Yes	1	2.3
No	43	97.7

Table 25: Methods for Coliphage Testing Offered

Method	Number of Labs
Enrichment-Spot Plate	1
Single Agar Layer	1

Table 26: Potential Pricing Structures Offered for Coliphage Testing

Response 1	"Would consider – pricing not known at this time or method"
Response 2 (Clinton)	"These would not be contract tests – they would be performed for our City's reclaimed water"
Response 3	"We don't get requests for this procedure. We only add new tests if we can generate approximately $10K or more in revenue per year

Table 27: Number of Labs Currently Certified for Testing Reclaimed Water

Test reclaimed water?	Number of Labs	Percentage of respondents
Yes	7	23
No	23	77

Table 28: Interest of Private and Public NC Labs for Becoming Certified for Type Two Reclaimed Water Testing

Interested in becoming certified?	Number of labs
Yes	6

No	23

Table 29: Reasons for Not Being Interested in Becoming Certified for Analysis of Type Two Reclaimed Water

Reason	Number of labs
Cost-related	7
Time	3
Not enough employees	1
Additional equipment needed	1
Not set up for microbiology work	1
Quantity of work	1
Not required by NPDES permit	10
Not enough interest	2

None of the bulk reclaimed water utilities in the state of North Carolina currently produce type two reclaimed water. However, lab tests have indicated that the water utilities with more laboratory resources such as OWASA, Raleigh, and Cary, have final reclaimed water products that frequently had undetectable levels of microbes. All utilities expressed that there is little or no desire to produce and become certified for type 2 reclaimed water in the near future. The main reason for this is that many of these utilities do not service locations that would have use for such reclaimed water at this time. In Orange and Wake Counties, where the upgrade to type 2 would be most easy to implement, the majority of water users are residential. Cary sends 85% of its water to residences for lawn irrigation, 10-15% for cooling towers, and one location for toilets. OWASA provides reclaimed water exclusively to UNC-Chapel Hill and Raleigh serves virtually no agricultural users. Many utilities also perform their microbial testing in house. Cary, Raleigh, Durham and OWASA each have labs on site for which they can test their own water for microbiological quality. Rarely do any of these lab outsource their testing. If they do, it is for generally more complex tests for microbial pathogens that are not routinely analyzed for in their own lab. Durham and Pittsboro expressed some interest in producing NC type 2 water, but predicted that they were very far away from actually doing so. Neither municipality had a significant amount of agricultural users that they could service with this water.

Monroe produces type 1 reclaimed water, but also has potential to supply to agricultural users. However, their reclaimed water production is entirely based on demand. Demand for type 1 water has been so low to this point that they hardly produce any and have not even considered

upgrading to type two reclaimed water. The overall trends based on the responses the various water utilities surveyed illustrate that there simply is not much demand for type two reclaimed water at this time. Of all of the utilities contacted, only Cary actually charged its residents for reclaimed water at $3.68 per 1000 gallons.

4.4 Discussion

4.4.1 Detection of Coliphages

The research presented here is an important extension and adaptation of previous work on the development and validation of methods to detect coliphages in water and wastewater. Coliphage detection methods have been developed and collaboratively studied in the USA (sponsored by US EPA) for detection in ground water sources of drinking water and in Europe for more general use in water (sponsored by the EU and facilitated through the International Standards Organization (ISO). The initial development of US EPA Methods 1601 and 1602 to detect coliphages, including the evaluation of candidate methods, the initial drafting of the standard protocols that eventually became Methods 1601 and 1602, the organizing and managing of multi-lab performance validation studies of the methods and subsequent demonstration of the methods at public workshops was done previously in this laboratory with the collaboration of other labs (Sobsey et al, 2004a). To our knowledge, this lab is the first to evaluate coliphages as virus indicators of reclaimed water quality in NC and this study is the first systematic effort to identify and evaluate the performance of candidate coliphage methods to analyze NC type 2 reclaimed water (2004b). Other efforts have been made to evaluate methods for coliphage detection, but these studies have been done in Europe to support the development of European Union and ISO methods, not in the USA, not in NC and not for analysis of reclaimed water.

As US EPA Methods 1601 and 1602 have not been optimized for reclaimed water, these methods were adapted and evaluated for the detection of coliphages in type 2 reclaimed water and wastewater in North Carolina. The adaptations of these methods are presented in section 4.2.2.1 of this report. The SAL was employed as described in US EPA Method 1602 and enrichment-spot plate was employed as described in US EPA method 1601, but was modified from a presence-absence test on a single sample volume to a quantal test using multiple sample volumes to estimate an MPN concentration. Additional candidate *E. coli* hosts for coliphage detection were also included. As presented in Tables 7 and 9, there were no significant differences in coliphage detection between the two coliphage methods. The only exception being for *E. coli* Famp in Table 7, where the sample size was small (n=11) and there were multiple non-detect values. In Table 9, when the sample size increased and there were fewer non-detect values, the detection of male-specific/F+ coliphages by *E. coli* Famp was no longer significantly different between the two methods. As coliphage detection by each method is not significantly different between the two methods, it is possible to use either method to quantify coliphages in reclaimed water. However, several other factors are important in the evaluation of these two methods including costs, complexity, and time to results.

The single agar layer method is a 3-day procedure, requiring one day for prep-work and two days for the method itself to return results. This method requires relatively few supplies, including tryptic soy agar bacteriological medium, 150 mm petri dishes, 250 mL autoclavable bottles, 125

mL sample bottles, *E. coli* hosts, antibiotics according to *E. coli* host, $MgCl_2$, and waterbaths. Results are evaluated by counting lysis zones as plaque forming units per 100 mL, giving a discrete coliphage count per volume.

The two step enrichment-spot plate method is significantly more complex than the single agar layer method, as it involves additional steps before returning results. This method is a-4 day procedure, requiring one day for prep-work, and 3 days for the enrichment culture, spot plating, and reading of results. The two step enrichment-spot plate method uses more supplies than the SAL method including a 10X tryptic soy broth (in the enrichment step), centrifuge tubes of various sizes, 150 mm petri dishes, 250 mL autoclavable bottles, 125 mL sample bottles, *E. coli* hosts, antibiotics according to *E. coli* host, $MgCl_2$, and waterbaths. This method also requires the use of a microcentrifuge, which is a specialized piece of equipment used for centrifuging small tubes. If the microcentrifuge is not used in the evaluation of coliphages, it will be very difficult, or impossible to read the results from the experiment.

There are many similarities in the supplies and materials of the SAL and the two-step enrichment-spot plate methods, but when evaluating the methods in terms of ease of use, amount and kinds of laboratory equipment and supplies, number of procedural steps and time to results, the preferred choice is the SAL. Additionally, results are in the form of discrete counts (PFUs) per 100 mL rather than as quantal estimates of concentration MPNs, which is advantageous in terms of quantifying the number of coliphages present in a reclaimed water sample.

As there was no statistically significant difference in the detection of somatic, male-specific (F+), or total coliphages when comparing the single agar layer method and the two step enrichment-spot plate method, the main selection criteria are ease of use, cost, and time to result. In all of these areas, the single agar layer method is the most efficient method.

As coliphage testing has been mandated for type 2 reclaimed water in North Carolina, the relationship between somatic, male specific coliphages and individual coliphage hosts is an important aspect of host selection. US EPA methods 1601 and 1602 describe the use of both a somatic and a male-specific/F+ coliphage host for the quantification of coliphage concentrations in water samples. However, if a total coliphage host could be applied to water samples, then only one set of analyses would need to be conducted for each water sample – utilizing fewer laboratory supplies, and requiring less sample processing and analysis time.

The initial development and evaluation of *E. coli* CB390 as a total coliphage host was conducted by Guzmán et al., 2008, in which the transformation of *E. coli* WG5 (a somatic coliphage host) to an F^+ coliphage host was accomplished by insertion of an F-plasmid. This study reported no statistically significant differences in coliphage concentrations detected between *E. coli* CB390 and those detected by the sum of the ISO recommended somatic coliphage host (*E. coli* WG5) and the male-specific/F+ host *Salmonella typhimurium* WG49 (Guzmán et al., 2008). The results of Guzman et al. (2008) are similar to the data described in the Results section of this current report, with the main difference being the coliphage hosts used for the detection of somatic coliphages being US EPA-recommended *E. coli* CN13 and the male-specific/F+ host being *E. coli* Famp in the current study. Other groups have also used *E. coli* CB390 as a total coliphage host for reclaimed water applications as well as for hydrogen peroxide disinfection experiments

(Jebri et al., 2012; Souza et al., 2013). The Souza et al., 2013 study included both somatic (ΦX174) and male specific (MS2) coliphages as positive controls when using *E. coli* CB390, thus indicating that *E. coli* CB390 is able to detect both somatic and male-specific/F+ coliphages.

Other than the studies cited above, there is very little available literature on *E. coli* CB390, and much less information available on its abilities to quantify male-specific/F+ and somatic coliphages individually or together. However, from the available literature and from the results presented in this study, it is clear that *E. coli* CB390 will be a coliphage host to consider for use because several sources have shown that it is able to quantify coliphage concentrations at a similar level to that of the sum of a somatic coliphage and a male specific/F+ coliphage host. This is an important application of a genetically created coliphage host, because 'total' coliphage hosts efficient at detecting both somatic and male-specific/F+ coliphages are not likely to be readily available naturally. The few known coliphage hosts that can detect both somatic and male-specific/F+ coliphages, such as *E. coli* C3000 and C3322 are not as effective in detecting both somatic and male-specific/F+ coliphages according to the results of the studies reported here.

As *E. coli* CB390 is intended to quantify both male-specific/F+ and somatic coliphages, additional testing is needed to further validate the ability of this host to give efficient detection of male-specific/F+ coliphages. It is clear from the results of this and other studies that *E. coli* CB390 detects somatic coliphages at levels similar to that of somatic coliphage host *E. coli* CN13. However, with the observed low levels of male-specific/F+ coliphages detected in most of the reclaimed water samples of this study, it is still unclear whether the *E. coli* CB390 host is detecting male-specific/F+ coliphages efficiently. Additional testing is recommended to further compare the detection of male-specific/F+ coliphages by its specific host *E. coli* Famp and by the total coliphage *E. coli* CB390 in reclaimed water and wastewater samples.

4.4.2 *Clostridium perfringens*

The research of this study validates and extends previous work that has been done on the detection of *C. perfringens* in surface waters, ground waters, and wastewater. Several previous studies have attempted to identify an optimum medium for detection of *C. perfringens* in various potentially contaminated water sources. However, many of the previous studies have either been found to be inconclusive or conflicting amongst each other. Furthermore, many of the previous studies on selective *C. perfringens* media have been performed on media which are either no longer frequently used or for the purpose of culturing *C. perfringens* from food samples. As early as 1974, Hauschild and Hilsheimer (1974) attempted to explore the performance of selective *C. perfringens* media at culturing food-borne *C. perfringens* colonies. They compared TSC agar along with 5 other agars and concluded that TSC was the most satisfactory at enumerating presumptive positive colonies. Since then, Mead (1985), de Jong et al. (2003), and Byrne et al. (2008), have all reached similar conclusions that TSC performs at least comparatively well compared to the other agar media that were tested for enumerating *C. perfringens* from food samples.

