Evaluation of Three Different Selective Media for Enumeration of *Clostridium perfringens* in Untreated and Treated Wastewater

A.J. Karon

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Approved by:

Mark Sobsey, PhD

Jill Stewart, PhD

Lisa Casanova, PhD

ABSTRACT

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(Under the direction of Mark Sobsey)

Current and emerging legislation in North Carolina and other regions calls for the enumeration of *Clostridium perfringens* as a surrogate indicator for protozoan parasites in various types of waters. Past studies that have evaluated selective media for the detection of this bacterium have provided limited, conflicting, and inconclusive results. In this study membrane filtration was used to enumerate *C. perfringens* as culturable spores or total culturable cells in 19 samples of untreated and 25 samples of partially treated wastewaters on three candidate media, Tryptose Sulfite Cycloserine Agar (TSC), CP ChromoSelect Agar (CCP), and membrane *Clostridium perfringens* Agar (m-CP) in parallel, and the results were compared. Presumptive isolates from each agar were further subjected to phenotypic confirmation tests for acid phosphatase production and stormy fermentation to determine the performance of each agar. The CCP agar was determined to have the highest enumerative capacity of total *C. perfringens* cells when compared to both TSC agar and m-CP agar (p-value < 0.05), but there was no significant difference in its ability to detect spores when compared to TSC agar (p-value >0.05). The overall specificity of CCP agar as determined by agreement of results from both confirmation tests was 0.81, while the specificity of TSC agar was only 0.28. Based on its performance, ease of preparation and use and consistency of colony characteristics, CCP agar is recommended as the preferred medium for *C. perfringens* enumeration in wastewater.

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INTRODUCTION

As water scarcity becomes a more prevalent global issue in the face of climate change and water source depletion, the need for alternative sources of water for drinking, industrial purposes, and agriculture is becoming increasingly important. One type of alternative water resource that is gaining interest world-wide, and especially in drought-prone areas, is treated wastewater or reclaimed water. The state of North Carolina serves as one example of a state looking to utilize reclaimed water to ensure a more sustainable future for its water resources going forward. In 2011, North Carolina passed legislation revising the regulation of reclaimed water that specified a new, higher quality reclaimed water with expanded allowable uses of such reclaimed water for agricultural and industrial purposes (subchapter 02U – Reclaimed Water) (NC DENR, 2011). Following this action, in 2014, the North Carolina legislature again expanded the allowable uses for this reclaimed water to include mixing with source waters for potable drinking water supplies at an approved ratio to then be further treated to produce drinking water (Session Law 2014-113 Senate Bill 163).

The N.C. sponsored legislation enabling expansion of allowable uses for reclaimed water also established specific quality guidelines for the higher quality reclaimed water, named type 2 reclaimed water, as the only category of reclaimed water that would be allowed for these expanded uses (subchapter 02U – Reclaimed Water) (NC DENR, 2011). In order to meet the criteria for Type 2 reclaimed water, these waters had to be treated by tertiary treatment and dual disinfection (i.e., chlorine disinfection and UV disinfection or allowable substitutes), and have more extensive quality testing than previously necessary for other treated wastewaters or type 1 reclaimed water. Included among these new quality tests was testing the type 2 tertiary treated wastewater for *Clostridium perfringens*, a spore-forming bacterium that serves as a surrogate

indicator for protozoan parasite pathogens. For water to be considered type 2 grade, reclaimed water treatment had to demonstrate a greater than 4 $\log_{10}$ reduction of *C. perfringens* from raw sewage and have a geometric mean of no more than 5CFU/100 mL with a daily maximum of 25 CFU/100 mL of treated water (subchapter 02U – Reclaimed Water,NC DENR, 2011).

While these new regulations contained specifications in regard to the ultimate quality of the water being created to satisfy the type 2 quality requirements, there was no mention of the appropriate methods to be used for proper enumeration of *C. perfringens* in reclaimed water and wastewater samples. Furthermore, US EPA methods and Standard Methods for the Examination of Water and Wastewater do not provide or cite official documentation for the best or acceptable methods for enumerating *C. perfringens* in treated wastewater samples such as those that will be produced in North Carolina in the coming years. Several methods have been described by both US EPA and Standard Methods for the Examination of Water and Wastewater for *C. perfringens* enumeration in other types of waters such as surface waters and drinking waters, but the performance of these methods has not been evaluated, adapted, performance-validated, documented and certified for this specific application to reclaimed water. Additionally, new methods for enumeration of *C. perfringens* in water samples continue to be developed, but there has been either poor or little proper documentation of any methods being adequate or superior to any of the others available. This leads to confusion and uncertainties about how to properly measure *C. perfringens* in both treated wastewaters and other types of waters that urgently needs to be clarified. Analytical methods that are documented to be accurate and effective are needed to enable entities such as North Carolina state water utilities as well as other stakeholders world-wide who wish to enumerate *C. perfringens* as an indicator for protozoan parasites in various wastewaters, treated wastewaters and other waters.

OBJECTIVE

The purpose of this research was to measure the effectiveness of three different available selective agars for enumeration of *C. perfringens* using membrane filtration to determine the best methods for measuring this bacterium in wastewater and treated wastewater samples. These agars would then be further validated by subjecting presumptive *C. perfringens* colony isolates from them to several different confirmation tests to provide a more rigorous assessment of the performance of the selective agars.

REVIEW OF THE LITERATURE

Clostridium perfringens

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming, rod-shaped, non-motile, sulfite-reducing bacterium that inhabits the intestinal tract tracts of humans and other animals, is shed fecally and is present in raw sewage at concentrations of 10,000-100,000 CFU per 100 mL (Fujioka 1985, Bisson and Cabelli 1980, Payment and Franco 1993; Sorenson 1989). It is also found widespread in the environment, although its sources when present in the environment are uncertain (Petit 1999). It is commonly found in the enteric tracts of humans and animals and some strains or variants can be pathogenic under certain circumstances and cause gastrointestinal illness (Petit 1999; McClane 1996). *C. perfringens* has been recognized as an indicator of fecal contamination of water for over 100 years (Klein and Houston 1899). Vegetative cells and spores have been found in varying proportions in sewage waters before and

after treatment, with vegetative cells found in higher proportions in untreated wastewater, but spores found in higher proportions after heat or chlorine treatment (Bisson and Cabelli 1980). *C. perfringens* was proposed as a possible fecal indicator for protozoan parasites such as Giardia and Cryptosporidium in fecally contaminated water primarily because of its ability to produce spores that survive for long periods of time in the environment and are relatively resistant to disinfection processes like the cysts and oocysts of protozoan parasites (Klein and Houston 1899; National Academy of Sciences, 2004; Payment and Franco, 1993; Bisson and Cabelli 1980).

Previous studies have also identified *C. perfringens* vegetative cells and/or spores in concentrations statistically correlated to enteric viruses, *Giardia* cysts and *Cryptosporidium* oocysts in surface water samples (Payment and Franco 1993, Ferguson et al. 1996). Studies have further demonstrated that detection of no *C. perfringens* in surface waters had a positive predictive values for absence of pathogens in the same waters (Rimhanen-finne et al. 2004). *C. perfringens* spores have been detected in increased quantities in water after sewage overflow events (Ferguson et al. 1996) and in soils after widespread flooding of animal agriculture areas (Casteel et al., 2006). Though it is most frequently used as an indicator of protozoan parasites, in some cases *C. perfringens* in surface water have had no correlation with the presence of protozoan cysts or oocysts (Rimhanen-finne et al. 2004). However, there have been objections to use of *C. perfringens* as a fecal indicator because of a historical lack of a reliable way to quantify it as well as the persistence of spores in the environment (specifically in soil and sediment) that may not correlate with the actual degree of fecal contamination in surface waters (Fujioka et al. 1985; Davies 1995; Cabelli 1978). Despite these concerns about its value and effectiveness as a fecal indicator microorganism, *C. perfringens* in water and wastewater are now typically and

reliably detected and quantified by membrane filtration methods using specific plating media following the success of the methods originally developed by Bisson and Cabelli (1980).

Clostridium perfringens Agar Media Comparison

Several previous studies have attempted to compare the best methods for detection of *C. perfringens* in various settings including both food and various water sources. Three agars are currently produced and widely available for use in a lab setting that claim to select for *C. perfringens* from water samples. These agars are membrane *Clostridium perfringens* agar (m-CP) produced by Oxoid and other commercial sources, *Clostridium perfringens* ChromoSelect agar (CCP) produced by Fluka, and tryptose-sulphite-cycloserine agar (TSC) produced by EMD Millipore and other commercial sources. In addition to using the direct count method of membrane filtration, there is also a most probable number multiple fermentation tube method, several pour-plate methods, and a pour tube method (Bisson and Cabelli 1979). These other methods were not considered in this study for direct detection and quantification because they were not as accessible or easy to use in the context of water testing labs in North Carolina. Additionally, non-membrane filtration methods have been noted to suffer from deficiencies that limit their use in detection from water and wastewater samples such as the need for sub-culturing for confirmed identification, the increased difficulties in analyzing large volumes of water, the somewhat reduced precision of most-probable-number methodologies compared to colony counts, and unclear growth appearance or colony morphologies for definitive identification (Bisson and Cabelli 1979). Selective methods for *C. perfringens* are all based on demonstrating

the known property of the bacteria for sulphite reduction that many *Clostridia* species share (Bisson and Cabelli 1979).

There are a few key differences that characterize each of the selective agars evaluated in this study. The m-CP agar uses sucrose as a nutrient and requires supplemental additives: indoxyl-β-D-glucoside, phenolphthalein diphosphate, polymyxin B and D-cycloserine. The agar produces yellow/beige colonies that will turn pink after exposure to ammonia fumes. The TSC agar uses sodium metabisulfite and ferric ammonium citrate to indicate sulfite reduction and D-cycloserine antibiotic as a supplement that inhibits the growth of some other bacteria that could cause interference. It produces black colonies with a yellow halo. The CCP agar uses ammonium iron (III) citrate, a chromogenic mixture, L-cysteine hydrochloride, magnesium heptahydrate, soy peptone, sucrose, tris buffer, tryptose, yeast extract and D-cycloserine as an antimicrobial supplement. The colonies this agar grows will turn green in color after an hour of exposure to an aerobic environment, following anaerobic incubation for colony growth (Manafi 2013).

Several culture methods exist for enumerating *C. perfringens* from food origins. Studies have attempted to quantify and explain the differences among the various media that are used to recover this bacterium from food samples. Of the three selective agars that are used for membrane filtration, only TSC has been used to culture *C. perfringens* from food sources (Byrne et al 2007, De Jong 2003, Hauschild & Hilsheimer 1973, Mead 1985). Food microbiology studies have assessed TSC in comparison to SFP, OPSP, RCA, BHI, SCA, and DCA agars, in addition to TYD-C, DRCM, PEM, and LS liquid media (Byrne et al 2007, De Jong 2003, Hauschild & Hilsheimer 1973, Mead 1985). Although differing slightly in each experimental setup, each study concluded that TSC was either most useful or equally useful to any of the other methods and media for recovery and enumeration of *C. perfringens* bacteria (Byrne et al 2007,

De Jong 2003, Hauschild & Hilsheimer 1973, Mead 1985). Two of these studies differed in use of TSC agar from working with water samples in that they used a pour plate or spread plate method instead of membrane filtration (Hauschild and Hilsheimer 1973, De Jong 2003). There were conflicting findings among the researchers as some found that TSC agar was limited in its ability to allow *C. perfringens* to form spores, while others found that it was better for recovery of spores (Byrne et al. 2007, Mead 1985). Another stated limitation of the TSC agar was that it was found to be a hospitable growth medium for other *Clostridia* species and therefore confirmatory testing would be required (Mead 1985).

In water samples, *C. perfringens* has been cultured using the TSC, m-CP, and CCP agars that have been previously described. However, there have also been studies documenting the culturing of these bacteria with a flourogenic TSC medium (TSCF), TSN, SPS, and WB agars (Araujo 2004, Sartory 1985). The majority of these studies have been conducted on water bodies used for drinking water, partially treated drinking water, or fully treated drinking water. One study by Sartory (1985) compared TSC and m-CP agars for *C. perfringens* in partially treated sewage and other low quality waters. Only one previous study has compared TSC, m-CP, and CCP agars, but included a flourogenic substrate in the TSC agar and analyzed only waters used for potable purposes both from the source and after treatment (Manafi et al. 2013). Results from this study demonstrated that no statistically significant difference was found among the three agars with respect to their abilities to enumerate presumptive *C. perfringens* colonies. Use of the m-CP media was determined to be onerous for the purpose of identifying presumptive positive colonies, while the TSC agar with fluorogenic substrate was difficult to count when high contamination was present. The CCP agar was identified as both the most specific agar and the least difficult to read when compared to the other two media.