However, the earliest study comparing different agar media by membrane filtration methods to detect *C. perfringens,* by Sartory (1985), compared mCp and TSC agar media and found that TSC recovered higher numbers of *C. perfringen* than did mCp in sewage samples. The study

also noted that mCp was overall more specific for culturing *C. perfringens* colonies. This previous study provides findings similar to those of this current study in that TSC was in general, more capable of enumerating presumptive positive *C. perfringens* colonies than was mCp. In 1998, Sartory et al. again compared the performance of mCp and TSC agar media, this time in environmental and partially treated drinking waters instead of sewage. Their results supported the previous findings that TSC was a superior agar medium for recovery of *C. perfringens* in contaminated water samples. In addition, they found that mCp had higher rates of both false-positivity and false-negativity. While, our study found high rates of false-positivity for mCp on pasteurized samples, this finding was not found for other samples analyzed, for which the mCp agar medium either performed similarly or better based on false-positive and negative rates than did the TSC agar medium. Araujo et al. (2004) compared both TSC and mCp agar media among other media for *C. perfringens* selectivity when analyzing groundwater samples for *C. perfringens* spores. They did not find TSC to be the best medium among the others tested and further found that mCp was inferior to the other media tested in its ability to recover *C. perfringens* spores. However, in a paper describing the development of mCp medium, Bisson and Cabelli (1979) reported higher recovery of vegetative *C. perfringens* with mCp than with TSC in a range of water and wastewater samples. Most recently, Manafi et al. (2013) did a comprehensive study comparing the newly developed chromogenic medium CP ChromoSelect agar medium to mCp agar medium and to TSC agar medium supplemented with a fluorogenic substrate component for detection of *C. perfringens* in drinking water and found CS agar to be the most effective of the three agar media.

Although the findings of these previous studies have all varied, there are several key points regarding all of them in relation to the study of this report. Common factors found in several of the previous studies that were replicated by the results of this present study are that mCp medium was found to be inferior to TSC medium and that CP ChromoSelect medium was found to be superior to mCp medium. Furthermore, all studies that performed confirmatory tests on presumptive *C. perfringens* isolates found at least some false-positive or false-negative colonies using each of the agar media. However, magnitude of false-positivity and false-negativity was often inconsistent from one study to another. It is noteworthy that the rates of confirmation of colonies in this present study expressed as prevalence of false-positives and false-negatives, often varied from week to week for each agar medium. In addition other factors sometimes contributed to issues in assessing false-negativity with the TSC agar. When large volumes of highly contaminated water samples were filtered onto membranes that were plated on TSC agar, the presumptive negative colonies frequently outnumbered the presumptive positives colonies, but such colonies were often later confirmed as positive. Hence, interferences associated with the presence of many colonies on a membrane and possibilities for cross contamination among colonies could have compromised the validity of these confirmatory analyses. Another important detail to acknowledge about the previous studies is that very few of them analyzed for both vegetative cells and spores and few analyzed wastewater samples. In this current study samples from various stages in the sewage treatment process were analyzed and both spores and vegetative cell were targeted for detection. Additionally, the current study is the only one that has compared TSC agar, mCp agar, and CP ChromoSelect agar simultaneously reclaimed water and wastewater. Therefore, this current study advances the body of knowledge about *C. perfringens* detection in ways that previous studies have not. *C. perfringens* testing has been mandated for type 2 reclaimed water in North Carolina. Therefore, analyzing samples from wastewater treatment systems and differentiating the recovery of spores and vegetative cells is of

high importance in exercising sound judgment in selecting the proper medium for future analysis of type 2 reclaimed water samples. Furthermore, the recent legislation allowing the use of type 2 reclaimed water for potable reuse further increases the importance of testing samples by methods and media that provide the highest recovery rate and greatest accuracy in *C. perfringens* detection for the protection of the public health. Overall the superior ability CP ChromoSelect agar medium to detect and quantify *C. perfringens* in pre-sand filtered water makes it the preferred choice to recommend to the State of North Carolina for analysis of *C. perfringens* in reclaimed water to determining standards compliance and to seek certification for is use as an agar medium for membrane filtration.

4.4.3 Laboratory Survey

Prior to the survey conducted among private and public water microbiology analytical labs in North Carolina, no previous study had been done either formally or informally to determine the market potential for type 2 reclaimed water testing. In regards to the survey, it is important to note that many of the labs that responded were either run by the public wastewater utility that they served or were contracted by the utility that they served. As a result, the prevalence of disinterest in offering both the *C. perfringens* and coliphage tests could be interpreted in several ways. Because a minority of private microbiology labs in the state answered the questionnaire, we do not know for sure if there are others of them that do in fact or could offer these tests. However, several of the private labs that did respond do not offer water testing at all and showed no interest in doing so in the future. Additionally, of the sample of 44 labs contacted, many of which do extensive water testing, the vast majority do not perform and are not interested in performing either of these tests. Therefore, it is unlikely that many, if any, of the non-responding labs offer these tests either or would be interested to do so. To gain more information on lab capacity and interest in *C. perfringens* and coliphage analysis of type 2 reclaimed water and wastewater a future study is recommended to collect data from both private and public labs throughout the state to get a better idea of the market potential for these tests. Offering such testing may now be of greater interest with the potential for type 2 reclaimed water to be used as source water for potable drinking water, with further water treatment. .

The lack of interest and capacity in *C. perfringens* and coliphage analysis found at the time of this questionnaire is likely related to several factors. First, the production of type 2 reclaimed water in North Carolina is still in its infancy. Only very recently has the State of NC taken steps to expand the potential uses for reclaimed water. As a result no utilities are currently prepared and incentivized to produce type 2 reclaimed water at this time. The major reclaimed water producers who are capable of doing so have no customers for type 2 reclaimed water. As evidenced by the correspondence with NC type 1 reclaimed water bulk providers, they would have to invest in costly infrastructure improvement in order to reach customers likely to want and use type 2 waters for non-potable purposes. The lack of type 2 water production along with the relatively recent regulatory implementation of this new, higher standard of reclaimed water quality provides explanation for why so few labs are interested in investing in establishing and providing these tests. However, two main factors could cause this prevailing disinterest to change quickly. First, many of the labs already have the necessary equipment to perform these tests. If a greater demand for *C. perfringens* or coliphage testing were to arise, many of these labs would simply have to purchase new reagents, a relatively inexpensive investment when

compared to purchasing equipment. Second, the survey was carried out before the approval of type 2 reclaimed water for potable use by the State of NC legislature. Now that this major step to expand the allowable use of reclaimed water as source water for potable drinking water has been taken, it may encourage the development of and demand for reclaimed water use. As a result, many of the labs that had previously been uninterested in performing these new microbial tests may now be much more interested in implementing and providing them. Similarly, many water and wastewater utilities that previously showed no interest in becoming certified to produce high quality reclaimed water may now be more interested as well, given the potential new use for type 2 reclaimed water. They would need testing for these microorganisms to become available in order for their type 2 reclaimed water systems and its product water to become certified. Because of the recent changes in legislation and policy, it would be advisable to determine if the opinions have changed about implementing and offering such tests in support of producing high quality type 2 reclaimed water since this previous inquiry.

4.5 Summary

4.5.1 Coliphage Detection

4.5.1.1 Host Choice for Coliphage Detection

Both somatic and male-specific/F+ coliphages were found at relatively high concentrations in raw sewage in this study. Hence, both of these coliphage groups should be considered for analysis in wastewater and reclaimed water to evaluate and monitor the performance of systems to produce type 2 reclaimed water and the quality of type 2 reclaimed water. To simplify and streamline the analysis of both somatic and male-specific/F+ coliphages in reclaimed water, the use of a single host to detect both of them simultaneously is recommended. Based on the studies of this report, the most suitable 'total coliphage' *E. coli* host is *E. coli* CB390. In both SAL and enrichment-spot plate methods, there were no significant differences in coliphage detection between results of *E. coli* CB390 and the sum of the *E. coli* CN13 and *E. coli* Famp results by both the SAL and enrichment spot plate methods (see Table 9). However, because the male-specific/F+ coliphage concentrations detected by *E. coli* Famp were low in comparison to the concentrations of somatic coliphages detected by *E. coli* CN13 in samples of reclaimed water, it is possible that the greater numbers of somatic coliphages overshadow or mask the presence of the male-specific/F+ coliphages on the CB390 host. To some extent, it was possible to address this issue by the analysis of coliphages the raw sewage samples. In raw sewage both the somatic coliphages detected on *E. coli* CN13 and the male-specific/F+ coliphages detected on *E.coli* Famp were detectable at similar levels and both of these coliphage types could be detected in raw sewage on total coliphage host *E. coli* CB390 by both the SAL and enrichment-spot plate methods. While we are confident in recommending *E. coli* CB390 as a preferred host bacterium to detect total coliphages in reclaimed water and wastewater, further investigation is recommended to more comprehensively determine if *E. coli* CB390 can detect both F+ male-specific and somatic coliphages adequately in a range of reclaimed water and wastewater sources in NC and elsewhere.

4.5.1.2 Coliphage Detection Method

Based on the studies of this project, there were no significant differences in the detectability of coliphages in reclaimed water or wastewater by the Single Agar Layer or Two Step Enrichment-Spot Plate assays, as summarized in Tables 8 and 10. Therefore, according to the results of this project, either coliphage detection method, the single agar layer method (SAL) or the two step enrichment-spot plate method, could be used for the quantification of coliphages in reclaimed water.

As presented in the Appendix 6.2.1, during the Workshop and Hands-on Training, workshop participants were given a survey and asked to rate preferences for each coliphage detection method. The single agar layer method was selected by a slight margin as a result of its use of fewer laboratory supplies, and specialized equipment (such as centrifuges). However, many participants were more familiar with the techniques involved in the performance of the two step enrichment-spot plate method, including the preparation of broth culture media, agar plates, and analyzing MPN data for determination of coliphage concentration.

Overall, the SAL method is the recommended over the enrichment-spot plate method because requires fewer steps to perform, takes less time to give results and is consistent with the methods described by US EPA Method 1602 and in Standard Methods for the Examination of Water and Wastewater. The only departures from the information and specification of these standard reference manuals on coliphage detection is the recommendation that both somatic and male-specific/F+ coliphages be analyzed for simultaneously on a single host as total coliphages and that such simultaneous detection be on total coliphage host *E. coli* CB390.

4.5.1.3 Limitations

Limitations of this study include (1) the limited number of reclaimed water sources studied (5 utilities in North Carolina), (2) the low presence and detectability of male specific (F+) coliphages in both pre UV and reclaimed water samples compared to somatic coliphages that may have interfered with their detection and quantification, (3) the limited number of samples analyzed (n=37), and (3) the need for further validation of *E. coli* CB390 in other wastewater and reclaimed water samples. Although abundant in raw sewage, male-specific/F+ coliphages were generally detected at lower concentrations than somatic coliphages in reclaimed water and there were many non-detect (zero values) present in the data set. Therefore, the detection of total coliphages as the sum of the detection on male-specific/F+ host *E. coli* Famp plus somatic coliphage detection on *E. coli* CN13 may not have been adequately representative in determining the concentration of total coliphages in reclaimed water samples. However, there are few other recommended male specific/F+ coliphage hosts and *E. coli* Famp is the standard host to analyze for male-specific/F+ coliphages in water and wastewater in the United States. As the sample size was limited for this study with n=37 total samples, additional sampling and analysis to obtain more results will be important for further verification and validation of the results of this study. Additionally, total coliphage host *E. coli* CB390 also requires further validation of its usefulness in simultaneously detecting both somatic and male-specific/F+ coliphages. Additional testing should be conducted with this host to further characterize its ability to simultaneously quantify both types of coliphages in water and wastewater.