Most studies comparing the available agars were concerned mainly with the m-CP and TSC media. Bisson and Cabelli (1979) first developed the m-CP membrane filtration method. At the time, TYI agar pour plates (using the Bonde pour tube method) were used for enumeration of *C. perfringens* in water samples (Bisson and Cabelli 1979). Using the m-CP agar that they developed, they were able to effectively select *C. perfringens* from the water samples using membrane filtration and found counts on these plates to be 10-20% lower than by using the traditional method, but with higher accuracy (93% confirmation of positives, 98% confirmation of negatives) (Bisson and Cabelli 1979). They also noted a colony counting limit between 85 and 120 on the upper end and low precision when 20 or fewer colonies were observed. Finally, they found that the agar was not as successful at culturing the bacteria if 24 hours or more time had passed between the time the bacteria were added to the water sample and the time that the assay was conducted (Bisson and Cabelli 1979).

Following the 1979 study, several other researchers have evaluated the m-CP agar that Bisson and Cabelli developed as well as the TSC agar that had been developed earlier. Some of the earlier work found TSC and m-CP to have comparable abilities to enumerate *C. perfringens* when analyzing untreated, partially treated, and known highly polluted surface waters. Both agars have been observed to have greater than 90% confirmation of presumptive positives with few or no false-negative colonies (Sartory 1985). Additionally, it was noted that TSC was significantly easier to use and a fraction of the price of m-CP agar (Sartory 1985).

Other researchers quantifying the difference between media for culturing *C. perfringens* have analyzed drinking water or source water that would later be treated for drinking or in lab engineered water samples. A 1998 study found the m-CP agar to be more selective than TSC for recovery of vegetative cells in spiked samples, but that TSC had a higher capacity to culture both

vegetative cells and spores in these samples (Sartory 1998). These findings were in contrast to previous findings from food based studies that found TSC a poor medium for culturing spores. However, the findings in this study were later validated by Maheux et al. (2013) who found high rates of confirmation of isolates previously cultured on m-CP agar from engineered waters with a panel of known strains of *C. perfringens* and also natural *C. perfringens* present in sewage that was added to test water.

In natural water sources for drinking and recreational use, TSC has been observed to have greater success at accurately detecting the presence of *C. perfringens* in water samples that have lower concentrations of the bacteria (Sartory, 1998; Manafi 2013). Researchers have also noted various levels of success with TSC when confirming isolates, ranging from 68% to 87% confirmation (Aurajo 2004; Sartory 1998). Further, researchers using TSC have had even broader ranges of conflicting results when confirming presumptive negative colonies, ranging from 100% confirmation of negatives to only 31.9% confirmation of negative colonies (Aurajo 2004; Sartory 1998). Only one study has evaluated this agar for its ability to detect both spores and vegetative cells (Sartory 1998). Evaluation of m-CP in the same waters has had similar mixed results in which some researchers have empirically found both low (<50%) and high (>95%) confirmation rates of presumptive positive and presumptive negative colonies when subjecting isolates to phenotypic biochemical testing (Burger *et al.* 1984; Sartory 1998; Maheux 2013; Armon and Payment 1987).. However, in several studies m-CP has been noted as a less preferred agar to TSC (Sartory 1998; Araujo 2004; Manafi et al. 2013). M-CP agar has been noted to have significantly lower enumerative capacity to TSC and observed to have high rates of false-positivity and false-negativity for both spores and vegetative cells (Sartory 1998; Araujo 2004). Researchers have noted that positive m-CP colonies are also sometimes difficult to

identify based on the varying shades of pink observed after exposing colonies to potassium hydroxide (Sartory 1998, Manafi et al. 2013).

Only one peer-reviewed study has evaluated CCP agar. Manafi et al. (2013) compared the CCP, m-CP and TSC agar containing a fluorogenic substrate (TSCF) to detect spores from drinking water samples and partially treated drinking water samples. They found *C. perfringens* on CCP and the TSCF agars were easy to count and subculture. However, they found all three agars comparable at quantifying the bacteria in their samples. However, they selected CCP as the preferred agar because of its ease of preparation and use as well as the ease in identifying positive colonies from the agar.

It is apparent that the studies conducted to this point have given an inadequate evaluation of the best methods for detection of *C. perfringens* from wastewater and treated wastewater samples via membrane filtration. The bulk of studies that have evaluated the agars of interest have been related to drinking water and not wastewater. Additionally, results have been conflicting and the error ranges for confirmation of presumptive positive colonies have varied widely. According to most of the literature, TSC appears to be a preferred agar to m-CP, but very little work has been conducted to evaluate the performance and identify the possible strengths and weaknesses of the newer CCP medium. The review of the current literature validates the need for more extensive work to be done evaluating which of these agars is most suitable for labs wishing to enumerate *C. perfringens* to meet new quality standards regarding reclaimed or other water sources.

Phenotypic Confirmation Tests

Phenotypic confirmation of *C. perfringens* is traditionally based on tests that demonstrate its lack of motility, ability to reduce nitrate, ferment lactose, and cause liquefaction of gelatin (Sartory 2006, Environment Agency UK, Eisgruber 2000). However, researchers have pointed out that these processes are labor intensive and time consuming and the results of some of these tests are at times unreliable (Cato et al. 1986; Barrow and Feltham 1993; Eisgruber 2000). These traits can be tested for by inoculation into motility-nitrate medium (test for motility and nitrate reduction) and lactose-gelatine medium (test for lactose fermentation and gelatine liquefaction) (Eisgruber 2000). Another phenotypic confirmation test can be done by the reverse CAMP test which uses streaking of presumptive *C. perfringens* isolates on sheep blood agar as described by Hansen and Elliott (1980). A third confirmation test utilizes an iron milk based medium in which *C. perfringens* produces stormy fermentation (Abeyta 1985; Erickson and Deibel 1978). Each of these confirmation tests require incubation of at least 24 hours. The tests have also been evaluated on previously isolated strains of *C. perfringens* and all have produced results demonstrating different levels of sensitivity or specificity below 100% (Hauschild and Hilsheimer 1974; Abeyta 1985; Eisgruber et al. 2000; Mead 1985; Hansen and Elliott 1980). Nitrate-motility media has been found to exhibit sensitivity as low as 78% and iron-milk media have been found to exhibit sensitivities as low as 71%, while the reverse CAMP test has exhibited higher observed sensitivity of 94% or greater (Hauschild and Hilsheimer 1974; Mead 1985; Eisgruber *et al.* 2000; Hansen & Elliot 1980).

The time consuming nature and level of imprecision in these confirmation tests led to the development of a new rapid phenotypic confirmation test that evaluated presumptive *C. perfringens* for acid-phosphatase production. Developed by Ueno et al. (1970), this test exposes presumptive colonies to a mixture of naphtyl phosphate disodium salt, Fast Blue B salt and

acetate buffer. Confirmation is based on an observed color change when a colony that produces acid-phosphatase is exposed to the reagent. This reaction takes place in minutes and has been validated multiple times as an acceptable alternative to other phenotypic confirmation tests based on testing with previously confirmed *C. perfringens* strains (Ryzinska-paier et al. 2011; Ueno et al. 1970; Wohlsen et al. 2006; Eisgruber 2000; Sartory 2006; Mead 1981). Several studies have identified sensitivities for this test to be around 95%, particularly for isolates obtained from cultures of environmental waters, indicating that the tests is usually very accurate for positive identification of isolates (Eisgruber 2000; Mead 1981; Sartory 2006; Adcock and Saint 2001).

METHODS

Sample Collection

Treated and untreated wastewater effluents were collected at five wastewater treatment/water reclamation 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.

Initially, seven secondary treated sewage effluent samples were collected from two wastewater treatment plants (A and B) before sand filtration, and analyzed for *Clostridium perfringens*. These samples were collected between May and July of 2013. Many of the final tertiary treated and dual disinfected effluent samples had no detectable *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* 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*. These same treatment plant sample locations yielded sufficient concentrations of *Clostridium perfringens* at all five wastewater treatment facilities for collected samples that were partially treated samples as indicated above.

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. These samples were collected between August 2013 and August 2014. During this time, 20 secondary treated effluent samples, 5 untreated sewage samples, and 10 final treated reclaimed water samples were analyzed. A third round of sampling for only reclaimed water and untreated sewage took place between February 2015 and July 2015 in which 14 samples of both types were analyzed. Overall, 19 untreated sewage samples, 25 secondary treated effluent samples, and 25 tertiary treated, dual disinfected reclaimed water samples were analyzed. The summary of the samples is displayed in Table 2 and the sequence of sampling can be found in Appendix 1 in Tables 12 and 13. 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 both free chlorine and UV disinfection in the production of reclaimed water. Only a single disinfection treatment by UV radiation was used at plant C

Table 1. Summary of Wastewater Treatment Processes and 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

Table 2. Number and type of samples analyzed by treatment plant

Treatment Plant	Untreated Raw Sewage (Number of Samples)	Secondary Treated Effluent before Sand Filtration (Number of Samples)	Tertiary Treated Reclaimed Water (Number of Samples)	TOTALS
A	4	8	6	18
B	3	5	5	13
C	4	4	5	13
D	4	5	5	14
E	4	3	4	12
TOTALS	19	25	25	70

Sample handling

Treated and raw wastewater grab samples were collected from the appropriate WWTP sampling points in sterile polypropylene bottles, and kept chilled in coolers with ice during transport to Chapel Hill. Sampling points were the same as those used by the treatment plants in order to allow for the utilities' own analysis of water samples during the various stages of the treatment process. The samples were stored at 4°C upon arrival at the laboratory. *Clostridium perfringens* assays were performed on the day of or the day following sample collection. Samples were collected and analyzed between 5/14/2013 and 04/06/2015.

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' in Appendix 2 which is intended to be an easy-to-follow, step-by-step protocol that laboratory analysts may use to perform the analyses. Following each assay, other confirmatory analyses were done to identify false-positive and false negative presumptive *C.*

perfringens colonies obtained by initial membrane filter analysis. As many as five individual presumptive positive and presumptive negative *C. perfringens* colonies from each of the three *C. perfringens* agar media tested were selected from a given sample and purified by re-streaking onto non-selective Columbia agar media to obtain isolated colonies. Purified colony isolates were then subjected to a test for acid-phosphatase (AP) production as described by Sartory et al. (2006). Later on, samples analyzed on this project were subjected to a second confirmatory test of stormy fermentation (SF) in tubes of iron milk medium 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* (m-CP) agar, CP ChromoSelect Agar (CCP), and Tryptose Sulfite Cycloserine (TSC) agar. TSC agar medium was obtained from EMD Chemicals Inc. TSC agar was prepared by adding 3.9 grams of TSC agar base /100 mL of deionized water in a 500 mL bottle, autoclaving for 15 minutes and cooling to keep molten. The m-CP 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 molten 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 m-CP 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 m-CP Selective Supplement, per 100 mL of

molten agar base medium. 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°C until use.

In order to perform the various confirmation tests, the following reagents were required: 2% ferrous sulfate solution and canned evaporated milk for the iron milk medium, deionized water, glacial acetic acid, sodium acetate (anhydrous), 1-naphtyl phosphate disodium salt, and Fast Blue B Salt for the acid phosphatase test, as well as and Columbia agar base. 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 aseptically 12 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 was 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, m-CP agar. Based on more recent evidence of the inferior performance of the MF method when using m-CP medium, two alternative *C. perfringens* MF media, TSB and CP ChromoSelect agar, were evaluated in parallel with modified m-CP (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 wastewater. These analyses focused 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, to facilitate comparisons of agar media performance 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 m-CP, 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 some colonies of other colors are scored as positive as well) or green in color, respectively, and can be directly counted. On m-CP 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 culturable 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 (m-CP, TSC and CP ChromoSelect).

Samples of reclaimed water, untreated wastewater, and secondary treated wastewater before sand filtration from the five wastewater treatment plants indicated previously were analyzed by membrane filtration using the three different agar media and were compared on the basis of *C. perfringens* detected as spores only or spores plus vegetative cells. Using aliquots of 1 mL (mixed with phosphate buffer), 5 mL, 10 mL, 20 mL, or 100 mL per sample, depending on the expected contamination level and sample type, the various samples were vacuum filtered through a 0.45 μm pore size, 47 mm diameter cellulose ester membrane filters. Each sample volume was plated in triplicate. Reclaimed water and raw sewage samples were filtered for only one sample 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 by 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 these 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.

Acid-phosphatase Confirmation Method

Upon counting colonies 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 from membrane filters were streaked initially onto separate non-selective Columbia agar medium plates (as many as five presumptive positive and five presumptive negative colonies per sample type, per agar medium) 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 latter 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 medium 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. *C. perfringens* and other sulfite-producing clostridia are positive for stormy fermentation. Results were recorded accordingly.