4.5.2 *Clostridium perfringens*

4.5.2.1 Agar Medium Selection

From the membrane filter analysis on the different *C. perfringens* agar media it is clear that there are significant differences among the various agar media in their ability to detect *C. perfringens*. Even when adjusting by the selected metric for false-positivity, the statistical significance of the differences among the agar media did not change. Although there were few significant differences between the agar media in reclaimed samples and raw sewage samples, these results were limited by small sample sizes and inconsistent recovery of colonies on a per sample basis. Of the three agar media, the CP ChromoSelect agar gives significantly higher recovery of presumptive *C. perfringens* colonies from pre-sand treated water samples than the other two selective agar media. For pasteurized samples both the CP ChromoSelect and TSC agar media recovered significantly more *C. perfringens* colonies per sample than did the mCp agar medium. However, there were no significant differences between the CP ChromoSelect and TSC agar media for this type of sample. These results suggest that CP ChromoSelect agar medium is the most reliable of the agar media at recovering, quantifying and correctly identifying *C. perfringens* in reclaimed waters and wastewaters.

4.5.2.2 Limitations

There are several important limitations to this analysis for agar media performance to detect and quantify *C. perfringens* in reclaimed waters and wastewaters. Only 5 raw sewage samples were analyzed, which is a sample size far too small to draw confident conclusions in regards to the ability of the candidate agar media to detect and quantify *C. perfringens* in this kind of sample. Thus, it is not possible to draw the conclusion that any of the agars performed significantly different from any of the other agars in detection of *C. perfringens* for this type of sample. Another limitation is that for some agar media, an estimated concentration value was used to replace the result on an agar medium plate that had either no growth or too numerous to count. These assigned values of lower (no detection) and upper (TNTC) censored results may have overrepresented or underrepresented the ability of the agar medium to detect and quantify *C. perfringens* in reclaimed water and wastewater. In addition, estimated values for adjusted recovery of *C. perfringens* by each agar medium were based on the assumption that if a presumptive positive colony tested positive for both confirmatory tests, then it was a true positive *C. perfringens* colony. However, this assumption is limited by the fact that human error limits the ability to correctly read the acid-phosphatase test. The acid-phosphatase test relies on the human eye to verify that material from a colony has turned purple when exposed to the reagent, but differentiating between purple as a positive and brown as a negative can sometimes be hard to visualize correctly. As a result, some colonies could have been counted as testing negative when they were actually positive and vice-versa. Furthermore, the assumption that stormy fermentation detects *C. perfringens* is compromised by the fact that other sulfite-reducing clostridia species besides *C. perfringens* produce this reaction. As a result, positivity from the stormy fermentation test does not assure that the colony examined actually was *C. perfringens* and not another sulfite-reducing clostridium. Finally, the fact that there was no statistically significant association between the two tests for several of the isolates is a limitation because either confirmatory test could have been scored incorrectly. If there is no association and concordance between the two confirmatory tests, then assuming that a colony testing positive for

both tests is truly positive for *C. perfringens* could be a false assumption to make. From the data obtained, the extent to which the two confirmatory tests are associated and in agreement for different presumptive *C. perfringens* colony isolates differed among the different samples. There were also limitations in performing the Chi-square tests to measure the association between the two confirmation tests. This is due to the fact that numerical values in the contingency tables were often smaller than a typical Chi-square test provides a reliable statistical outcome on whether the association between the variables is significant. Therefore, there is uncertainty as to whether significant associations were found between the two confirmation tests for some samples sets analyzed. Further statistical analysis is recommended to better determine this relationship.

4.5.3 Laboratory Survey

As documented by the trends in survey data collected and analyzed above, it is not clear that many wastewater treatment plants are currently able to expand their microbial testing or water treatment capabilities and capacities to produce type 2 reclaimed water. However, if demand for such water were to increase, such that the production of this water were to become profitable or otherwise financially justifiable, then it is likely that water utilities would be willing to produce and sell both type 1 and type 2 reclaimed water and that the demand for microbial analysis of such water would also increase.

4.6 Conclusions

4.6.1 Coliphages

Based on the results of this study, including information collected during the reclaimed water microbiology methods workshop in February 2014, the coliphage method preferred for ease of use is the single agar layer method. This method is less time intensive and requires fewer laboratory supplies to complete, when compared to the two step enrichment-spot plate method, while providing results that are not statistically different from those of the two step enrichment-spot plate method. The two step enrichment-spot plate method requires the use of a microcentrifuge and other specialized laboratory equipment that may or not be available to groups currently, or looking to produce reclaimed water. Additionally, the confirmation of positives and negatives in the two step enrichment-spot plate method requires a level of skill that may not be present in many laboratories. Comparison of the performance of both the SAL and enrichment-spot plate methods using multiple total coliphage hosts, *E. coli* CN13 for somatic coliphages and *E. coli* Famp for male specific/F+ coliphages showed no significant difference between the two methods. As a result, the conclusion can be made that the single agar layer (SAL) method is the preferred method when compared to the two step enrichment-spot plate method.

From the results of this study was no significant difference in the detection of somatic coliphages and male-specific/F+ coliphages by the use of the *E. coli* CB390 host to detect both groups simultaneously as total coliphages and the sum of the separate detection of somatic coliphages by *E. coli* CN13 and male-specific/F+ coliphages by *E. coli* Famp. Therefore, the use of *E. coli* CB390 for the simultaneous detection of both somatic and male-specific/F+ coliphages as “total’

coliphages is a recommended approach to detect coliphages in NC type 2 reclaimed water and raw sewage to meet the coliphage requirements of the NC type 2 reclaimed water regulation.

4.6.2 *Clostridium perfringens*

From the data of this study it is clear that the three agar media to detect and quantify *C. perfringens* by the membrane filter method vary in detection rates for both pasteurized and unpasteurized samples of pre-sand filtered water. Of the agar media tested, CP ChromoSelect appears to be the best performer of the three. While, it does not differ significantly from TSC in *C. perfringens* spore detection in pasteurized samples, comparative results for several other samples and other factors indicate that CP ChromoSelect is the preferred choice of agar medium for detecting *C. perfringens* in reclaimed water and wastewater. Based on ease of use, ChromoSelect is also the best agar medium choice. ChromoSelect agar medium can be made with only one supplement and can be boiled instead of autoclaved, which are simpler requirements that those for preparing the other two agar media. In addition, to read colonies, the only extra step to take is to allow the plates to be exposed for 1 hour to an aerobic environment after incubation for the colonies to develop their distinctive color indicative of *C. perfringens*. The mCp agar medium requires multiple supplements, autoclaving, and exposure to caustic and irritating potassium hydroxide fumes to confirm colonies. This is a much more laborious and time-consuming process that makes mCp harder to work with.

TSC agar is comparable in ease of use with ChromoSelect, but does require autoclaving. For colony identification, ChromoSelect and mCp agar media are equally easy to identify. ChromoSelect yields clearly green-tinted positive colonies and mCp, when exposed to potassium hydroxide yields clear pink colonies. Negatives for these agars are a distinct purple or blue respectively. However, TSC agar medium has a wide range of colony types and color that grow and many of its presumptive negative colonies are found to be *C. perfringens* as demonstrated by the results of confirmatory testing of presumed negative colonies. TSC presumptive positive *C. perfringens* colonies are black dots with a yellow halo. However, there are also many colonies that have lighter dots surrounded by yellow halos and yellow dots, both of which often test positive through confirmation testing.

In addition, membrane filtering of larger volumes of unpasteurized water samples frequently resulted in lower presumptive positive colonies in the TSC agar. This could be due to competition from other microbes. Regardless, the ability to correctly distinguish *C. perfringens* colonies on TSC agar medium is most difficult on this agar medium. Because of the limitations of TSC agar medium for distinguishing *C. perfringens* colonies and its high rate of false-negativity, as well as the limitations of mCp agar medium due to difficulties of media preparation and lower detection rates, CP ChromoSelect agar medium emerges the most effective agar medium for *C. perfringens* detection. ChromoSelect has the most favorable attributes of an ideal agar medium and therefore it is the preferred agar for the detection of for *C. perfringens* in reclaimed water samples.

4.6.3 Laboratory Survey

As indicated by the survey private and public microbiology laboratories conducted during this study, water utilities are currently not equipped to either produce type 2 reclaimed water or to test for either coliphages or for *Clostridium perfringens.* As a result, it is concluded, that without significant demand increases or changes in legislation, reclaimed water testing capacity and the expansion of reclaimed water infrastructure systems may be slow to develop. However, since the inception of this project (and since this survey was conducted), new legislation has passed through the NC legislature allowing for the combined use of type 2 reclaimed water with surface waters for potable reuse. This could have an impact on the overall perception of both water utilities and private labs that are using or who were considering the use of these microbial methods for NC type 2 reclaimed water analysis. Therefore, it is concluded that more surveys need to be conducted to assess new attitudes, perceptions and interest in on type 2 reclaimed water and how this will impact both microbial testing procedures and capacities and type 2 reclaimed water infrastructure expansion in North Carolina.

4.7 Recommendations

4.7.1 Coliphages

As evaluated and further discussed in the previous sections of this report, the use of the single agar layer method and the use of *E.coli* CB390 for the detection of total coliphages as the sum of both somatic and male-specific/F+ coliphages is an effective means of quantifying coliphage concentrations in raw sewage, partially treated, and NC type 2 reclaimed water samples. Therefore, it is recommended that the single agar layer method and *E.coli* CB390 as a total coliphage host be used for the quantification of these microbes in wastewater and reclaimed water samples of this type to meet the requirements of the NC type 2 reclaimed water regulation. It is further recommended that a standard protocol and Standard Operation Procedure of this method be developed from the information and materials in this report and submitted to the NC Department of Environment and Natural Resources as a candidate method to be certified for use to analyze type 2 reclaimed water samples and wastewater samples to meet the performance requirements of the NC regulation for type 2 reclaimed water. It is further recommended that this protocol be submitted to the US EPA for consideration for further evaluation and eventual approval through the US EPA Alternative Test Procedure Protocol process (US EPA, 2010).

4.7.2 *Clostridium perfringens*

As presented in the results and analyses of this study, *C. perfringens* in reclaimed water and wastewater can be detected by standard membrane filter methods using one of several candidate agar media. However, there are challenges and limitations to the use and effectiveness all of the available agar media evaluated in this study. From the results presented and discussed in this report, CP ChromoSelect agar is recommended for use in the detection of *Clostridum perfringens* in reclaimed water, based on as results indicating comparatively higher or similar detection rates compared to the other agar media tested, the ease of is preparation and use and the least ambiguity and uncertainty in correctly identify and counting presumptive positive colonies. Although, it appears that CP ChromoSelect agar medium has moderate rates of false-negativity and false-positivity, these rates are generally comparable with the other agar media evaluated. Additionally, the true rates of false-negativity and false-positivity for these agars are still uncertain as there were inconsistent associations between the two *C. perfringens* confirmation

tests used in this study, acid phosphatase and stormy fermentation. As a result, the recommendation of this study is the use the agar medium that was most capable of consistently producing presumptive colonies with distinctive color, without requiring additional use of a reagent to visualize these colonies, which in this case was CP ChromoSelect agar medium. The ease of preparation and use for this agar medium was also an advantage.

4.7.3 Laboratory Survey

The survey conducted of NC water and wastewater microbiology labs for their ability and interest to perform *C. perfringens* and coliphage analysis of reclaimed water and waste water samples was informative and indicated little lab capacity and little interest in performing these analyses. However, with new legislation recently passed that allows the use of type 2 reclaimed water in North Carolina as source water for potable reuse in drinking water, it is recommended that additional surveys be conducted to reevaluate interest in both coliphage and *Clostridium perfringens* methods.