Clostridium perfringens Data Analysis Procedures

The data collected on *C. perfringens* concentration were analyzed initially using the GraphPad Instat statistical package from GraphPad Software. All data were 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. These tests were performed on direct count results obtained through membrane filtration on the three previously described selective media. The analysis was conducted for both pasteurized and unpasteurized samples (vegetative cells and spores and only spores) from diluted raw sewage, partially treated sewage (before sand filtration) and tertiary treated reclaimed water. To compare the concentrations of *C. perfringens* detected by TSC and CCP agars in raw sewage samples, a Wilcoxon matched-pairs signed-ranks test was used. An alpha value of 0.05 ($P < 0.05$), was used to establish statistical significance. The data from these tests are displayed in Tables 4-6 below. In addition to these statistical tests used to determine if the agar media differed significantly in

their ability to detect *C. perfringens* colonies, several descriptive figures were generated. 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 R.Studio (R Studio Team 2015).

In Microsoft Excel, plots were created displaying the sequential samples collected over time as $\log_{10}$ concentrations of total cells and only spores (Figures 3, 4, 7, 8). Time intervals between samples in these plots were not representative of actual time intervals. Bar charts of arithmetic average $\log_{10}$ concentrations were constructed in Excel in order to demonstrate difference in detection of presumptive *C. perfringens* total cells and spores by agar medium for each of the treatment plants (Figures 9-12). Bar charts were also constructed to show comparative $\log_{10}$ reductions of *C. perfringens* spores and total cells as detected by each of the agars based on arithmetic averages of $\log_{10}$ concentrations measured from untreated sewage and final reclaimed water analyzed from each of the treatment plants (Figures 13-14).

For analysis of the confirmation test results, data were analyzed in Microsoft Excel and Stata 14 (StataCorp 2015). In Excel, the data were analyzed to determine the extent to which each *C. perfringens* confirmation test result agreed with what the isolate was considered presumptively (either *C. perfringens* positive of negative) when originally observed and the colony isolated from its respective agar medium. Microsoft Excel was then used to generate sensitivities, specificities, positive predictive values, and negative predictive values. The confirmation tests were also compared against each other using McNemar's test in Stata14 (StataCorp 2015) to determine if the results of the tests were significantly different from each other.

RESULTS

The geometric mean of total *C. perfringens* concentrations in secondary treated effluent as detected by the TSC, CCP, and m-CP media were 263 CFU/100 mL (SD: 147), 673 CFU/100 mL (SD:670), and 167 CFU/100 mL (SD: 263) respectively (Table 3). The median *C. perfringens* values were 285 CFU/100 mL, 636 CFU/100 mL and 185 CFU/100 mL for each respective agar (Table 3 and/or Figure 1). For the same secondary treated samples, the ranges of *C. perfringens* concentrations on TSC agar were from 6 to 615 CFU/100 mL, on CCP agar from 161 to 2285 CFU/100mL, and on m-CP agar from 11 to 855 CFU/100 mL (Table 3 and/or Figure 1). TSC had the smallest range for detection of total *C. perfringens* cells in secondary treated wastewater (Figure 1). CCP had the largest minimum and maximum detection of all agars, and also had several observations that were outliers from the other samples (Figures 1 and 3). The results for concentrations of total *C. perfringens* in unpasteurized samples of secondary treated effluent as detected on the different agar media are summarized in Appendix 1 in Table 13 and they are displayed in sequential samples collected over the sampling period as $\log_{10}$ concentrations in Figures 3 and 4 for total cells and spores, respectively. The intervals between samples are not representative of actual time intervals as samples were not taken over uniform time periods.

The results for *C. perfringens* spore concentrations in pasteurized secondary treated sewage samples as detected on the three agar media tested are shown in Appendix 1 in Table 14. In these pasteurized secondary treated sewage samples, the geometric mean and median concentrations of *C. perfringens* spores for TSC, CCP, and m-CP agars were 176 (SD: 134) and 233 CFU/100 mL, 332 (SD: 441) and 267 CFU/100 mL, and 16 (SD: 48) and 21 CFU/100 mL respectively (Table 3 and Figure 2). *C. perfringens* spore concentration of pasteurized secondary

treated effluent ranged from 2 to 452 CFU/100 mL for TSC, 42 to 1535 CFU/100 mL for CCP, and 1 to 206 CFU/100 mL for m-CP agar media, respectively (Table 3 and Figure 2). Both CCP and m-CP agars had weeks in which the observed concentration of presumptive *C. perfringens* spores were outliers with respect to the other secondary treated effluent samples that were analyzed (Figure 2). The overall range of spore concentrations as detected by m-CP was much narrower than that of the other two agars (Figure 2). The results for *C. perfringens* spore concentrations in pasteurized secondary treated sewage samples as detected on the three agar media tested are shown in Appendix 1 in Table 14.

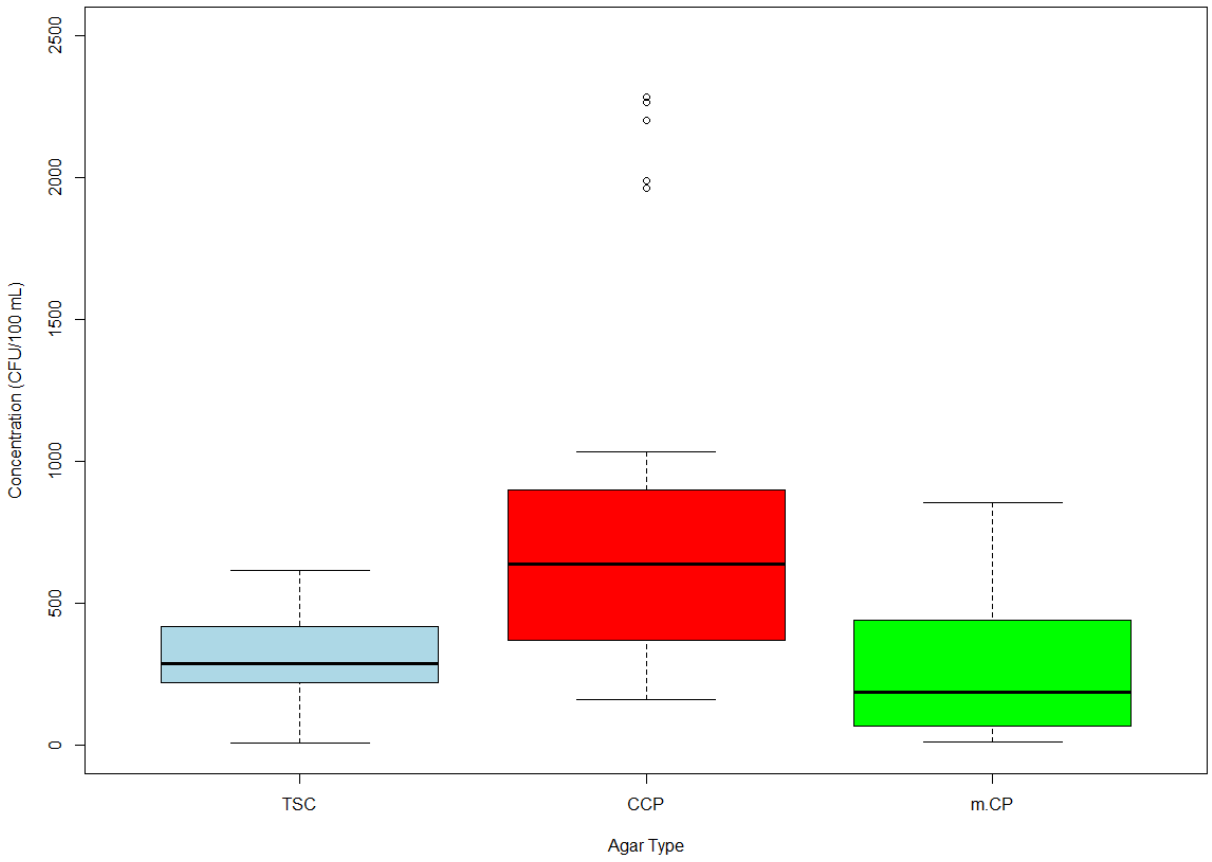

Figure 1. Box and Whisker plot of the distribution of mean concentrations of *C. perfringens* in secondary treated sewage samples for the TSC, CCP, and m-CP agars (n=25).

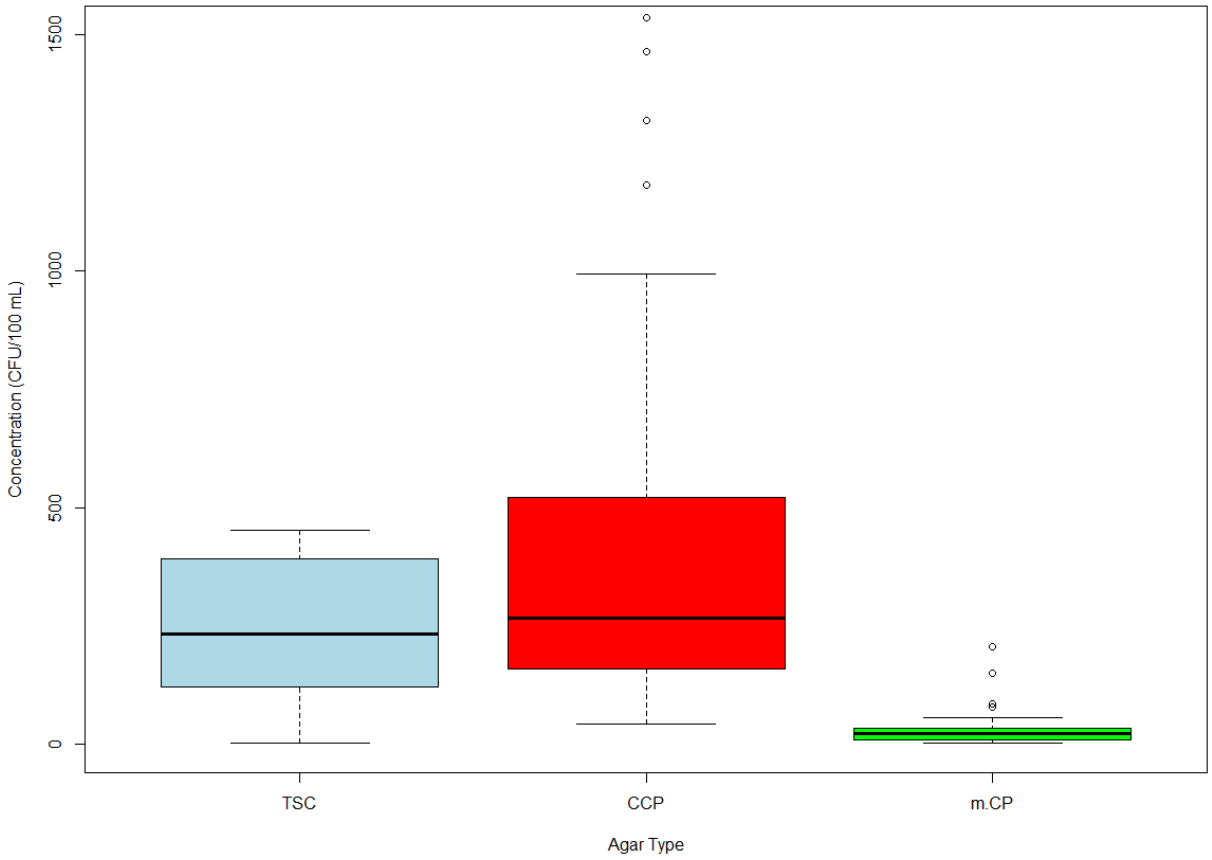

Figure 2. Box and Whisker plot of the distribution of mean concentrations of *C. perfringens* spores in pasteurized secondary treated sewage samples for the TSC, CCP, and m-CP agars (n=25).