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6 Appendices

6.1 Alphabetical Abbreviations and Symbols

ANOVA: Analysis of Variance

AP: Acid-phosphatase confirmation test

C. perfringens: Clostridium perfringens

CFU: colony-forming unit

CS: CP ChromoSelect agar medium

Dept.: Department

DI: Deionized water

E. coli: *Escheria coli*

ESP: enrichment spot plate

EPA: United States Environmental Protection Agency

Log phase: exponential growth phase of *E.coli*

mCp: Membrane *Clostridium perfringens* agar medium

$MgCl_2$: Magnesium Chloride

mL: milliliter

M: molar

µm: micrometer (micron)

µM:micromolar

MPN: Most Probable Mumber

NC: North Carolina

NCDENR: North Carolina Department of Environment and Natural Resources

PBS: Phosphate buffered saline

PFU: Plaque-froming unit

SAL: single agar layer

SF: Stormy Fermentation confirmation test

TNTC: Too numerous to count

TSA: tryptic soy agar

TSB: tryptic soy broth

TSC: Tryptose Sulfite Cycloserine

US EPA: United States Environmental Protection Agency

WWTP: Waste water treatment plant

6.2 Presentations, Publications, and Technology Transfer

This work was presented at the 2014 Water Resources Research Institute Annual Conference, the 2014 University of North Carolina Water Microbiology and Health Conference, and the 2014 American Society for Microbiology Annual General Annual Meeting.

There have been no publications from this work in the peer-reviewed literature to date.

A workshop and hands-on training session in methods for analysis of coliphages and *Clostridium perfringens* in reclaimed water was held in the laboratory of Dr. Mark Sobsey at the Gillings School of Global Public Health of the University of North Carolina in Chapel Hill on February 21, 2014. Representatives from these nine regional entities attended:
Durham County Department of Public Health
Eastern Band of Cherokee Indians
Town of Holly Springs
Johnston County Public Utilities
North Carolina DENR Division of Water Resources Water Quality Programs Laboratory
Onslow County
City of Raleigh Neuse River Laboratory
Secure Resources, PLLC
Shellfish Sanitation and Recreational Water Quality NC Division of Marine Fisheries Office

The purpose of the workshop was to introduce the most effective of the candidate methods for analysis of *Clostridium perfringens* bacteria and coliphage viruses in Type 2 reclaimed water to potential method users and other interested entities and individuals. Registered participants were provided with a document discussing fecal indicator microorganisms and assay methods prior to the meeting as well as bench sheets providing detailed step-by-step procedures for each of the candidate protocols. The workshop consisted of an opening presentation about fecal indicator organisms in general and two of the indicators for Type 2 reclaimed water in particular, *Clostridium perfringens* bacteria and coliphage viruses. Following a discussion period, the

candidate enumeration methods for both microorganisms were demonstrated by the three UNC students who had performed most of the sample analyses in the course of the project. The workshop was concluded with hands-on training for all of the participants with each of the candidate analysis methods. Arrangements were made for interested participants to receive continuing education unit credits for attending this workshop.

Participants were requested to fill out a questionnaire at the end of the workshop. The participants expressed an opinion strongly in favor of the use of the CP ChromoSelect Agar for the enumeration of *Clostridium perfringens* by the membrane filtration method, since the presumptive *C. perfringens* green colonies yielded by this medium were more distinct than the black colonies produced by the alternative candidate culture medium, tryptose sulfite cycloserine (TSC) agar. The participants favored the single agar layer technique for the enumeration of coliphage viruses over the most probable number enrichment-spot plate method by a slight margin.

6.2.1 Workshop and Hands-on Training – February 21, 2014

Background document:

Fecal Indicators and Methods to Detect them in Water and Wastewater for Workshop and Hand-on Training in Methods for Analysis of Coliphages and *Clostridium perfringens* in Reclaimed Water, February 21, 2014

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Introduction

Fecal indicator microorganisms and their importance

Fecal indicator microorganisms are microorganisms that are plentiful in the human and often the animal intestinal tract and are shed in large number in feces. Most of these fecal indicator microbes are not themselves harmful but they indicate the possible presence and concentrations of pathogenic (disease-causing) bacteria, viruses and protozoan parasites that can infect humans (and some animals) and pose human health risks of infection, illness and possibly death. There are many different bacterial, viral and protozoan pathogens potentially present in sewage and fecally contaminated water, and it is difficult, time-consuming, and expensive to test directly for the presence of such a large variety of pathogens. Therefore, we test for fecal indicators instead.

Measuring fecal indicator microorganism concentrations is a convenient way to determine how much microbial fecal contamination is in wastewater or water or how effective water and

wastewater treatment processes are in removing or destroying these microorganisms. It is believed that concentrations of fecal indicator microorganisms in water and wastewater are predictive of the concentrations of human fecal pathogens, the risks of exposure to such fecal pathogens and their associated risks of causing infection and disease. Based on this assumption, the presence and concentrations of fecal indicator microbes are measured either routinely or for special studies to monitor water and wastewater quality for management purposes.

Members of several bacteria groups present commonly in human and animal fecal waste are used as indicators of possible fecal or sewage contamination. Therefore, their presence in water, wastewater and treated wastewater suggests that pathogenic microorganisms (especially bacterial pathogens of intestinal or fecal origin) might also be present and that ingestion of other exposure to such water or wastewater might be a health risk. Sources of fecal contamination to surface waters include wastewater treatment plants, on-site septic systems, domestic, agricultural and wild animal manure, and storm runoff or by-pass and combined sewer overflows.

Fecal indicator bacteria types

The most commonly tested fecal bacteria indicators are total coliforms, fecal coliforms, and *Escherichia coli* (*E. coli*). *E. coli* is a specific bacteria species within the coliform/fecal coliform groups. There are many serotypes and strains of *E. coli*, most of which are harmless inhabitants

of the lower intestinal tract but some are pathogenic and can cause serious illness and death. The fecal coliforms are a broader group of related fecal bacteria (including E. coli) that are considered to be of mostly fecal origin. However, some of these bacteria may come from non-fecal sources and may occur in the environment from such sources as woody vegetation. The broadest group of coliforms is the total coliform group. While it includes *E. coli* and the fecal coliforms, the total coliform group also includes a variety of other bacteria that are often of non-fecal, environmental origins, such a vegetation and soil. Thus, the usefulness of total coliforms as an indicator of fecal contamination depends on the extent to which the bacteria species found are fecal and human in origin. As this is difficult to determine, it is preferred to test for fecal coliforms or, even better, just *E. coli* as more reliable evidence of fecal contamination.

Other fecal bacteria present in human and animal feces and present in large numbers in sewage are the fecal streptococci and enterococci. Like total coliforms and fecal coliforms, fecal streptococci may include species of bacteria that are from non-fecal sources in the environment. Enterococci are a subgroup within the fecal streptococcus group and are considered more specific to fecal contamination. Based on human health effects (epidemiological) studies, the US EPA recommends enterococci as the best indicator of health risk in salt water used for recreation and as a useful indicator in fresh water as well.

Another fecal indicator bacterium commonly present is human and animal fecal wastes and sewage is *Clostridium perfringens*. This is an anaerobic bacterium that grows well only under conditions where oxygen is absent (anaerobic or highly reduced conditions). It forms hardy spores that are resistant to water and wastewater treatment processes and persistent in the environment. It has been suggested as a possible indicator of the responses to treatment of hardy protozoan parasite cysts and oocysts of the intestinal pathogens *Cryptosporidium parvum* and *Giardia lamblia*.

Fecal indicator viruses: the coliphages

Coliphages are viruses that infect *E. coli* bacteria and possibly other similar bacteria. These viruses are also present in human fecal contamination, sewage and fecally contaminated water. As viruses, coliphages resemble human pathogenic viruses. They are similar in size, composition, persistence in water and wastewater and in their reductions by water and wastewater treatment processes. Like the coliform group of bacteria and the fecal streptococci/enterococci group of bacteria, coliphages are diverse. They belong to different taxonomic groups and differ in their size, shape, composition and structural organization. In this way coliphages are like the human enteric viruses, which are also taxonomically diverse and differ in their size, shape, composition and structural organization. Based on how they infect *E. coli* host cells, coliphages are divided into two main groups, somatic coliphages that attach directly to the outer cell layer and F+ male-specific coliphages that attach directly to the hairlike surface appendages, called pili of F+ male host cells.

Types of methods and test formats to quantify fecal indicators in water and wastewater

There are only a few basic methods and test formats for analyzing water and wastewater samples for fecal indicator microorganisms with culture-based techniques: (1) enumeration by membrane

filter methods, (2) enumeration by agar plating methods and (3) broth culture enrichments to quantify by most probable number methods.

Membrane filtration methods

The membrane filtration method involves filtering one or several different-sized portions of the water sample through membrane filters (with standard diameter and pore sizes) to retain bacteria/microbes on the filter surface, then placing each filter on a selective nutrient medium in a petri plate, incubating the plates at a specified temperature for a specified time period to allow for bacterial/microbial growth, and subsequently counting the colonies that have grown on the filter (See Figure 1)

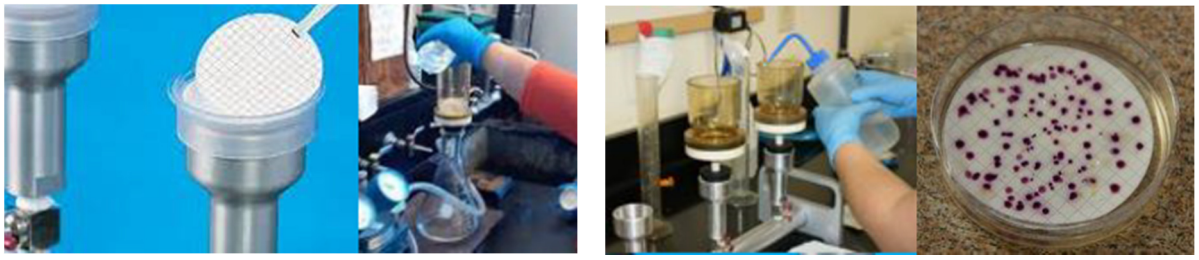

Figure 1. Membrane filter method to enumerate bacteria in water and wastewater

By counting the number of colonies of a distinctive appearance that grow on the membrane filter after incubation for the volume of sample filtered through the filter, it is possible to calculate the concentration of bacteria per unit volume of sample, usually expressed in number of colony-forming units of bacteria per 100 mL. This method varies for different bacteria types based on, for example, the nutrient medium type, incubation temperature, the number and types of incubations, etc.). The membrane filter method has also been adapted to the enumeration of coliphages, but that method will not be covered in this workshop. In this workshop the membrane filtration method will be applied to the enumeration of *Cl. perfringens* in wastewater and water and several candidate culture media for such enumeration will be compared.

Enumeration of coliphages in water and wastewater by single agar layer (SAL) method (pour plate plaque assay in agar medium-host cell culture)

Coliphages can be enumerated in water and wastewater samples by combining the water sample with *E. coli* host bacteria and molten culture medium and pouring the mixture into a culture plate. After overnight incubation, coliphages present that infected host cells produce localized areas of infection of the confluent growth of *E. coli* in the agar medium, resulting in the formation of clear zones of lysed *E. coli* cells called plaques. From the number of plaques counted in the culture plate and the volume of sample analyzed in the culture plate, the coliphage concentration is computed, usually per 100 mL of sample (see Figure 2.)

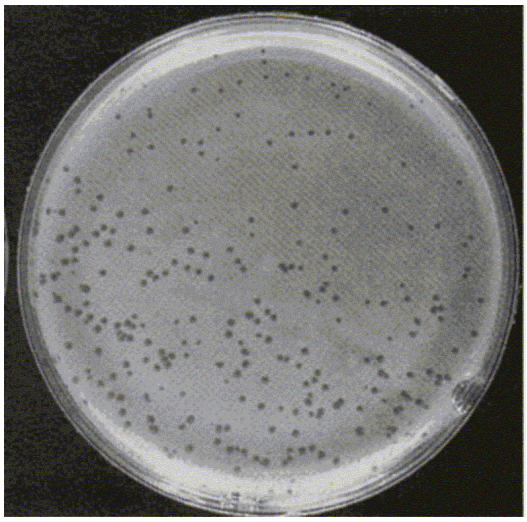

Figure 2. Coliphage plaques in a lawn of *E. coli* in a culture plate.

Quantal (multiple volume) broth culture methods for bacteria and coliphages in water

Fecal bacteria and coliphages can be quantified by multiple-volume broth culture methods. Traditionally, coliform, fecal coliform and *E. coli* bacteria have been quantified by such methods using multiple volumes in so-called fermentation tubes. The methods involve adding specified quantities of the sample to tubes containing a nutrient broth, incubating the tubes at a specified temperature for a specified time period, and then looking for the development of gas and/or turbidity that the bacteria produce and for some, a specific color change or a fluorescent product visible under long wavelength UV light (see Figure 3). The presence or absence of growth, gas, color or fluorescence in each tube is used to tally positive and negative tubes for each sample volume and calculate an index known as the Most Probable Number (MPN) concentration.

Figure 3. Multiple tube method to quantify bacteria by the most probable number (MPN) method

The MPN method has been adapted to the quantification of coliphages in water and wastewater. In this adaptation, a volume of water is supplemented with bacteria culture medium and *E. coli* host bacteria, and then this mixture is divided into different volumes in replicate. These volumes are incubated overnight to allow for viral infection, replication and lysis of bacterial host cells. If any coliphages are present in the volume of enrichment culture, the coliphages infect and lyse

the bacteria host cells to produce very high concentrations of the coliphage in the enrichment culture volume. After overnight cultivation, a small amount of each enrichment culture volume is

spotted onto the surface of a culture plate of agar medium containing *E. coli* host. After incubation, those spots from enrichments in which coliphages grew will show clearing (lysis) of the lawn of *E. coli* host bacteria in the plate. Based on the numbers of broth culture enrichment volumes that are positive and negative for coliphage lysis in their spot, the MPN concentration of coliphages is calculated (see Figure 4.)

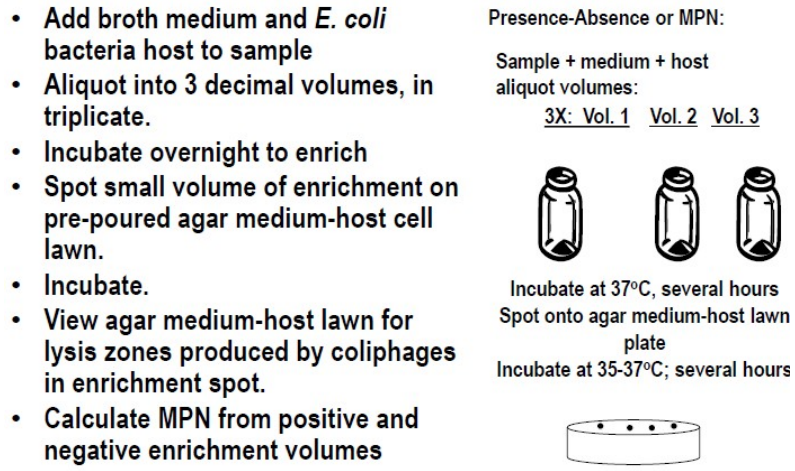

Figure 4. Enrichment-Spot plate MPN method to recover and quantify coliphages in large volumes of water and wastewater: quantitative EPA Method 1601

Fecal indicator microorganisms and the NC regulations for reclaimed water

N.C. is looking increasingly to reclaimed water as a water resource with many beneficial uses to better meet growing water needs, such as in agriculture and industry. In 2011 N.C. passed revised reclaimed water regulations (subchapter 02U – Reclaimed Water) (NC DENR, 2011) that expands reclaimed water uses in part by establishing a higher quality reclaimed water, type 2, for a wider range of beneficial uses. To address risks from disease-causing pathogens in human wastewater, the rules for Type 2 reclaimed water specify effluent quality and treatment performance requirements not only for fecal bacteria, such as *E. coli*, but also for coliphages as fecal indicator viruses and for the spore-forming bacterium *Clostridium perfringens* as a surrogate or indicator for protozoan parasite pathogens, such as *Giardia* and *Cryptosporidium*. Design criteria for wastewater treatment must achieve 6 $\log_{10}$ reductions of *E. coli*, 5 $\log_{10}$ reductions of coliphages and 4 $\log_{10}$ reductions of *Clostridium perfringens* and effluent quality for type 2 reclaimed water must meet a geometric mean of 3/100 mL for *E. coli* and 5/100 mL for both coliphages and *Clostridium perfringens*. For all of these indicators, and the daily maximum concentration is 25/100 mL.

This new rule for NC type 2 reclaimed water now creates the need for effective, practical, accessible and affordable methods to detect and quantify coliphages and *Clostridium perfringens* in wastewater and type 2 reclaimed water effluent in order to determine if effluent microbial quality standards and specified $\log_{10}$ reduction requirements as treatment design criteria are being met. Methods for coliphages and *Clostridium perfringens* have been developed and are described by US EPA and/or in Standard Methods for the Examination of Water and

Wastewater, both of which are sources of analytical methods accepted by the State of N.C. (APHA, 2005; US EPA 2001a; 2001), as well as by other sources (for example, the International Standards Organization). However, these available analytical methods for coliphages and *Cl. perfringens* are diverse, they were not specifically developed, performance-validated, written up in a specific form or approved by NC certification for application to NC type 2 reclaimed water. Therefore, the performance of the candidate methods for coliphages *and Cl. perfringens* for use to analyze reclaimed water has not been adequately evaluated, performance-validated, been well-documented and become certified for this context and use.

Also, new and improved methods for analysis of coliphages and *Clostridium perfringens* in water and wastewater continue to be developed, sometimes claiming improved performance and sometimes not supported by method performance validation data at all. Currently, potential users of coliphage and *Clostridium perfringens* analysis methods who want to apply them to N.C. type 2 reclaimed water or wastewater are faced with a wide and confusing range of options, none of which are either clear or well-established and adequately documented in performance for this purpose. Additionally, users, regulators and managers face uncertainties about which coliphages to measure and which methods to use to analyze for *Cl. perfringens*, as such details were not specified in the revised reclaimed water rule.

Candidate coliphage methods for reclaimed water

There are two main groups of coliphages, somatic, which infect host cells by attaching directly to the host cell outer layer, and F+ male-specific, which attach to the pili (fimbriae) surface appendages of only those host cells that are F+ male-specific (see Figure 5).

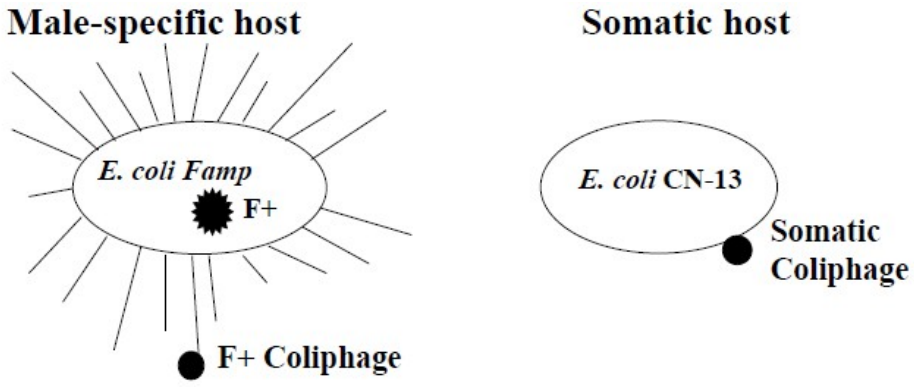

Figure 5. Left: Male-specific coliphage and host bacterium. Right: Somatic coliphage and host bacterium

Both groups of coliphages are abundant in raw sewage (about 10,000 to 100,000 per 100 mL), but they may differ in survival and they may be reduced to different extents different by wastewater treatment and disinfection processes. Little information is available on the presence of these two different groups of coliphages in type 2 reclaimed water or the effects of type 2 reclaimed treatment processes to reduce their levels in wastewater. Under the current rule and because of the uncertainty about which coliphage groups may persist in treatment process for reclaimed water, it

is probably prudent and most protective of public health to measure both coliphage groups in NC type 2 reclaimed water. Currently, these two main groups of coliphages are analyzed separately on different *E. coli* host bacteria. However, with the right choice of *E. coli* bacteria host, it is possible to measure both somatic and male-specific coliphages together on a single *E. coli* host, thereby reducing the workload and effort to detect and quantify "total" (F+ and male-specific) coliphages in NC type 2 reclaimed water and wastewater. Given the current lack of clarity of the NC reclaimed water rule on which coliphages to measure and the lack of guidance or regulatory requirement on what coliphage method(s) to use, it is probably advisable to measure at first all 3, (1) somatic coliphages, (2) F+ male-specific coliphages and (3) simultaneously both somatic and F+ male-specific coliphages, before making a decision about which coliphages and which methods for them are most appropriate for use under the rule.

Candidate *Clostridium perfringens* methods for reclaimed water

Clostridium perfringens are spore-forming anaerobic bacteria resent in raw sewage at about 10,000-100,000 per 100 mL. *Cl. perfringens* in water and wastewater are typically detected and quantified by membrane filtration methods using specific plating media. In the USA, the standard medium has been mCp agar (or broth), which has been modified by reducing the concentration of the expensive ingredient indoxyl β-D-glucoside to 60 mg/L (1/10 the initial recommended concentration) to reduce cost without affecting method sensitivity (Bisson and Cabelli, 1979; Armon and Payment, 1988). However, using modified mCp medium, *Cl. perfringens* detection requires a confirmatory step in which the bacterial colonies on the plates are exposed to ammonium hydroxide fumes to determine if they produce a characteristic pink color that confirms their identity. This step is extra work and must be done under a fume hood, making it less practical for routine use in many labs. In addition, the mCp medium contains several heat-sensitive ingredients that must be made up sterile and then added aseptically after the medium base is autoclaved, which is another inconvenience that adds cost to performing the analysis. Furthermore, studies document mCp medium as inferior to other media for detection of *Cl. perfringens* in water by membrane filtration. Sartory et al. (1998) documented that TSC (Tryptose Sulphite Cycloserine) agar gave better recoveries of *Cl. perfringens* than did mCp medium, resulting in the selection of TSC medium for the ISO method for *Cl. perfringens* now widely used in many other countries. TSC gives distinctive black *Cl. perfringens* colonies on TSC medium and its superior detection to mCp medium has been reported by others (Arujo et al., 2004). More recently another medium for *Cl. perfringens* detection in water by membrane filtration has been developed, CP ChromoSelect agar (Sigma Aldrich; Fluka), that is easy to make, gives distinctive green colonies of *Cl. perfringens* and provides detection comparable to or better than other methods against which it has been compared. Base on their reported effective performance and/or their acceptance as standard methods for official use, these three different media appear to be preferred candidates to consider and further evaluate for detection of *Cl. perfringens* in NC type 2 reclaimed water and wastewater.

Another issue to consider for detecting *Cl. perfringens* in NC type 2 water and wastewater is whether to detect spores only or spores plus vegetative cells. For the use of *Cl. perfringens* as a protozoan parasite indicator or surrogate, it is the *Cl. perfringens* spores that are most like the cysts and oocysts of protozoan parasites, in terms of environmental persistence and resistance to water and waste treatment processes. However, some studies of water and wastewater microbial

reductions by treatment processes have analyzed for one or the other of *C. perfringens* spores only or spores plus vegetative cells or for both spores only and spores plus vegetative cells (Sartory et al., 1986; Payment et al., 1993; Wen et al, 2009). To detect the spores only, the water samples are first heated to a temperature between 60 to 80 degrees C for 15 to 30 minutes prior to analysis in order to kill off the vegetative cells. This heat treatment leaves only the spores to be detected by membrane filtration and culture. For current purposes, the measurement of both spores and vegetative cells as well as spores only seems advisable, given the current lack of information about the occurrence and alternative treatment reductions of *Cl. perfringens* in wastewater and NC type 2 reclaimed water.