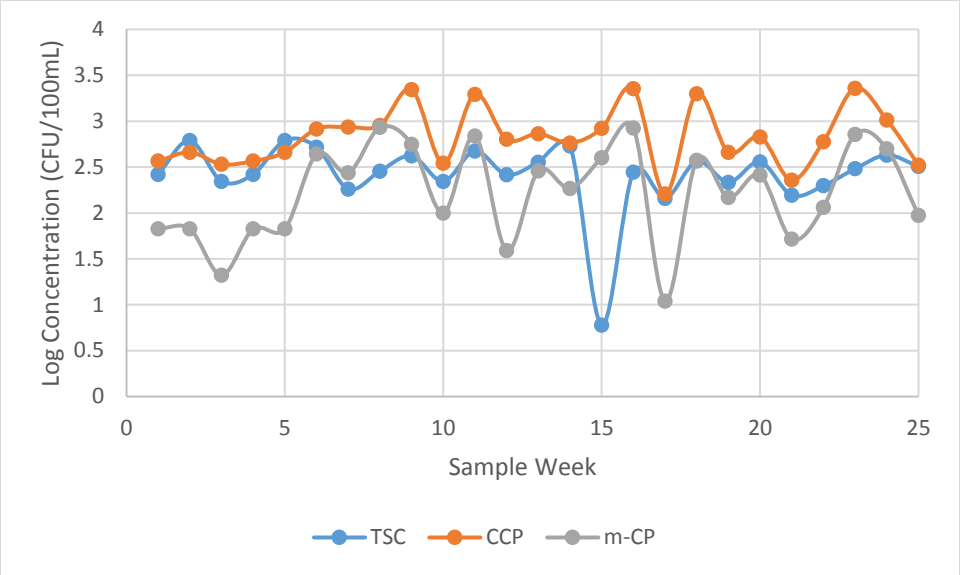

Figure 3. Log concentrations of total *C. perfringens* in secondary treated sewage effluent by sample week as detected by the TSC, CCP, and m-CP agars

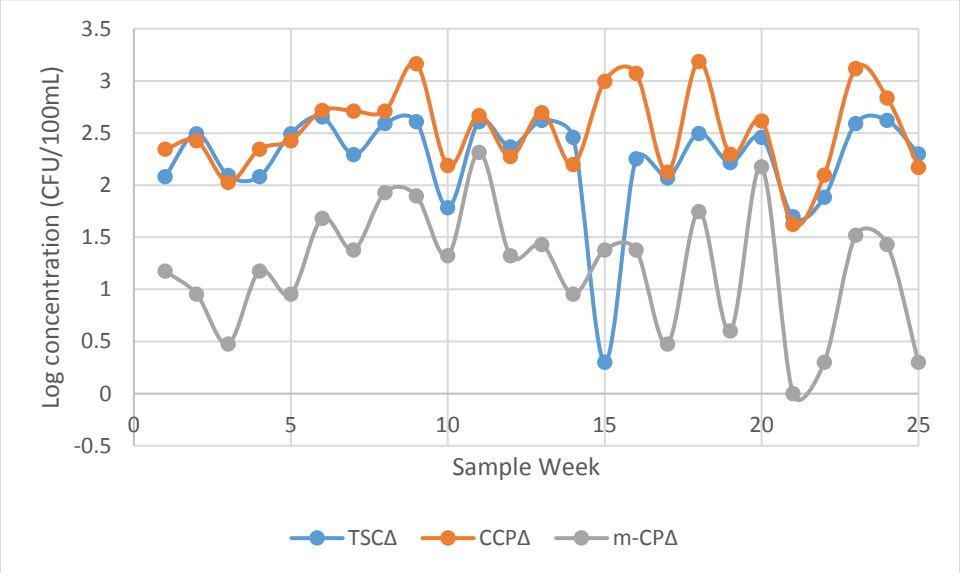

Figure 4. Log concentrations of *C. perfringens* spores in pasteurized secondary treated sewage effluent by sample week as detected by the TSC, CCP, and m-CP agars

After sampling and analyzing 11 untreated raw sewage samples, m-CP agar was determined to have significantly lower detection capacity of both total cells and spores than the other two agars. As a result, analysis of these samples with m-CP agar was halted (Figures 5-8). In untreated sewage samples, the geometric average *C. perfringens* concentrations were $5.36*10^4$ CFU/100 mL (SD: $4.91*10^4$), $7.73*10^4$ CFU/100 mL (SD: $7.71*10^4$), and $1.54*10^4$ CFU/100 mL (SD: $1.55*10^4$) for TSC, CCP, and m-CP agars respectively (Table 3 and Figure 5). The median *C. perfringens* concentrations as detected by each of the agars in the same untreated sewage samples were $5.33*10^4$ CFU/100 mL, $6.33*10^4$ CFU/100 mL, and $1.67*10^4$ CFU/100 mL for TSC, CCP, and m-CP agars, respectively (Figure 5 and Table 3). The ranges of total cell concentrations of *C. perfringens* in these same sewage samples on TSC were from $1.2 *10^4$ to $1.47 *10^5$ CFU/100 mL, on CCP from $3.50*10^4$ to $3.58*10^5$ CFU/100 mL, and on m-CP from $5.56*10^3$ to $5.97*10^4$ CFU/100 mL. The distributions of total cell detection by TSC and CCP were skewed upwards for untreated sewage samples (Figure 5). Similar to what was seen when analyzing the secondary treated effluent, CCP agar had the highest maximum and minimum concentration detected from the samples of the three media tested and the m-CP agar had the narrowest range of detection of total cells in raw sewage (Figure 5). The results for total *C. perfringens* concentrations in samples of untreated sewage as detected on the different agar media are summarized in Appendix 1 in Table 13 and they are shown in sequential samples collected over the sampling period as $\log_{10}$ concentrations in Figures 7 and 8 for total cells and spores, respectively. The intervals between samples not representative of actual time intervals as samples were not taken over uniform time periods.

In the pasteurized untreated sewage samples, the average and median concentrations of spores on TSC, CCP, and m-CP agar media were $3.27*10^4$ (SD: $3.19*10^4$) and $4.3*10^4$ CFU/100

mL, $3.87*10^4$ (SD: $7.64*10^4$) and $4.17*10^4$ CFU/100 mL, and $4.73*10^3$ (SD: $1.40*10^2$) and $3.3*10^3$ CFU/100 mL respectively (Table 3 and Figure 6). *C. perfringens* spore concentrations in these samples of pasteurized untreated sewage ranged from $8.3*10^2$ to $1.3*10^5$ CFU/100 mL on TSC, $1.7*10^3$ to $3.2*10^5$ CFU/100 mL on CCP, and $5.6*10^2$ to $1.8*10^4$ CFU/100 mL on m-CP, respectively (Table 3). Both CCP and TSC agars had weeks in which the observed concentration of presumptive *C. perfringens* spores were outliers with respect to the other untreated sewage samples that were analyzed (Figure 6). Again, the overall range of spore concentrations as detected by m-CP was much narrower than that of the other two agars (Figure 6). The distribution of spore detection from pasteurized untreated raw sewage was skewed upward for both the CCP and m-CP agars (Figure 6). The individual weekly results for *C. perfringens* spore concentrations in pasteurized raw sewage samples as detected on the three agar media tested can be found in Appendix 1 in Table 14.

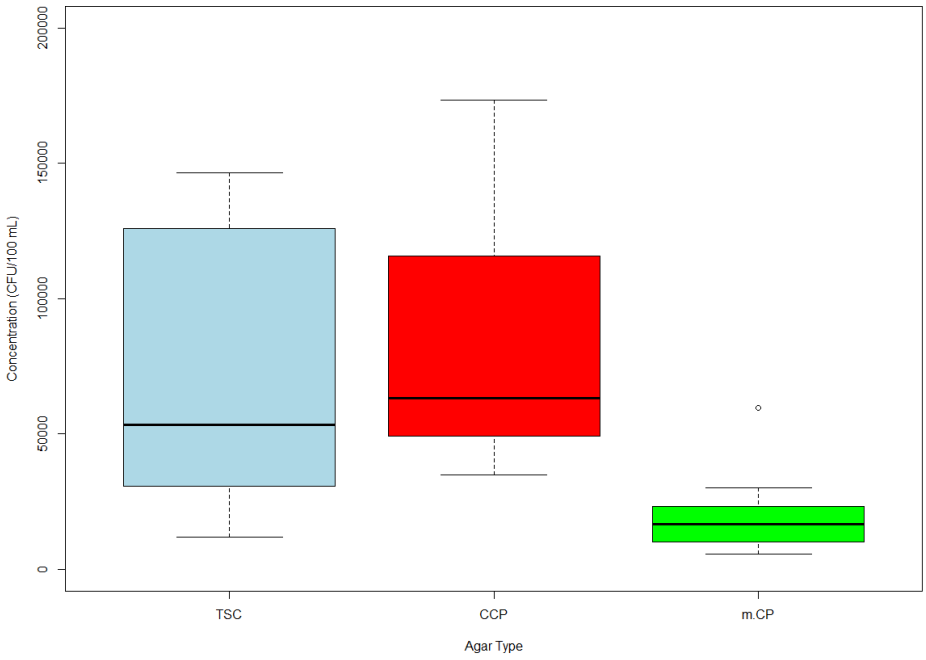

Figure 5. Box and Whisker plot of the distribution of mean concentrations of *C. perfringens* in untreated sewage samples for the TSC, CCP, and m-CP agars (n=19).

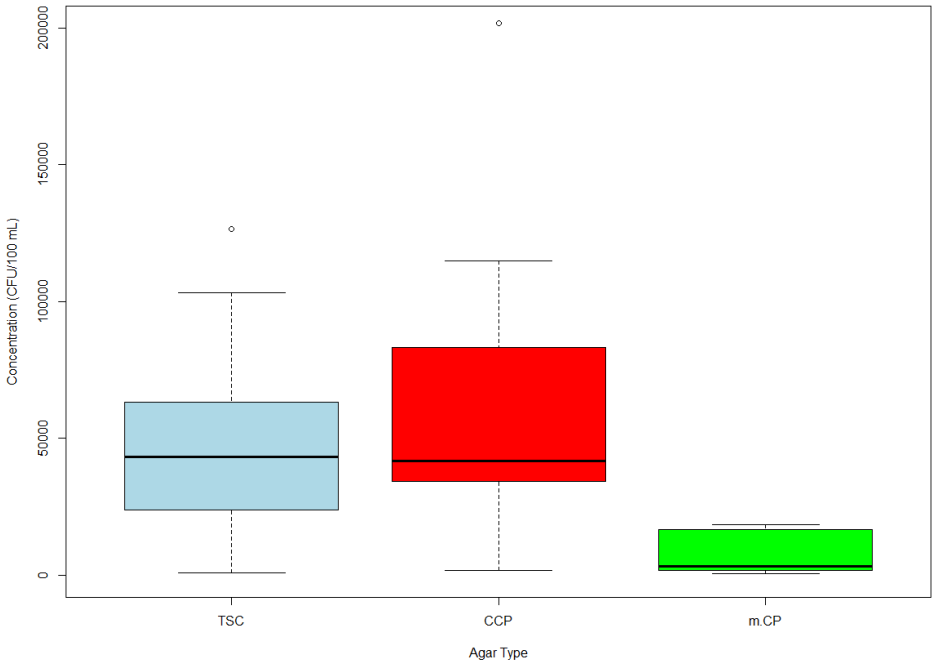

Figure 6. Box and Whisker plot of the distribution of mean concentrations of *C. perfringens* spores in pasteurized untreated sewage samples for the TSC, CCP, and m-CP agars (n=19).

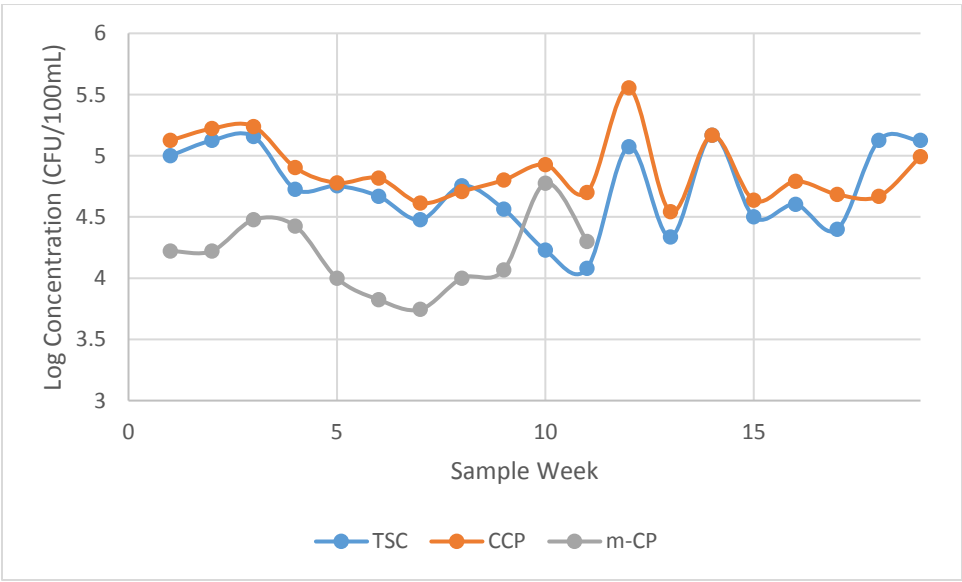

Figure 7. Log concentrations of total *C. perfringens* in untreated sewage by sample week as detected by the TSC, CCP, and m-CP agars (n=19)

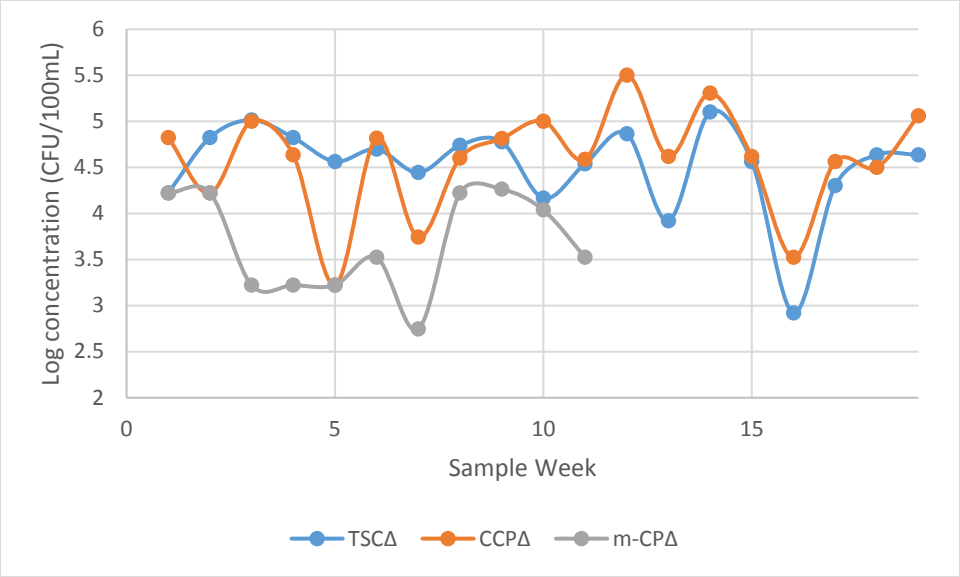

Figure 8. Log concentrations of *C. perfringens* spores in pasteurized untreated sewage by sample week as detected by the TSC, CCP, and m-CP agars (n=19)

For reclaimed water, no *C. perfringens* colonies were detected in the samples from the four treatment plants with combined chlorine and UV disinfection. In three samples from treatment plant C, with only UV disinfection of tertiary treated sewage, *C. perfringens* colonies were detected on all three agar media, with TSC enumerating 40, 70, and 22 CFU/100 mL, CCP enumerating 20, 40, and 46 CFU/100 mL, and m-CP enumerating 3, 10, and 39 CFU/100 mL, respectively.