Development of Standard Operating Procedures for detection of *C. perfringens* and coliphages in type II treated wastewater (reclaimed water)

Methods protocols or standard operating procedures (SOPs) specific for the detection of coliphages and *Cl. perfringens* in type 2 NC reclaimed water or wastewater are not available and none have been submitted to or formally accepted by the State certification program for such analysis. Hence, there is a need for a systematic and thorough study to (1) identify and screen the established, candidate and emerging methods for analysis of coliphages and *Clostridium perfringens* in N.C wastewater and reclaimed water, (2) evaluate the best of these methods for their performance and other characteristics (complexity, time to results, costs, etc.), (3) subject the best of these methods to a multi-laboratory performance validation study, (4) write up the methods in readily accessible and end-user friendly forms as protocols or SOPs, (5) submit the methods to the N.C. methods certification program, (5) seek approval of the methods through the US EPA Alternative Test Procedure (ATP) Program and (6) make the methods known and available to potential users in N.C. though workshops and other means of outreach, communication and dissemination. This workshop and hands-on training is intended to address some of these needs on coliphage and *Cl. perfringens* detection and quantification methods.

Coliphage bench sheet:

Workshop and Hands-on Training – February 21, 2014
University of North Carolina-Chapel Hill
Department of Environmental Sciences and Engineering
Laboratory of Mark D. Sobsey
Support by the NC Water Resources Research Institute

Quantification of Somatic and Male-specific Coliphages in Water and Wastewater

Escherichia coli (*E. coli*) and other "coliform" bacteria are plentiful in feces and sewage. Many different viruses capable of infecting *E. coli*, called coliphages, are also plentiful in feces and sewage. Some of these coliphages have become favorite subjects and tools for virus research in the areas of biochemistry, molecular biology and genetics. The natural history or ecology of these coliphages and their usefulness as indicators of fecal contamination are becoming better understood. Various coliphages can be readily detected and quantified in raw, domestic sewage, treated sewage effluents, reclaimed water, sewage polluted water, foods and other environmental samples. They are considered useful viral indicators of fecal contamination of water, foods and other environmental media. Some groups of coliphages also have potential uses to track or identify sources of fecal contamination as being either human or non-human in origin. Coliphage detection can be done by (1) direct plating on their host bacteria using the plaque technique, which is analogous to a "pour plate" technique for bacteria, (2) by enrichment in broth culture followed by confirmation of positive broth culture by transferring some of it to a host bacteria lawn in agar medium and incubating for development of a zone of lysis and by several alternative membrane filter methods.

In this workshop we will demonstrate two methods to detect and quantify somatic and male- specific coliphages in water and wastewater: the single-agar layer (SAL) plaque assay and the enrichment- spot plate Most Probable Number (MPN) method.

Coliphage procedure - **Two-step Enrichment-Spot Plate Method**
A Lab Benchsheet Based on EPA Method 1601
EPA Method 1601: http://www.epa.gov/nerlcwww/documents/1601ap01.pdf

General:

***E. coli* Host**	**Detectable Coliphages**	**Antibiotics***
Famp	F+ male specific	Streptomycin-ampicillin
CN13	Somatic coliphages	Nalidixic Acid
CB390	Both	Ampicillin

***100x antibiotic stock solutions and their preparation**
0.15g ampicillin sodium salt + 0.15g streptomycin sulfate into 100 mL DI water; sterile filter with 0.22µm filter; aliquot in to small volumes; store frozen.
1g nalidixic acid sodium salt into 100mL DI water; sterile filter with 0.22µm filter; aliquot into small volumes; store frozen.
0.15g ampicillin sodium salt into 100 mL DI water; sterile filter with 0.22µm filter; aliquot into small volumes; store frozen.

Day 1:
Grow up overnight hosts in 25mL TSB (tryptic soy broth)
Materials required:
- 25mL 1x TSB
- Appropriate 100x antibiotics
- Sterile wooden rods (single use only), or sterile metal loop for cell transfers (must be flamed between uses).
- 3 – 50mL sterile shaker (Erlenmeyer) flasks (sterile 100-mL bottles can substitute)

Procedure:
- Add 25mL TSB and 0.25mL antibiotic to flask labeled with specific *E. coli* host.
- Using a sterile wooden rod or sterile metal loop, scrape a small amount of frozen cells from a frozen stock of *E. coli* and inoculate the TSB with the cells.
- Cap the flask or bottle, and incubate at 37°C on a shaker tray (90-100rpm) for 18-24 hours (overnight).

Day 2: (NOTE: The work of this day is what the workshop will focus on for this method)

Turn on a water bath to 45-48°C.
Start log phase *E. coli* host cultures (4 hours prior to assay):
Materials:
- 50mL 1x TSB
- Appropriate 100x antibiotics

- 3 – 125mL sterile bottles or shaker flasks
- Overnight *E. coli* host stock

Procedure:

- Set up and label 3 - 125mL bottles or flasks with each *E. coli* host, date & time, log phase, and initials.
- Add 50mL TSB and 0.5mL appropriate antibiotic to each bottle of flask.
- Add 0.5mL mixed overnight culture of *E. coli* host to appropriate bottles.
- Incubate at 37°C on shaker tray for 4-5 hours.

Prep Work / Labeling

Materials:

- 150mm petri plates (For 3 *E. coli* hosts, 3 plates):
  - One plate per *E. coli* host
- Enrichment spot plate agar tubes
  - 3 - 50mL tubes, 1 per host
- Enrichment spot plate tubes
  - 3 -50mL tubes per host, sterile
  - 3 - 8mL tubes per host, sterile
  - 3 – 1.5mL tubes per host, sterile
- 3 – 125mL sterile bottles or flasks

Procedure:

- Draw square grids on 3 plates for Enrichment Spot Plate Assay (Need at least 9 spots to plate grids of at least 3 x 3). Within the grid, label three spots each as 32mL, 3.2mL, and 0.32mL. Label plate with enrichment assay, host, and date.
- Label enrichment tubes: For each host label 3 - 50mL tubes, 3 - 8mL tubes, and 3 - 1.5mL tubes. Make sure to label each tube with host and volume.
- For the 3 – 125mL sterile bottles or flasks, 3 bottles with enrichment, host, and water sample type.

Make agar (1.5 hours before assay):

Label on bottle	**Minimum Bottle size**	**Agar volume**	**Agar Strength**
Enrichment assay	250 mL	90 mL	1x

Autoclave at 121°C for 20 minutes on liquid cycle, with caps slightly loosened. After cycle, transfer agar bottles to 45-48°C water bath until assay. Make sure water level in the waterbath is at least as high as the level of the agar in the bottle.

Enrichment Assay – step 1

Materials:

- Labeled plates, agar tubes, enrichment tubes, and 125 mL sterile bottles from previous step
- Log phase *E. coli* hosts (*E. coli* Famp, *E. coli* CN-13 and *E. coli* CB390)
- Appropriate 100X antibiotics
- 4M $MgCl_2$
- 10x TSB

Procedure: For each labeled bottle:
1. Measure 100 mL of water sample into a sterile 125 mL or larger bottle labeled with the sample designation and the host *E. coli* strain.
2. Add 1.25 mL of 4M magnesium chloride
3. Add 5 mL of 10X tryptic soy broth.
4. Add 1 mL of appropriate 100X antibiotic solution.
5. Add 0.5 mL of a log phase *E. coli* host culture.
6. Thoroughly mix. Immediately dispense 0.32 mL, 3.2 mL and 32 mL into labeled triplicate sterile tubes. This step must be completed within 10 minutes to avoid the possibility that coliphages may infect *E. coli* cells and form progeny viruses before being dispensed in the individual volumes for the MPN assay format.
7. Incubate the tubes overnight at 37°C. Mixing on a rotary shaker is not required.

Make enrichment-spot plate agar plates
Plates may be made after agar has cooled to ~45°-48°C in water bath.
1. Pipet 3.0 mL of each log phase *E. coli* host strain culture into separate sterile, labeled 50 mL centrifuge tubes.
2. Add 0.3 mL of appropriate 100X antibiotic solutions to tubes.
3. Slowly pipet 30 mL of molten (tempered to 45-48 ºC) TSA into one tube, and mix thoroughly but gently to avoid producing bubbles.
4. Gently pour the agar mixture into a sterile 150 mm petri dish labeled with the name of that host strain.
5. Repeat for each E. coli host strain. Allow plates to harden and dry for 15 minutes.

Store plates inverted at 4°C overnight.

Day 3:

Enrichment Assay -step 2
- Take enrichment spot plates out of 4°C refrigerator; let warm to room temperature (warming under a biological safety hood is preferred).
- Label enough 1.5mL centrifuge tubes to match quantity of other enrichment tubes (3 per host-volume combination). Label with host and enrichment volume (ex: 'Famp, 32mL')

1. Take a 0.32mL sample from each enrichment tube (there are 9 total per sample) and transfer to corresponding 1.5mL, labeled centrifuge tube.
2. Microfuge samples at 10k rpm for 5 min, making sure to balance the microfuge.
3. From the supernatant, spot plate 10uL of each aliquot onto a grid spot on the appropriate enrichment plate (preferably do this under the hood), changing pipette tips between every spot.
4. Allow spots to soak into agar for 15-20 minutes, or until spots can no longer be seen clearly on the agar surface. Cover and invert plates, incubate at 37ºC for 18-24 hours.

Day 4:
Read enrichment results

Positive zones of lysis will be clear or will show a clear ring around the edge of the spot. Sometimes bacteria have grown within the spot and the lysis caused by coliphages appears as a clear "halo" around the central region of bacteria growth within the spot. Record results, input to an MPN calculator and determine statistical MLE concentration (MPN concentration).

Coliphage procedure - **Single Agar Layer Method**

A Lab Bench sheet Based on EPA method 1602
EPA method 1602: http://www.epa.gov/nerlcwww/documents/1601ap01.pdf

General:

***E. coli* Host**	**Detectable Coliphages**	**Typical Plaque Appearance**	**Antibiotic***
Famp	F+ male specific	Small, circular	Strep-amp (streptomycin and ampicillin
CN13	Somatic coliphages	Medium to large, circular	Nalidixic Acid
CB390	Both	Varies	Ampicillin

*See footnote to table of two-step enrichment-spot plate method

Day 1:

Grow up overnight *E. coli* hosts in 25mL TSB
Materials required:

- 25mL 1x TSB
- Appropriate 100x antibiotics
- Sterile wooden rods (single use only), or sterile metal loop for cell transfers (must be flamed between uses).
- 3 – 50mL sterile flasks or bottles

Procedure:

- Add 25mL TSB and 0.25mL antibiotic to flask labeled with *E. coli* host.
- Using a sterile wooden rod or sterile metal loop, scrape a small amount of frozen *E. coli* cells from a frozen stock and inoculate the TSB with the cells.
- Cap the flask or bottle, and incubate at 37ºC on a shaker tray (90-100 rpm) for 18-24 hours (overnight).

Day 2: (NOTE: The work of this day is what the workshop will focus on for this method)

Turn on two waterbaths, one set to 37ºC and the other to 45-48ºC.

Start log phase *E. coli* hosts (4 hours prior to assay):

Materials:

- 50mL 1x TSB
- Appropriate 100x antibiotics
- 3 – 125mL sterile bottles or flasks
- Overnight *E. coli* host stock

Procedure:

- Set up and label 3 - 125mL bottles or flasks with each *E. coli* host, date & time, log phase, and initials.
- Add 50mL TSB and 0.5mL appropriate antibiotic to each bottle or flask.
- Add 0.5mL mixed overnight *E. coli* host culture to appropriate bottles or flasks.
- Incubate at 37°C on shaker tray for 4-5 hours.