Table 3. Central tendency statistics and ranges for concentrations of presumptive *C. perfringens* detected by TSC, CCP, and m-CP agars in pasteurized and unpasteurized samples of untreated sewage and secondary treated effluent.

Central Tendency Statistic and Range	Unpasteurized Untreated Sewage (n=19)			Pasteurized Untreated Sewage (n=19)		
	TSC	CCP	m-CP	TSC	CCP	m-CP
Geometric Mean (CFU/100 mL)	5.36E+04	7.73E+04	1.54E+04	3.27E+04	3.87E+04	4.73E+03
Median (CFU/100 mL)	5.33E+04	6.33E+04	1.67E+04	4.33E+04	4.17E+04	3.33E+03
Minimum Value (CFU/100 mL)	1.20E+04	3.50E+04	5.56E+03	8.33E+02	1.67E+03	5.56E+02
Maximum Value (CFU/100 mL)	1.47E+05	3.58E+05	5.97E+04	1.27E+05	3.17E+05	1.83E+04
	Unpasteurized Secondary Treated Effluent (n=25)			Pasteurized Secondary Treated Effluent (n=25)		
	TSC	CCP	m-CP	TSC	CCP	m-CP
Geometric Mean (CFU/100 mL)	263	673	167	176	332	16
Median (CFU/100 mL)	285	636	185	233	267	21
Minimum Value (CFU/100 mL)	6	161	11	2	42	1
Maximum Value (CFU/100 mL)	615	2285	855	452	1535	206

Figures 9-12 depict the arithmetic average $\log_{10}$ concentrations and standard errors of *C. perfringens* total cells in treated effluent and raw sewage and *C. perfringens* spores only in treated effluent and raw sewage, respectively, as detected on each agar medium in samples from in each treatment plant. According to figures 9 and 11, CCP agar has a higher arithmetic mean of $\log_{10}$ concentrations of total cells and spores than that of TSC or m-CP in secondary treated effluent in all treatment plants. The standard errors of CCP and TSC $\log_{10}$ arithmetic mean concentrations also overlap for several of the treatment plants in both samples. According to Figures 10 and 12, CCP agar and TSC agar have higher arithmetic mean of $\log_{10}$ concentrations of total cells and spores than that of m-CP for all treatment plants, but the standard errors of CCP

and TSC for these concentrations by treatment plant frequently overlap with each other.

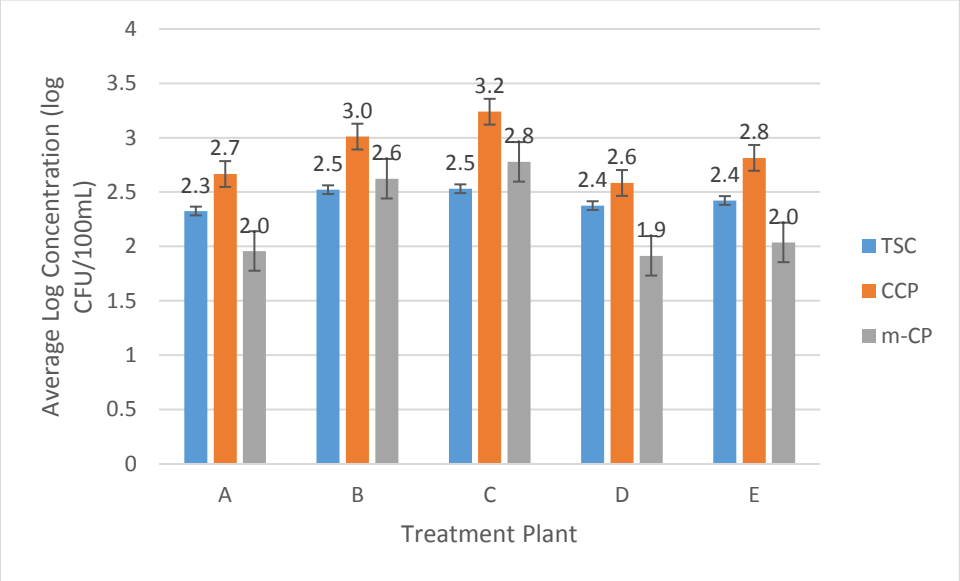

Figure 9. Average $\log_{10}$ concentrations of total *C. perfringens* with standard errors as detected by the TSC, CCP, and m-CP agars in secondary treated effluent for each treatment plant from which samples were analyzed (n=25)

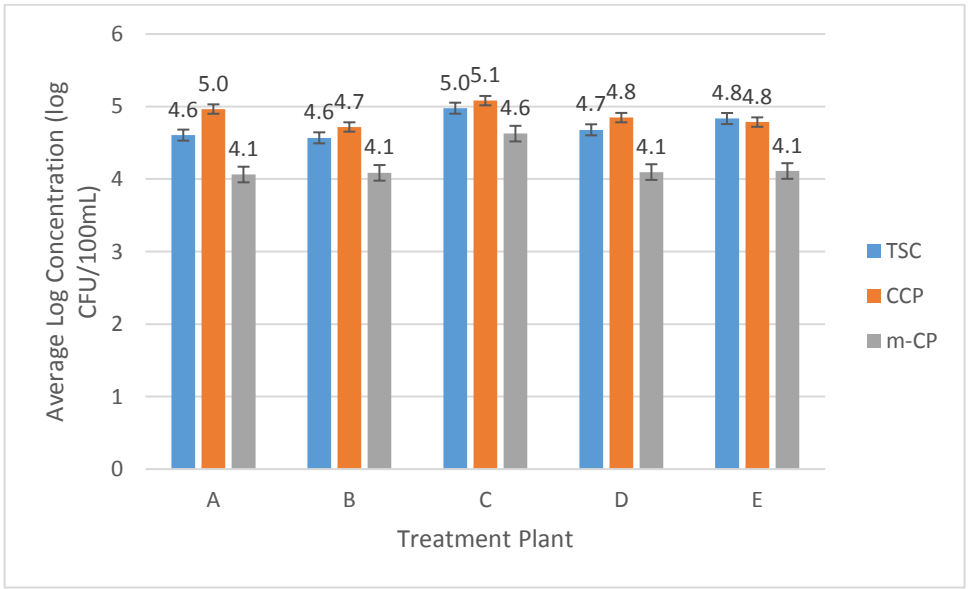

Figure 10. Average $\log_{10}$ concentrations of total *C. perfringens* with standard errors as detected by the TSC, CCP, and m-CP agars in untreated sewage for each treatment plant from which samples were analyzed (n=19)

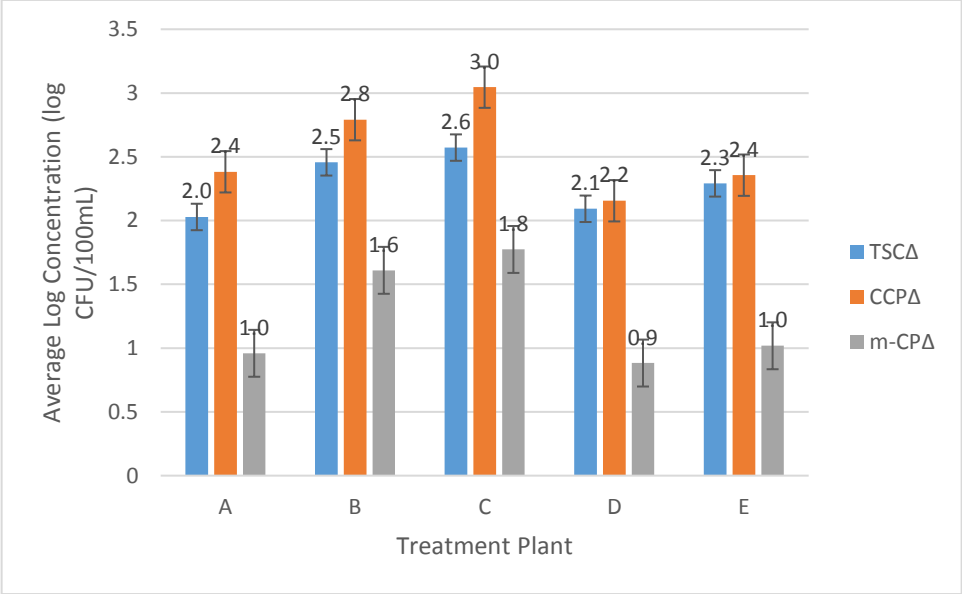

Figure 11. Average $\log_{10}$ concentrations of *C. perfringens* spores with standard errors as detected by the TSC, CCP, and m-CP agars in pasteurized secondary treated sewage effluent for each treatment plant from which samples were analyzed (n=25)

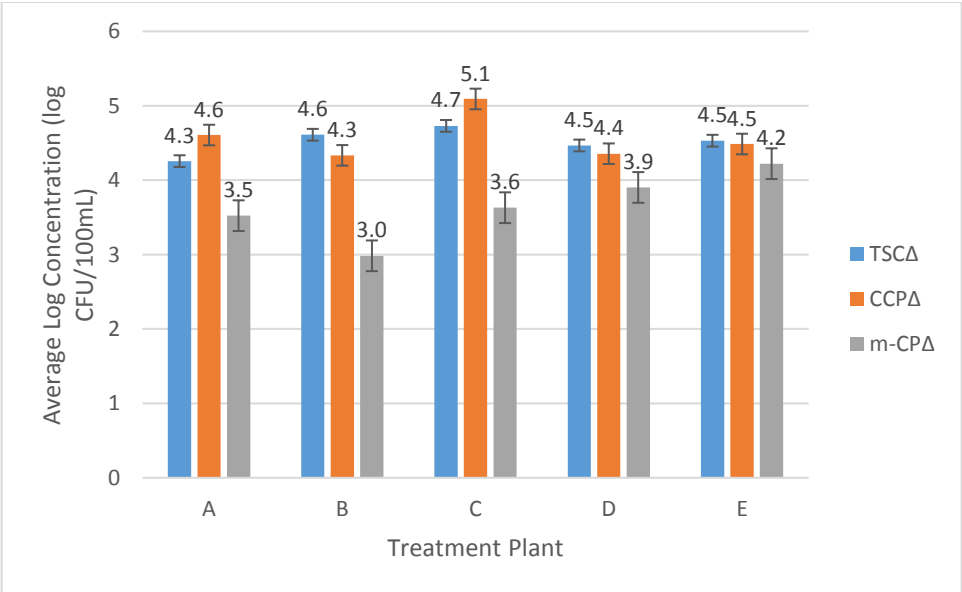

Figure 12. Average $\log_{10}$ concentrations of *C. perfringens* spores with standard errors as detected by the TSC, CCP, and m-CP agars in pasteurized untreated sewage for each treatment plant from which samples were analyzed (n=19)

To determine the $\log_{10}$ reductions of total *C. perfringens* cells and *C. perfringens* spores at each treatment plant, the average $\log_{10}$ concentrations of the tertiary treated reclaimed water (tertiary treated reclaimed water concentrations in Tables 13 and 14) were subtracted from the average $\log_{10}$ concentrations of the untreated sewage. All treatment plants' tertiary treatment process included dual disinfection with UV and chlorine except for plant C which used single disinfection with UV radiation. The average $\log_{10}$ reductions of total *C. perfringens* cells for each of the treatment plants based on the CCP agar, which had the highest enumeration levels of *C. perfringens*, were 4.60, 4.57, 4.02, 4.68, and 4.83 for treatment plants A, B, C, D, and E respectively (Figure 13). Likewise, The average $\log_{10}$ reductions of *C. perfringens* spores for each of the treatment plants based on the CCP agar, which had the highest enumeration levels of *C. perfringens*, were 4.61, 4.33, 4.31, 4.36, and 4.49 for treatment plants A, B, C, D, and E respectively (Figure 14).

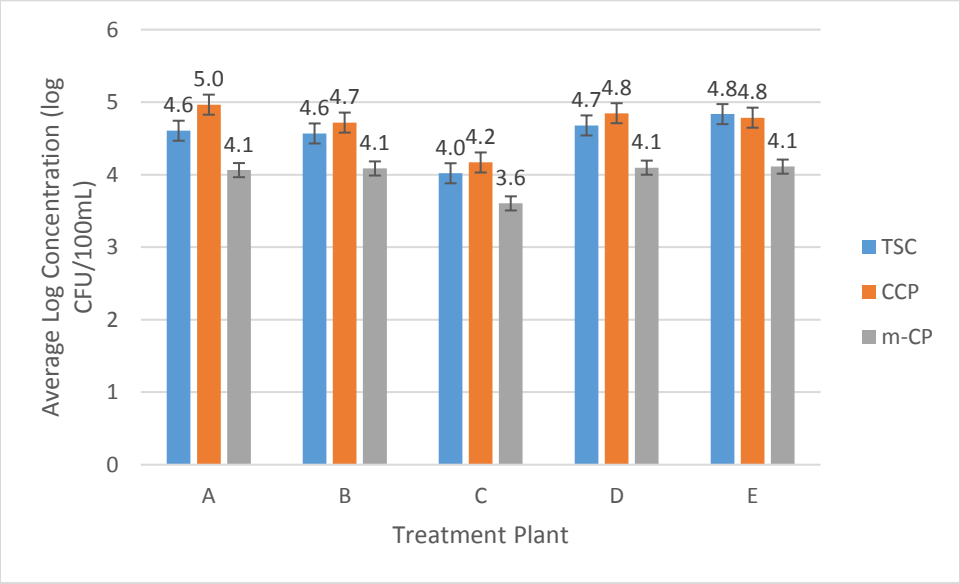

Figure 13. Average $\log_{10}$ treatment reductions of total *C. perfringens* with standard errors as detected by the TSC, CCP, and m-CP agars for each treatment plant from which samples were analyzed

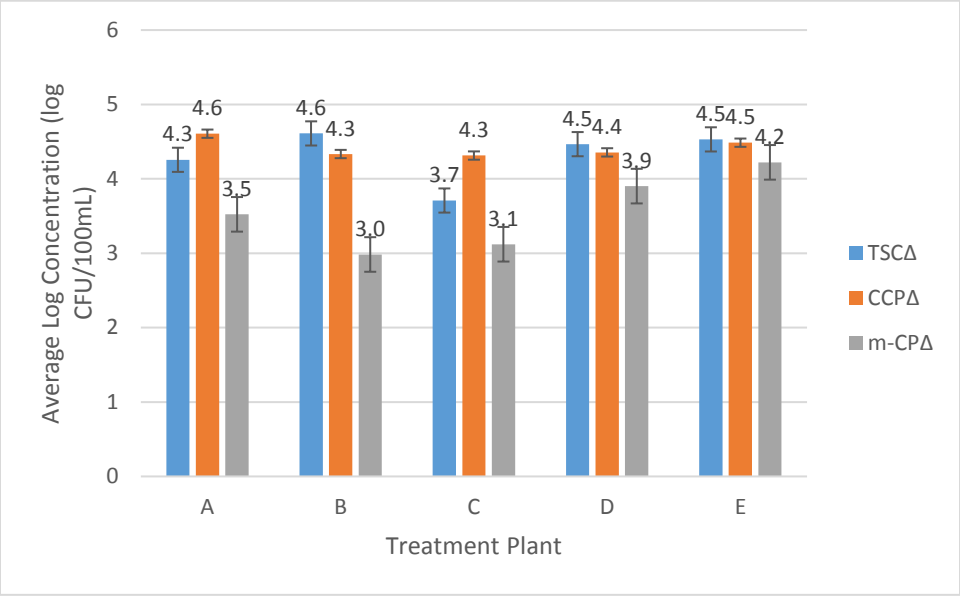

Figure 14. Average $\log_{10}$ treatment reductions of *C. perfringens* spores with standard errors as detected by the TSC, CCP, and m-CP agars for each treatment plant from which samples were analyzed

A Friedman nonparametric test was used to compare the concentrations of *C. perfringens* total cells and only spores as detected by the TSC, CCP, and m-CP agars in secondary treated wastewater (Tables 4 and 5) and raw wastewater (Table 6). The results from these statistical tests suggested that statistically significant differences in concentrations of *C. perfringens* total cells and spores were detected by the three agars. Following the Friedman test, a Dunn Multiple Comparison post-test was performed to compare each of the agar pairs individually. In the secondary treated sewage sample, the CCP agar was found to have a statistically significantly greater capacity to enumerate total *C. perfringens* cells compared to the TSC and m-CP agar (Table 5; p-value<.001). Both the CCP and TSC agar were found to have a statistically significantly higher detection rate of *C. perfringens* spores than the m-CP agar (Table 5; p-value

<0.001). However, there was no statistically significant difference between the TSC and CCP agars for capacity to detect *C. perfringens* spores (Table 4; p-value >0.05).

Compared across the first 11 raw sewage samples, both the TSC and CCP agars were found to detect significantly higher concentrations of spores and total cells of *C. perfringens* than the m-CP agar. Following these statistical analyses of these samples, the next 8 untreated sewage samples were analyzed only with the CCP and TSC agars and not the m-CP agar. Following the sample analyses, a Wilcoxon matched pairs signed-rank test was performed to compare the difference in *C. perfringens* spores and total cell concentrations in untreated sewage, detected by the TSC and CCP agars. As shown by the results in Table 5, the CCP agar detected a statistically significant higher concentration of total *C. perfringens* cells in raw sewage than did TSC agar (p = 0.0015). However, there was no statistically significant difference between the two agars in *C. perfringens* spore concentration detection in raw sewage (p = 0.2101).

Table 4. Results of Friedman test comparing the matched concentrations of total *C. perfringens* and spores in secondary treated sewage effluent as detected by TSC, CCP, and m-CP agars

Sample type	**Friedman test p-value (p-value < 0.05 is significant)**
2^0 treated sewage, no treatment	<0.0001
2^0 treated sewage, pasteurized	<0.0001

Table 5. Results of the Dunn Multiple Comparison post-test comparing the matched concentrations of total *C. perfringens* and spores in secondary treated sewage effluent as detected by TSC, CCP, and m-CP agars

Agar 1	**Agar 2**	**Sample type**	**Dunn Multiple Comparison p-value (p-value < 0.05 is significant) (raw data)**	**Rank Sum Difference (raw data)**
TSC	**CCP**	2^0 treated sewage	<0.001	-30
TSC	**m-CP**	2^0 treated sewage	>0.05	9
CCP	**m-CP**	2^0 treated sewage	<0.001	39
TSC	**CCP**	2^0 treated sewage Δ	>0.05	-12
TSC	**m-CP**	2^0 treated sewage Δ	<0.001	30
CCP	**m-CP**	2^0 treated sewage Δ	<0.001	42

Δ-denotes pasteurization of sample at 65º C for 15 minutes

Table 6. Results of the Wilcoxon matched-pairs signed-ranks test comparing the concentrations of total *C. perfringens* and spores in untreated sewage as detected by TSC and CCP agars

Agar 1	**Agar 2**	**Sample type**	**Wilcoxon matched-pairs signed-ranks test p-value**	**Rank Sum Difference (raw data)**
TSC	**CCP**	Raw Sewage	0.0015	-105
TSC	CCP	Raw Sewage Δ	0.2101	-63

Δ-denotes pasteurization of sample at 65º C for 15 minutes

Presumptive positive and negative *C. perfringens* colony isolates for pre-sand filtered samples and untreated sewage samples were collected from each of the three test agar media for both pasteurized and unpasteurized samples. A test for acid-phosphatase production (AP) was performed on colony isolates from each of the agar media and the Stormy Fermentation (SF) test in iron-milk media tubes was performed as well. In total, 533 presumptive isolates (275 presumptive positive colonies and 258 presumptive negative colonies) from pasteurized and unpasteurized secondary treated effluent and 303 presumptive isolates (171 presumptive positive colonies and 132 presumptive negative colonies) from pasteurized and unpasteurized untreated sewage were subjected to both confirmation tests (Table 7). Table 7 contains the summary of the number of presumptive positive and negative isolates tested by both confirmation tests for each agar medium in the two types of samples, pasteurized and unpasteurized. Table 8 displays a contingency table with the aggregate overall number of times that the two confirmation tests agreed and disagreed with each other for both presumptive positive isolates and presumptive negative isolates from all samples, both pasteurized and unpasteurized.

The results of the two *C. perfringens* confirmation tests had varying levels of agreement among the agar media. Additionally, the results of the tests for acid-phosphatase production and for stormy fermentation in iron-milk media differed from each other for many of the presumptive isolates tested. Because of the differing results, specificities and sensitivities were generated

based on each individual confirmation test and an agreement between the two confirmation tests. To evaluate the agar media on the basis of their selectivity, sensitivities, specificities, positive predictive values (PPV) and negative predictive values (NPV) were calculated for each agar medium based on the results of the two confirmation tests for both pasteurized and unpasteurized raw sewage, secondary treated effluent, and total combined samples. All of the sensitivities and specificities calculated under the different scenarios are displayed in Table 9. The overall sensitivities of TSC, CCP, and m-CP agar media as determined by agreement of both confirmation tests on presumptive positive and presumptive negative *C. perfringens* isolates from both types of sewage samples were 0.81, 0.78, and 0.81 respectively. For the same agar media, their overall specificities as determined by agreement of both confirmation tests on *C. perfringens* isolates from both types of samples were 0.28, 0.81, and 0.97 respectively. The sensitivities of the TSC, CCP, and m-CP agar media determined by the same method, but for combined pasteurized samples were 0.79, 0.81, and 0.63 while the specificities were 0.23, 0.65, and 0.98 respectively.

All PPV and NPV values calculated under the different scenarios are displayed in Table 10. The overall PPV of TSC, CCP, and m-CP agar media as determined by agreement of both confirmation tests on presumptive positive and presumptive negative *C. perfringens* isolates from both types of sewage samples were 0.53, 0.83, and 0.97 respectively. For the same agar media, their overall NPVs as determined by agreement of both confirmation tests on *C. perfringens* isolates from both types of samples were 0.59, 0.75, and 0.82 respectively. The PPVs of the TSC, CCP, and m-CP agar media determined by the same method, but for combined pasteurized samples were 0.53, 0.76, and 0.97 while the NPVs were 0.50, 0.72, and 0.74 respectively (Table 10)

Table 7. Summary of the number of presumptive isolates tested for acid phosphatase production and stormy fermentation in iron milk from each agar type and sample type

Sample	Number of Presumptive Isolates Tested								
	Agar	Negative	Positive	Agar	Negative	Positive	Agar	Negative	Positive
Secondary Treated Effluent	TSC	50	50	m-CP	39	47	CCP	46	50
	TSCΔ	49	50	m-CPΔ	35	28	CCPΔ	39	50
Raw Sewage	TSC	30	30	m-CP	22	30	CCP	21	30
	TSCΔ	24	30	m-CPΔ	17	21	CCPΔ	18	30

(Δ-designates that the sample plates on this agar was pasteurized)

Table 8. Contingency Table with results from AP and SF Reactions of total combined presumptive positive and negative *C. perfringens* colonies in pasteurized and unpasteurized samples

	SF+	SF-	Total
AP+	436	58	494
AP-	85	257	342
Total	521	315	836

Table 9. Sensitivities and specificities of each agar as determined by agreement of presumptive isolates with phenotypic confirmation testing for acid phosphatase production or stormy fermentation in untreated sewage and secondary treated effluent samples