Prep Work / Labeling

Materials:

- 150mm petri plates (For 3 *E. coli* hosts, need total of 18 plates):
  - For SAL: 5 plates per *E. coli* host, 1 plate per negative control *E. coli* host
- SAL tubes
  - 3 - 50mL tubes, 1 per *E. coli* host
- Negative Control tubes
  - 3 - 50mL tubes, 1 per *E. coli* host
- 3 – 125mL sterile bottles or flasks
- PBS (phosphate buffered saline) or Standard Methods phosphate buffer (Part 9050)

Procedure:

- Label SAL plates in sets of 5 (5 replicate plates per *E. coli* host) with SAL, *E. coli* host, date
- Label 3 negative control plates with negative control, *E. coli* host, date
- For the 3 – 125mL sterile bottles or flasks, label 3 bottles with SAL, *E. coli* host, water sample type

Label on Bottle or Flask	**Minimum Bottle or Flask Size**	**Agar volume**	**Agar Strength**
CN13	250mL	100mL	2x
Famp	250mL	100mL	1x
CB390	250mL	100mL	2x
Negative Control	250mL	60mL	2x

Autoclave at 121°C for 20 minutes on liquid cycle, with caps slightly loosened. After cycle, transfer agar bottles to 45-48°C water bath until assay. Make sure water level in the

waterbath is at least as high as the level of the agar in the bottles or flasks.

Negative Control plates

1. Warm a 125 mL bottle or flask with 100mL sterile PBS or DI water and thermometer to 37°C.
2. Transfer the bottle to 45-48ºC waterbath. Time how long until PBS reaches 43ºC. Record this time, as it will serve as a temperature control for the other SAL samples that will go through this heating process.
3. In the 50 mL *E. coli* host negative control tube combine 20 mL of the PBS, 2 mL of the log phase host culture, 0.4 mL of the appropriate antibiotic solution, 0.1 mL of 4M magnesium chloride and 20 mL of 2x TSA. Gently mix, and pour into a single 150 mm petri dish. Repeat this step for each *E. coli* host, for a total of 3 negative control plates.

Single Agar Layer Assay

Materials:

- Labeled 125mL bottles or flasks, sterile
- Labeled plates (5 per host), sterile
- Labeled 50mL centrifuge tubes, sterile
- Log phase *E. coli* host broth cultures
- 100x antibiotics
- 4M $MgCl_2$

Procedure:

1. Measure 100mL water sample into 3 separate bottles for flasks. Add 2mL appropriate antibiotic, and 2.5mL $MgCl_2$.
2. Transfer bottles or flasks to the 37ºC water bath. Allow bottles to equilibrate to temperature.
3. Transfer a bottle or flask from 37ºC water bath to the 45-48ºC water bath. Once it reaches 43ºC (as determined by the timing of the negative control PBS sample), remove it from the water bath, add 10mL log phase *E. coli* host, and add this to the appropriate labeled 100mL volume of 2x molten agar in a bottle or flask.
4. Gently mix (swirl), and pour approximately 42 mL aliquots into each of the 5 labeled 150mm plates. Measure out the 42mL aliquots by using the labeled SAL + host 50mL tube to estimate the volume. Make sure all of the sample + agar medium and E. coli host mixture is poured into the plate. Mix plate very gently by tilting and swirling to avoid bubbles, which can mimic or obscure plaques.
5. Allow plates to harden and then dry for 15 minutes, preferably under the hood. Making sure plates are dry will help reduce condensation in the plates, which can smear or mimic plaques on the agar.
6. Repeat steps 1-3 for each *E. coli* host
7. Cover, invert, and incubate plates at 37ºC for 18-24 hours.

Day 3:

Read and Record SAL Results

Record total plaques observed on each plate. Plaques may vary in size, but should all be

circular. Add up plaques from all plates for each *E. coli* host and the corresponding sample volumes to determine the plaque-forming units (PFU)/100mL observed using each *E. coli* host.

Clostridium perfringens bench sheet:

Clostridium perfringens Membrane Filtration Procedure
CP ChromoSelect, TSC and mCp agars

Based on and adapted from US EPA Method 1103.1
(http://www.epa.gov/nerlcwww/documents/1103_1sp02.pdf)

The enumeration of *Clostridium perfringens* in water and wastewater by a simple membrane filtration method was first described in 1979 by Bisson and Cabelli of US EPA. They reported the development of a new medium called m-CP for use in enumerating *Cl. perfringens* in water by membrane filtration. However, the use of m-CP agar has disadvantages, including the need to expose the colonies to ammonia using ammonium hydroxide, which prevents subculturing the *Cl. perfringens* colonies, and it is hazardous to handle. The red color of the *Cl. perfringens* colonies also tends to fade after exposure to the ammonia, which also compromises confirmation. Recent studies suggest that m-CP medium may not be as good as other media for *Cl. perfringens* enumeration in water, such as the widely used Tryptose–Sulphite-Cycloserine (TSC) agar medium. The enumeration of *Cl. perfringens* by ISO methods is based on the use TSC agar and m-CP agar was rejected for use. However, TSC agar has been reported to give excessive and variable blackening of the peripheral colonies on membranes, which makes colony counting at lower dilutions difficult and leads to false positives. More recently, a chromogenic medium, CP Chromo Select, has been developed to detect *Cl. perfringens* by membrane filtration. It gives distinctive green colonies of *Cl. perfringens* and the agar is reported to be more reliable and easier to handle than m-CP and TSC agars. The green color does not diffuse in the agar medium and confirmation is not required since the green coloration is specific for *Cl. perfringens*.

General

Agar	**Detectable Bacteria**	**Supplements**
CP ChromoSelect	*Cl. perfringens*	D-cycloserine
TSC	*Cl. perfringens*	D-cycloserine
mCp	*Cl. perfringens*	D-cycloserine, polymyxin B sulfate, ferric chloride, phenolphthalein diphosphate, Indoxyl-β-D-glucoside, mCp Selective Supplement

Day 1
Prepare agar plates for assay
Materials required:
- 60 mm petri dishes
- 500 mL media storage bottles

- 10 mL pipets
- TSC agar base
- CP ChromoSelect agar base
- mCp agar base
- D-cycloserine
- Deionized water
- 4.5% ferric chloride
- 0.5% phenolphthalein diphosphate
- Indoxyl β-D-glucoside
- mCp Selective Supplement
- Sterile H2O

Procedure:

TSC

- Weigh out 3.9 g/100mL TSC agar base on weighboat and dispense into 500 mL media storage bottle
- Using a graduated cylinder, pour appropriate amount of water (i.e. 100 mL for 3.9g) into storage bottle and shake or swirl until base is completely dissolved
- Autoclave on liquids setting for 15 minutes
- Remove bottle from autoclave and place in water bath to cool at ~50 C.
- Once, cool enough to touch (~15 mins), add .04g/100 mL of D-cycloserine and swirl until dissolved.
- Under a hood or using aseptic technique at the bench, pipet 5 mL of agar into 60 mm petri dishes
- store upside down in plastic bag in cold room until use
-

CP ChromoSelect

- Weigh out 6.28 g/100mL TSC agar base on weighboat and dispense into 500 mL media storage bottle
- Using a graduated cylinder, pour appropriate amount of water (i.e. 100 mL for 6.28g) into storage bottle and shake or swirl until base is completely dissolved
- Boil on hot plate and then place in water bath to cool at ~50 C.
- Once, cool enough to touch (~5-10 mins), add 0.04g/100 mL of D-cycloserine and swirl until dissolved.
- Under a hood or with aseptic technique at the bench, pipet 5 mL of agar into 60 mm petri dishes
- store upside down in plastic bag in cold room until use
-

mCp

- Weigh out 7.11 g/100mL mCp agar base on weighboat and dispense into 500 mL media storage bottle
- Using a graduated cylinder, pour appropriate amount of water (i.e. 100 mL for 7.11 g) into storage bottle and shake until base is completely dissolved
- Autoclave on liquids setting for 15 minutes
- Remove bottle from autoclave and place in water bath to cool at ~50 C.
- Once cool enough to touch (~15 mins), add 200 µl 4.5% ferric chloride/100 mL

agar, 2 mL .5% phenolphthalein diphosphate/100 mL agar, .8mL Indoxyl-β-D-glucoside/ 100mL agar, and 0.4mL mCp Selective Supplement/ 100mL agar
- Under a hood or using aseptic technique at the bench, pipet 5 mL of agar into 60 mm petri dishes
- Store upside down in plastic bag in cold room until use

Day 2

If analyzing pasteurized sample, turn on water bath to 65 C

Membrane Filtration Assay

Materials Required:
- One sterile 300 mL magnetic filter funnel per sample
- One 1-L filtration flask with thick-walled vacuum tubing
- One pair flat-bladed forceps
- 100% ethanol
- Millipore HAWG047S6 filter membranes (or equivalent), sterile, 0.45 um pore size, 47 mm diameter, six per sample plus two negative controls
- 60 mm petri dishes, each containing 5 mL of agar medium, six per sample plus two negative controls.
- phosphate-buffered saline (PBS) or Standard Methods phosphate buffer
- Anaerobic jar(s) with lid and clamp OR anaerobic bag
- Cello-Seal sealing grease (only necessary for jars)
- Anaerobic atmosphere - generating envelope(s)
- Dry Anaerobic Indicator Strip(s)
- Bunsen Burner
- Vacuum Source
- 44.5 C incubator

Pasteurization (optional):
- If pasteurizing, turn on water bath to 65 ^{o}C.
- Remove caps from bottles, dispense desired sample volumes into storage bottles of equal size, ensuring equal volumes in each bottle used (i.e. 3 bottles of 100 mL or 1 bottle of 300 mL), place caps back on bottles
- Dispense equivalent amount of sample or deionized water into another storage bottle of equal size, cover bottle opening with aluminum foil
- Place the bottles in the water bath and insert thermometer through foil covered control bottle
- Wait for control bottle to read 65^{o} C and then let sit for 15 minutes, Remove samples from water bath and place in a tub full of ice, samples are now ready to be used in membrane filtration procedure, only *Cl. perfringens* spores will be detected in analysis

Procedure:
- Label the bottoms of prepared agar dishes with the sample designation and the volume to be filtered.
- Place a sterile filter funnel into a filtration flask and connect the flask to a vacuum source.