		Agar Type and Sample Treatment					
Acid Phosphatase		TSC	CCP	m-CP	TSC Δ	CCPΔ	m-CPΔ
Secondary Treated Effluent	Sensitivity	0.86	0.86	0.87	0.86	0.90	0.82
	Specificity	0.34	0.91	1.00	0.32	0.79	1.00
Raw Sewage	Sensitivity	0.87	0.90	0.87	0.80	0.80	0.71
	Specificity	0.37	0.77	0.86	0.46	0.89	1.00
Combined Secondary Treated Effluent and Sewage	Sensitivity	0.86	0.88	0.87	0.84	0.86	0.78
	Specificity	0.38	0.94	0.99	0.36	0.82	1.00
Stormy Fermentation							
Secondary Treated Effluent	Sensitivity	0.92	0.92	0.87	0.90	0.96	0.75
	Specificity	0.32	0.81	0.98	0.38	0.74	1.00
Raw Sewage	Sensitivity	0.93	0.77	0.93	0.87	0.83	0.86
	Specificity	0.37	0.86	0.95	0.29	0.78	1.00
Combined Secondary Treated Effluent and Sewage	Sensitivity	0.93	0.86	0.90	0.89	0.91	0.80
	Specificity	0.34	0.82	0.97	0.35	0.75	1.00
Acid Phosphatase & Stormy Fermentation							
Secondary Treated Effluent	Sensitivity	0.82	0.82	0.81	0.84	0.90	0.68
	Specificity	0.26	0.79	0.98	0.24	0.64	1.00
Raw Sewage	Sensitivity	0.80	0.70	0.80	0.70	0.67	0.57
	Specificity	0.30	0.86	0.95	0.21	0.67	0.94
Combined Secondary Treated Effluent and Sewage	Sensitivity	0.81	0.78	0.81	0.79	0.81	0.63
	Specificity	0.28	0.81	0.97	0.23	0.65	0.98

(Δ-designates that the sample plates on this agar was pasteurized)

Table 10. Positive Predictive Values (PPV) and Negative Predictive Values (NPV) of each agar as determined by agreement of presumptive isolates with phenotypic confirmation testing for acid phosphatase production or stormy fermentation in untreated sewage and secondary treated effluent samples

		Agar Type and Sample Treatment					
Acid Phosphatase		TSC	CCP	m-CP	TSC Δ	CCPΔ	m-CPΔ
Secondary Treated Effluent	PPV	0.55	0.91	1.00	0.55	0.83	1.00
	NPV	0.65	0.84	0.94	0.63	0.84	0.88
Raw Sewage	PPV	0.59	1.00	0.96	0.60	0.87	0.92
	NPV	0.69	0.86	0.84	0.28	0.67	0.73
Combined Secondary Treated Effluent and Sewage	PPV	0.57	0.94	0.98	0.57	0.84	0.97
	NPV	0.67	0.85	0.87	0.57	0.78	0.82
Stormy Fermentation							
Secondary Treated Effluent	PPV	0.55	0.82	0.97	0.58	0.80	1.00
	NPV	0.76	0.90	0.89	0.63	0.93	0.83
Raw Sewage	PPV	0.56	0.88	0.96	0.55	0.83	1.00
	NPV	0.82	0.72	0.91	0.56	0.75	0.80
Combined Secondary Treated Effluent and Sewage	PPV	0.55	0.84	0.97	0.62	0.81	1.00
	NPV	0.79	0.83	0.89	0.61	0.87	0.82
Acid Phosphatase & Stormy Fermentation							
Secondary Treated Effluent	PPV	0.53	0.80	0.97	0.53	0.75	1.00
	NPV	0.59	0.80	0.84	0.60	0.84	0.80
Raw Sewage	PPV	0.53	0.88	0.96	0.53	0.77	0.92
	NPV	0.60	0.67	0.78	0.36	0.55	0.64
Combined Secondary Treated Effluent and Sewage	PPV	0.53	0.83	0.97	0.53	0.76	0.97
	NPV	0.59	0.75	0.82	0.50	0.72	0.74

(Δ-designates that the sample plates on this agar was pasteurized)

In order to determine whether there were significant differences among the rates of *C. perfringens* confirmation as determined by the confirmation test for acid-phosphatase production and stormy fermentation in iron-milk medium, the results of the confirmation tests were entered into contingency tables for each agar medium under the two different sample treatment types (pasteurized and unpasteurized). The results of these statistical tests are found in Tables 11 and 12. The contingency tables for these tests can be found in Table 8 and Tables 15-28 in Appendix 1. Before creating the contingency tables, a Wilcoxon-Mann-Whitney test was performed to

determine if the distribution of confirmation test results differed between the untreated sewage and secondary treated sewage samples. The test returned p-values of greater than 0.05 for both comparisons between the samples with both confirmation tests, indicating no statistically significant difference in confirmation test results between the two sample types (Table 11). Therefore, the sum of confirmation test results from both samples was used when constructing contingency tables for each of the agar media in order to increase sample size and thereby the reliability of the following statistical tests.

Table 11. Results of Wilcoxon-Mann-Whitney test to compare if distribution of confirmation test results differ between untreated raw sewage and secondary treated effluent.

Confirmation Test	z-score	p-value
Acid-Phosphatase	-1.03	0.302
Stormy Fermentation	-.569	0.570

The contingency tables were analyzed by McNemar's test to determine if the results of each *C. perfringens* confirmation test differed significantly. According to this statistical test, the two confirmation tests had significant differences in results when looked at in total (p-value = 0.024) (Table 12). However, the confirmation tests showed no significant differences when the results of exclusively presumptive negative isolates or the results of exclusively presumptive positive isolates were input to a contingency table. When disaggregated by agar medium and whether a sample was pasteurized, presumptive negative isolates from unpasteurized samples on CCP agar appeared to elicit significantly different results from the two confirmation tests (p-value = 0.011). No significant differences were found between the confirmation tests for any of the other agar media.

Table 12. Results of McNemar's test to compare the results of the phenotypic confirmation tests of presumptive isolates from each agar medium for both pasteurized and unpasteurized samples.

Presumptive test result for each agar medium	McNemar's chi^2 value	p-value
CCP-	6.40	0.011
CCP+	0.06	0.808
CCPΔ-	0.29	0.59
CCPΔ+	1.67	0.197
TSC-	0.69	0.405
TSC+	1.92	0.166
TSCΔ-	0	1
TSCΔ+	0.82	0.366
m-CP-	1	0.317
m-CP+	0.25	0.80
m-CPΔ-	1	0.317
m-CPΔ+	0	1
Combined presumptive positive	2.28	0.13
Combined presumptive negative	2.96	0.085
Total presumptive positives and negatives	5.10	0.024

(-: indicates presumptive negative isolates; +: indicates presumptive positive isolates; Δ: indicates that isolates came from pasteurized samples)

DISCUSSION

The results from the statistical tests comparing the performance of the agar media in quantifying *C. perfringens* by membrane filtration in raw and treated wastewater samples and the confirmation tests applied to presumptive positive and presumptive negative colony isolates demonstrate that there are significant differences among the agar media with respect to their ability to detect presumptive *C. perfringens* total cells and spores. The results of this study support previous findings that TSC is a superior agar to m-CP agar in its ability to detect and quantify *C. perfringens* in different types of water and wastewater samples (Sartory 1985;Sartory, 1998; Manafi 2013). The results of this study also demonstrate that CCP agar is

able to enumerate total *C. perfringens* cells at higher levels than the two other agar media tested in both types of wastewater samples analyzed, which has not been demonstrated in previous studies. This study found CCP agar to produce higher detection of *C. perfringens* spores than m-CP agar. *C. perfringens* spore detection by CCP agar also was comparable to TSC agar which is similar to the findings of previous work comparing CCP agar and TSC agar with a fluorogenic substrate (Manafi 2013).

While TSC and CCP agar may not have differed significantly in their ability to detect *C. perfringens* spores, the results of the two confirmation tests for presumptive *C. perfringens* colonies suggest that there may be differences in the reliability of these agar media to accurately detect biochemically and phenotypically confirmed *C. perfringens* colonies and true negative colonies. Assuming a conservative criterion of concordant results for both phenotypic confirmation tests in true negativity or positivity, the sensitivity and specificity for TSC agar was close to 80% and 25%, respectively, for total *C. perfringens* cells and only spores. The combined sensitivity and specificity for CCP agar based on these same criteria was about 78% and 81%, respectively, for total *C. perfringens* cells and about 81% and 65%, respectively, for *C. perfringens* spores. In contrast, the m-CP agar medium, had high specificity above 95%, but similar sensitivities of 81% for total *C. perfringens* cells and 63% for *C. perfringens* spores. The finding of somewhat reduced sensitivity in *C. perfringens* detection for the TSC agar has been noted previously (Araujo 2004; Sartory 1998). The high sensitivity of m-CP for *C. perfringens* has also been previously observed, but the lower specificity of m-CP for *C. perfringens* detection is in contrast to some previous findings (Bisson and Cabelli 1979). Overall, the values close to 80% specificity found for each of these agar media is in agreement with previous work that has found varying degrees of specificity among these agar media (Araujo 2004; Sartory 1998).

However, this study is the first to recognize that these similar specificities (0.81 for unpasteurized samples as determined by dual confirmation and 0.65 for pasteurized samples as determined by dual confirmation) are also found for *C. perfringens* detection by CCP agar in raw and treated wastewater, as they have been reported previously for other waters.

Although presumptive *C. perfringens* colony isolates were tested by phenotypic confirmation, there were moderate levels of disagreement between the two phenotypic confirmation tests, with 143 cases of discordant results between the confirmation tests of the 836 (17.1%) total isolates subjected to confirmation testing (Table 8). When looked at in the aggregate, there was a significant difference between the results of the two phenotypic confirmation tests according the McNemar's tests (p-value = 0.024). While molecular confirmation of these isolates was not done for this research, previous studies have found that the phenotypic confirmation tests are not always accurate, although the test for acid-phosphatase production has had higher levels of measured sensitivity than the test for stormy fermentation (Hauschild and Hilsheimer 1974; Mead 1985; Eisgruber et al. 2000; Hansen and Elliott 1980). These finding may explain why the two confirmation tests had varying levels of disagreement at times. In order to better assess the accuracy of these phenotypic confirmation tests and the true sensitivities and specificities of the test agar media, confirmation of the presumptive isolates should be done by additional biochemical testing and through molecular analyses, such as molecular characterization and identification by nucleic acid analysis and by protein targeted mass spectrometry, such as MALDI-TOF MS using identity comparison to robust databases.

In addition to the quantitative results for *C. perfringens* enumeration and phenotypic confirmation found in this study, there were also qualitative and user ease differences observed among the three agar media. The m-CP agar was the most difficult to prepare, use and read

results for. Medium preparation required the use of multiple medium additives after autoclaving the base medium and reading the results of the test required the exposure of plates with colonies to ammonium hydroxide fumes in order to cause a color change in presumptive positive colonies. This step had to be done under a hood to prevent the inhalation of fumes and after the color change initially took place, the colonies would rapidly revert back to their initial coloring. This made reading plates with high colony counts difficult to do. The TSC agar also presented some difficulties for accurate *C. perfringens* colony enumeration. Presumptive positive colonies for this agar are supposed to be black with a yellow halo, but there were often colonies that were varying shades of gray with a yellow halo, which were difficult to identify as either positive or negative. In accordance with the manufacturer's instructions, these colonies were counted as negative, but phenotypic confirmation testing suggested that these colonies were often likely to be *C. perfringens*. Further, TSC frequently cultured high levels of presumptive negative colonies that were yellow with no black or grey center, but these apparently negative colonies often tested as positive by one or both of the phenotypic confirmation tests. This resulted in the TSC agar having an unacceptably low sensitivity.

The CCP agar was generally the easiest to prepare and use, and gave reliable colony differentiation of presumptive positive and presumptive negative colonies based on their color. However, the process of reading results requires an additional 30-60 minutes after overnight incubation of plates to allow for the color change of positive colonies to occur. Regardless, the distinction between positive and negative colonies is very clear, as positives come in varying shades of green, while negative colonies are distinctly gray, purple, or bright blue. Additionally, the CCP agar is very easy to prepare as it is boiled and only requires a single supplement to make.

Aside from the difference in ease of use, there are differences in cost to consider among the agar media. The current list prices for these media on the Fisher Scientific website (04/25/16) are as follows: TSC agar sells for $237/500 g, m-CP sells for $233/500 g, and CCP agar sells for $393/250 g and is available from Sigma Aldrich. The considerable difference in price between the CCP agar medium and the other two agar media is important for consideration especially for labs with lower budgets, as they decide which medium and method is best to use.

There are also important qualitative aspects regarding the phenotypic confirmation tests that affect their ease of use. Stormy fermentation in iron-milk tubes is very easy to use and there is an easy distinction to observe between positive and negative tubes. The acid-phosphatase test is easy to perform and rapid but was more difficult to interpret because a positive confirmation by the test is based on a color change. The color change that occurs after exposing a presumptive colony to a reagent occurs over a variable amount of time between one and five minutes. However, after time passes, the color of the reagent eventually changes to brown regardless of the result of the test. The brown color can be difficult to differentiate from the purple color in a positively confirmed reaction. Thus, some of the disagreement between the two confirmation tests may be explained through observer error in reading the confirmation tests. However, it is also possible that some isolates positive by stormy fermentation are not all *C. perfringens*, as this test can also detect other sulphite reducing *Clostridium* species besides *C. perfringens*.