- Place flat-bladed forceps into a small beaker with 100% ethanol so that the tips are submerged.
- Sterilize forceps over Bunsen burner and allow for them to cool for several seconds, then carefully use them to remove a filter membrane from packaging
- Remove upper reservoir from filter funnel and place the membrane on the base of the filter funnel with forceps; replace the reservoir onto the base
- Pipet 10 mL of PBS (or Standard Methods phosphate buffer) onto membrane and filter apparatus, open vacuum line until all PBS (or Standard Methods phosphate buffer) has run through, remove membrane filter with sterile forceps and place onto one of the agar plates as a control
- Replace a new membrane filter on the apparatus using the same technique as before
- Starting with most dilute sample, pipet or pour appropriate volume of water sample in the reservoir, and open the vacuum line as before, Once the sample filtration is complete, rinse the walls of the reservoir with 5 mL of sterile phosphate-buffered saline (or Standard Methods phosphate buffer) to wash any residual sample through the filter. Shut off vacuum
- Using sterile forceps, slowly and carefully remove the membrane, and place it, gridded side up, on the layer of agar in a labeled 60 mm petri dish. To avoid air pockets between the agar and the membrane that can block nutrient flow through the membrane to the bacterial colonies on the upper membrane surface, apply the membrane slowly in a rolling motion from one side of the dish to the other. If air pockets are observed, the filter can be partially or completely re-positioned using the forceps.
- Sterilize the forceps by dipping into absolute ethanol contained in a small beaker, then exposing briefly to alighted Bunsen burner to set the ethanol alight. Allow to cool for several seconds
- Repeat previous five steps until all replicate subsamples of all volumes of a single sample have been filtered.
- Remove the filter base and reservoir and replace with another sterile filter funnel assembly before proceeding with the next sample
- Prepare two negative control samples as described above immediately after installing the first and last filter funnel assembly before the first and last samples are processed.
- Place the inverted petri dishes in an anaerobic jar or bag. If using jar, ensure the upper rim of the jar is lightly coated with Cello-Seal to make a gas -tight seal, preventing atmospheric oxygen from subsequently entering. Wet the blue tip of one dry anaerobic indicator strip with deionized water, and place it on top of the piled petri dishes so that the blue tip of the strip is exposed to the atmosphere on all sides, and is visible from the outside of the jar or bag. Open the foil container of a GasPak Anaerobe Container System envelope, and slide down one side of the jar or bag without disrupting the dishes. Promptly close the jar lid with a twisting motion to spread Cello-Seal grease or seal the anaerobic bag. If using jar, place clamp over jar lid, and seal firmly, but not so tight as to crack the lid.
- Place jar or bag with sample plates in 44.5 ^{o}C incubator overnight

Day 3

Read results of membrane filtration

- Confirm system incubated overnight anaerobically, previously blue tip of indicator strip should appear white
- Count colonies on TSC agar plates. Presumptive positive colonies will be black and have small yellow rings around them. Presumptive negative colonies will be yellow or yellow with faint gray spot in them. Count colonies on each membrane and record counts.
- CP ChromoSelect agar - Open anaerobic jar or bag and remove GasPak, replace open jar or bag into incubator for ~1 more hour, then count and record colonies on each membrane. Presumptive positive colonies will be of greenish hue, presumptive negative colonies will be other colors including purple, blue, turquoise, or mix of green and purple.
- mCp agar – Pour ammonium hydroxide into a plastic or glass container. Expose plates one at a time to ammonium hydroxide fumes for 30 seconds-1.5 minutes until colonies turn pink. Colonies that turn pink when exposed are presumptive positive colonies. All other colonies are presumptive negative. Count and record pink colonies.

Appendix 3. Composition and description of *Clostridium perfringens* culture media

In this project three different growth media were evaluated for the detection of *Clostridium perfringens* in reclaimed water by the membrane filter method. The three media have somewhat different compositions and provide detection of *Cl. perfringens* colonies based on distinctive colony color. In the case of one of the media, mCp agar, there is an added step of treating the colonies with ammonium hydroxide fumes to elicit a distinctive color change that is diagnostic for *Cl. perfringens*. The compositions of the three different media are shown in the table below.

Component	Tryptose Sulfite Cycloserine Agar	CP *ChromoSelect* Agar	Modified mCP Agar
Tryptose	15	20	30
Soy peptone	5	5	none
Yeast extract	5	15	20
Sucrose	none	3	5
Magnesium sulfate + 7 H_20	none	0.1	0.1
Ferric ammonium citrate	1	0.2	none
L-cysteine hydrochloride	none	1	1
Sodium disulfite	1	none	none
Tris buffer	none	1.8	none
Chromogenic mixture	none	1.73	none
Bromocresol purple	none	none	0.04

Agar	12	15	
Total grams of components per liter	39	62.8	71.1
Supplements			
D-cycloserine	0.4	0.4	0.4
Polymyxin B sulfate	none	none	0.025
Phenolphthalein biphosphate, tetra sodium salt	none	none	0.1
Ferric chloride + 6H2O	none	none	0.09
Indoxyl-ß-D-glucoside	none	none	

Description of Ingredients in the *Clostridium perfringens* media

Nutrients

Tryptose and peptone are enzymatic digests of protein which contain high concentrations of amino acids, trace elements and vitamins, all necessary for growth and replication of bacteria.

Peptone contains smaller peptide molecules, while tryptose contains more higher-weight peptides.

Yeast extract is a water-soluble extract of autolyzed yeast cells which is processed in a way to preserve B-complex vitamins. It also contains amino acids. Yeast extract is added to bacteriological media to stimulate growth.

Sucrose is a sugar which can be anaerobically fermented by *Clostridium perfringens*, forming acidic metabolic products which cause bromocresol purple to change to a yellow color. Many other *Clostridia* species cannot ferment sucrose, so those colonies are not yellow. Sucrose is included in mCP agar to provide differentiation of (yellow) *Clostridium perfringens* colonies from most other *Clostridia* species.

Salts

Magnesium sulfate is a component of CP ChromoSelect Agar and mCP agar because it has been found that the presence of magnesium ions enhances growth of *Cl. perfringens*.

Ferric ammonium citrate and ferric chloride are sources of iron, which is another enhancer of *Cl. perfringens* growth. Ferric ammonium citrate plays a major role in TSC agar. *Cl. perfringens* possesses sulfite reductase, an enzyme which reduces sodium disulfite, another component of TSC agar, to sulfide. Iron ions combine with sulfide to produce black colonies, which are counted as presumptive *Clostridium perfringens* colonies.

L-cysteine hydrochloride is a reducing agent which lowers the redox potential of the medium, enhancing growth of anaerobic bacteria like *Clostridium*.

A Tris buffer is included in CP ChromoSelect agar to stabilize the pH of the medium, since *Cl. perfringens* growth begins to be inhibited above pH 7.6.

Agar

Agar is a gelling agent derived from a polysaccharide of red algae. The standard concentration of agar in microbiological media is 1.5% or 15 grams per liter.

Selective agents

D-cycloserine is a broad-spectrum antibiotic which inhibits the synthesis of bacterial cell walls. It is effective against gram-negative bacteria, such as E. coli and other enteric bacteria.

Polymyxin B sulfate is another broad-spectrum antibiotic which is effective against many gram-negative bacteria like Pseudomonas and enteric bacteria, except for Proteus. It binds to and increases the permeability of bacterial cell membranes, causing uncontrolled water uptake and cell death. Polymyxin B is used in combination with D-cycloserine in mCP agar to inhibit a wider range of non-*Clostridial* bacteria. Two other selective conditions are utilized to inhibit non-*Clostridium* bacteria. The agar dishes are incubated in an anaerobic atmosphere and at an elevated temperature (44.5°C).

Chromogenic indicators

The composition of the CP ChromoSelect chromogenic mixture is a trade secret, described as containing "enzyme substrates, inhibitors and different promoters to protect injured cells, to improve recovery rate and to enhance growth." *Clostridium perfringens* colonies grown anaerobically on CP Chromoselect Agar for 24 hours at 44°C will be green in color, before and/or after aerobic incubation at 44° for an additional hour.

Bromocresol Purple is a pH indicator molecule which is yellow below pH 5.2 and purple above pH 6.8. It is used in mCP agar to indicate sucrose fermentation by *Clostridium perfringens*, which form yellow colonies.

Clostridium perfringens lacks an enzyme, ß-D-glucosidase, possessed by other *Clostridia* species. Bacteria that do possess this enzyme can hydrolyze the colorless substrate indoxyl-ß-D-glucoside, yielding indigo blue. Phenolphthalein is another pH indicator, being colorless below pH 8.2, and dark pink or fuchsia above pH 10.0. Phenolphthalein biphosphate is incorporated into mCP agar to act as a substrate for the enzyme acid phosphatase, which is possessed by *Cl. perfringens*, but not by certain other *Clostridia* species. When yellow presumptive *Cl. perfringens* colonies are exposed to ammonium hydroxide fumes following overnight incubation, cell colonies possessing the acid phosphatase enzyme will cleave the phosphate ions from phenolphthalein, allowing that molecule to turn pink in the presence of the highly alkaline ammonia fumes. This

reaction confirms that those colonies which turned from yellow to pink are *Cl. perfringens*. Colonies which remain yellow are other *Clostridia* species.

To summarize, *Clostridium perfringens* colonies grown on mCP agar, and exposed to ammonia fumes if yellow, have the following appearances due to the various combined color reactions:

	Sucrose fermentation +	Glucoside hydrolysis	Acid phosphatase activity
Cl. perfringens	positive = yellow +	negative = still yellow	positive = pink-red
Other Clostridia	positive = yellow +	negative = still yellow	negative = still yellow
Or	positive = yellow +	positive = blue/green	Not done
Or	negative = colorless +	positive = purple	Not done

6.3 Contingency Tables for *Clostridium perfringens* Analysis

Table 17: Summary of false-positivity Results from Confirmatory AP and SF Tests on Presumptive *C. perfringens* Colony Isolates of Pasteurized and Unpasteurized Samples Analyzed on TSC, CS and mCp Agar Media by Chi-square Analysis

Presumptive positive	Rate of false-positivity	Chi-square value	p-value
TSC	0.18	1.994	0.1579
CS	0.18	1.994	0.1579
mCp	0.19	8.252	0.0041
TSCΔ	0.16	28.187	<0.0001
CSΔ	0.10	9.78	0.0018
mCpΔ	0.32	2.029	0.1543

Table 18: Summary of False-negativity Results from Confirmatory AP and SF Tests on Presumptive *C. perfringens* Colony Isolates of Pasteurized and Unpasteurized Samples Analyzed on TSC, CS and mCp Agar Media by Chi-square Analysis

Presumptive negative	Rate of false-negativity	Chi-square value	p-value
TSC	0.74	20.415	0.0001
CS	0.21	5.307	0.0212
mCp	0.02	.02127	0.8841
TSCΔ	0.76	11.46	0.0007
CSΔ	0.35	3.359	0.0668
mCpΔ	0.03	8.493	0.0036

Table 30: Contingency Table for AP and SF Reactions of TSC Presumptive Positive *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP +	41	2	43
AP-	5	2	7
Total	46	4	50

Table 31: Contingency Table for AP and SF Reactions of TSC Presumptive Negative *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP +	30	3	33

AP-	4	13	17
Total	34	16	50

Table 32: Contingency Table for AP and SF Reactions of CP ChromoSelect Presumptive Positive *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP+	41	2	43
AP-	5	2	7
Total	46	4	50

Table 33: Contingency Table for AP and SF Reactions of CP ChromoSelect Presumptive Negative *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP+	3	1	4
AP-	6	37	43
Total	9	38	47

Table 34: Contingency Table for AP and SF Reactions of mCp Presumptive Positive *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP+	38	3	41
AP-	3	4	7
Total	41	7	48

Table 35: Contingency Table for AP and SF Reactions of mCp Presumptive Negative *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP+	0	0	0
AP-	1	47	48

Total	1	47	48

Table 36: Contingency Table for AP and SF Reactions of TSC Presumptive Positive *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	42	1	43
AP-	1	6	7
Total	43	7	50

Table 37: Contingency Table for AP and SF Reactions of TSC Presumptive Negative *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	27	7	34
AP-	4	12	16
Total	31	19	50

Table 38: Contingency Table for AP and SF Reactions of CP Chromoselect Presumptive Positive *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	45	0	45
AP-	3	2	5
Total	48	2	50

Table 39: Contingency Table for AP and SF Reactions of CP Chromoselect Presumptive Negative *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	5	4	9
AP-	6	27	33
Total	11	31	42

Table 40: Contingency Table for AP and SF Reactions of mCp Presumptive Positive *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	19	4	23
AP-	2	3	5
Total	21	7	28

Table 41: Contingency Table for AP and SF Reactions of mCp Presumptive Negative *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	1	0	0
AP-	0	34	34
Total	1	34	35