Limitations

Although the conclusions of this research as stated above are supported by the analysis of a large number of different wastewater samples, there are several limitations to this work. For some agar media, an estimated concentration value was used to represent 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 each respective wastewater sample.

Another limitation is that no true or "gold standard" sensitivity or specificity was able to be applied for each of the agar media as only phenotypic confirmation was used for presumptive isolates and for which results were sometimes found to be conflicting. The use of a molecular based confirmation analysis of presumptive *C. perfringens* isolates, such as MALDT-TOF MS or PCR followed by nucleotide sequencing, would have provided a more definitive basis for confirmation of true positives.

Human judgement subject to error limits the ability to correctly read the acid-phosphatase test which relies on visualization of a color reaction by the human eye to verify that material from a colony has turned purple when exposed to the reagent. However, 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 acid phosphatase 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.

Another limitation was encountered when attempting to extend these results to the use of these media and methods in evaluation of tertiary treated reclaimed water. All samples treated with UV and chlorine disinfection detected no *C. perfringens* in any of the samples. In the one

treatment plant without chlorination after UV irradiation, there were *C. perfringens* cells and spores detected in three of the samples, However, this is not a large enough sample size to truly compare the agar media against each other to evaluate their performance. Therefore, although it is possible to say that differences exist among the agar media with respect to their ability to detect *C. perfringens* in untreated wastewater and secondary treated wastewater, we cannot document if this is also true for detection of *C. perfringens* in tertiary treated and dual disinfected reclaimed water.

CONCLUSION

Though there are many factors to consider when deciding which agar medium is the best for identifying and quantifying *C. perfringens* in wastewater and treated wastewater, the results of these sets of experiments and analyses suggest that the CCP agar is the best option based on its ease of use and ability to differentiate and detect colonies. Furthermore, the relatively high confirmation rates of presumptive positive isolates as determined by the tests for acid-phosphatase production and stormy fermentation in iron-milk media support the reliability of the performance of this agar

However, the TSC agar may be a suitable alternative if the interested lab is looking only at presence of spores in their water samples. This may frequently be the case as the spores are the intended surrogate indicator for the presence of protozoan parasites in the water. Because no statistically significant difference was found between the ability of TSC and CCP agars to detect *C. perfringens* spores, it is likely that these two media can be used interchangeably for this

purpose. This could be useful for resource limited labs that may not be able to afford the more expensive CCP medium. However, the extremely low specificity determined for the TSC agar and the occasional occurrence of high counts of presumptive negative colonies suggests that when it is possible, the CCP agar medium is still preferred for use to obtain the most accurate level of *C. perfringens* spores concentrations.

Future Work

Several future topics should be investigated following the results of this research. Molecular confirmation is recommended to compare to positive confirmation of presumptive isolates by the two phenotypic confirmation tests used, acid phosphatase and stormy fermentation. Additionally, the TSC agar with the fluorogenic substrate that provides a rapid phenotypic phosphatase confirmation test should evaluated in untreated and treated sewage samples to compare its performance and determine whether it could be a feasible alternative to the CCP media and whether the specificity on this medium would be as low as its TSC counterpart. More work should also be done to compare the efficacy of these direct count methods for enumeration of *C. perfringens* with quantal most probable number methods such stormy fermentation in iron-milk tubes in the context of wastewater and treated wastewater as such studies have not previously been done.

Appendix 1: Raw Data Tables and Contingency Tables

Table 13. Concentrations (CFU/100 mL) of total *C. perfringens* cells as detected by TSC, CCP, and m-CP agars for each sample analyzed

Date	Sample Site	Sample Type	TSC	CCP	m-CP
5/16/2013	A	Pre-sand filter	264	367	67
5/23/2013	A	Pre-sand filter	615	458	67
5/28/2013	A	Pre-sand filter	221	339	21
6/18/2013	A	Pre-sand filter	264	367	67
6/25/2013	A	Pre-sand filter	615	458	67
7/23/2013	B	Pre-sand filter	521	824	439
7/30/2013	B	Pre-sand filter	182	864	273
8/6/2013	C	Pre-sand filter	285	900	855
8/20/2013	C	Pre-sand filter	418	>2203*	561
8/27/2013	D	Pre-sand filter	221	348	100
9/3/2013	D	Pre-sand filter	473	>1961*	691
9/10/2013	E	Pre-sand filter	261	636	39
9/24/2013	E	Pre-sand filter	358	730	288
10/8/2013	A	Pre-sand filter	539	579	185
5/13/2014	A	Pre-sand filter	6	836	400
5/20/2014	B	Pre-sand filter	279	>2264*	842
5/27/2014	D	Pre-sand filter	144	161	11
6/3/2014	C	Pre-sand filter	367	>1988*	375
7/1/2014	A	Pre-sand filter	215	458	148
7/8/2014	B	Pre-sand filter	360	673	258
7/15/2014	D	Pre-sand filter	156	227	52
7/15/2014	E	Pre-sand filter	198	598	115
7/22/2014	C	Pre-sand filter	303	>2285*	718
8/5/2014	B	Pre-sand filter	427	1033	500
8/12/2014	D	Pre-sand filter	321	333	94
7/15/2014	D	Raw Sewage	100000	133333	<16667**
7/15/2014	E	Raw Sewage	133333	166667	<16667**
7/22/2014	C	Raw Sewage	143333	173333	30000
8/5/2014	B	Raw Sewage	53333	80000	26667
8/12/2014	D	Raw Sewage	56667	60000	10000
2/09/2015	A	Raw Sewage	46667	65556	6667
3/3/2015	B	Raw Sewage	30000	41111	5556
3/17/2015	E	Raw Sewage	56667	51111	10000
3/24/2015	D	Raw Sewage	36667	63333	11667
3/31/2015	C	Raw Sewage	28917	>84583*	>59667*
4/6/2015	A	Raw Sewage	12000	50167	20000
5/26/2015	A	Raw Sewage	118333	358333	N/A
6/2/2015	E	Raw Sewage	21667	35000	N/A
6/11/2015	C	Raw Sewage	146667	146667	N/A

6/11/2015	B	Raw Sewage	31667	43333	N/A
6/15/2015	A	Raw Sewage	40000	61667	N/A
6/29/2015	D	Raw Sewage	25000	48333	N/A
7/7/2015	E	Raw Sewage	133333	46667	N/A
7/14/2015	C	Raw Sewage	133333	98333	N/A
5/13/2014	A	Reclaimed	0	0	0
5/20/2014	B	Reclaimed	0	0	0
5/27/2014	D	Reclaimed	0	0	0
6/3/2014	C	Reclaimed	40	20	3
7/1/2014	A	Reclaimed	0	0	0
7/8/2014	B	Reclaimed	0	0	0
7/15/2014	D	Reclaimed	0	0	0
7/15/2014	E	Reclaimed	0	0	0
7/22/2014	C	Reclaimed	70	40	10
8/5/2014	B	Reclaimed	0	0	0
8/12/2014	D	Reclaimed	0	0	0
2/09/2015	A	Reclaimed	0	0	0
3/3/2015	B	Reclaimed	0	0	0
3/17/2015	E	Reclaimed	0	0	0
3/24/2015	D	Reclaimed	0	0	0
3/31/2015	C	Reclaimed	22	46	39
4/6/2015	A	Reclaimed	0	0	0
5/26/2015	A	Reclaimed	0	0	N/A
6/2/2015	E	Reclaimed	0	0	N/A
6/11/2015	C	Reclaimed	0	0	N/A
6/11/2015	B	Reclaimed	0	0	N/A
6/15/2015	A	Reclaimed	0	0	N/A
6/29/2015	D	Reclaimed	0	0	N/A
7/7/2015	E	Reclaimed	0	0	N/A
7/14/2015	C	Reclaimed	0	0	N/A

*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.

Table 14. Concentrations (CFU/100 mL) of *C. perfringens* spores as detected by TSC, CCP, and m-CP agars for each sample analyzed

Date	Sample Site	Sample Type	TSCΔ	CCPΔ	m-CPΔ
5/16/2013	A	Pre-sand	121	221	15
5/23/2013	A	Pre-sand	312	267	9
5/28/2013	A	Pre-sand	124	106	3
6/18/2013	A	Pre-sand	121	221	15
6/25/2013	A	Pre-sand	312	267	9
7/23/2013	B	Pre-sand	452	521	48
7/30/2013	B	Pre-sand	197	515	24
8/6/2013	C	Pre-sand	391	515	85
8/20/2013	C	Pre-sand	406	>1464*	79
8/27/2013	D	Pre-sand	61	155	21
9/3/2013	D	Pre-sand	406	467	206
9/10/2013	E	Pre-sand	233	188	21
9/24/2013	E	Pre-sand	418	497	27
10/8/2013	A	Pre-sand	288	158	9
5/13/2014	A	Pre-sand	2	994	24
5/20/2014	B	Pre-sand	179	>1182*	24
5/27/2014	D	Pre-sand	117	133	3
6/3/2014	C	Pre-sand	313	>1535*	56
7/1/2014	A	Pre-sand	165	198	4
7/8/2014	B	Pre-sand	288	413	150
7/15/2014	D	Pre-sand	50	42	1
7/15/2014	E	Pre-sand	77	125	2
7/22/2014	C	Pre-sand	391	>1318*	33
8/5/2014	B	Pre-sand	418	688	27
8/12/2014	D	Pre-sand	200	148	2
7/15/2014	D	Raw Sewage	<16667**	66667	<16667**
7/15/2014	E	Raw Sewage	66667	<16667**	<16667**
7/22/2014	C	Raw Sewage	103333	100000	<1667**
8/5/2014	B	Raw Sewage	66667	43333	<1667**
8/12/2014	D	Raw Sewage	36667	<1667**	<1667**
2/09/2015	A	Raw Sewage	50000	65556	3333
3/3/2015	B	Raw Sewage	27778	5556	556
3/17/2015	E	Raw Sewage	55000	40000	16667
3/24/2015	D	Raw Sewage	60000	65000	18333
3/31/2015	C	Raw Sewage	14667	>100417*	10917
4/6/2015	A	Raw Sewage	34667	38667	3333
5/26/2015	A	Raw Sewage	73333	316667	N/A
6/2/2015	E	Raw Sewage	8333	41667	N/A
6/11/2015	C	Raw Sewage	126667	201667	N/A
6/11/2015	B	Raw Sewage	36667	41667	N/A

6/15/2015	A	Raw Sewage	833	3333	N/A
6/29/2015	D	Raw Sewage	20000	36667	N/A
7/7/2015	E	Raw Sewage	43333	31667	N/A
7/14/2015	C	Raw Sewage	43333	115000	N/A
5/13/2014	A	Reclaimed	0	3	0
5/20/2014	B	Reclaimed	0	0	0
5/27/2014	D	Reclaimed	0	0	0
6/3/2014	C	Reclaimed	70	17	3
7/1/2014	A	Reclaimed	0	0	0
7/8/2014	B	Reclaimed	0	0	0
7/15/2014	D	Reclaimed	0	0	0
7/15/2014	E	Reclaimed	0	0	0
7/22/2014	C	Reclaimed	83	10	3
8/5/2014	B	Reclaimed	0	0	0
8/12/2014	D	Reclaimed	0	0	0
2/09/2015	A	Reclaimed	0	0	0
3/3/2015	B	Reclaimed	0	0	0
3/17/2015	E	Reclaimed	0	0	0
3/24/2015	D	Reclaimed	0	0	0
3/31/2015	C	Reclaimed	22	46	39
4/6/2015	A	Reclaimed	0	0	0
5/26/2015	A	Reclaimed	0	0	N/A
6/2/2015	E	Reclaimed	0	0	N/A
6/11/2015	C	Reclaimed	0	0	N/A
6/11/2015	B	Reclaimed	0	0	N/A
6/15/2015	A	Reclaimed	0	0	N/A
6/29/2015	D	Reclaimed	0	0	N/A
7/7/2015	E	Reclaimed	0	0	N/A
7/14/2015	C	Reclaimed	0	0	N/A

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

	SF+	SF-	Total
AP +	65	4	69
AP-	9	2	11
Total	74	6	80

Table 16: Contingency Table for AP and SF Reactions of TSC Presumptive Negative *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP +	42	5	47
AP-	8	25	33
Total	50	30	80

Table 17: Contingency Table for AP and SF Reactions of CP ChromoSelect Presumptive Positive *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP+	62	9	70
AP-	8	2	20
Total	69	11	80

Table 18: 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-	54	9	63
Total	55	12	67

Table 19: Contingency Table for AP and SF Reactions of m-CP Presumptive Positive *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP+	60	7	67
AP-	9	1	10
Total	69	8	77

Table 20: Contingency Table for AP and SF Reactions of m-CP Presumptive Negative *C. perfringens* Colonies of Unpasteurized Samples

	SF+	SF-	Total
AP+	1	0	1
AP-	1	59	60
Total	2	59	61

Table 21: Contingency Table for AP and SF Reactions of TSC Presumptive Positive *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	64	4	68
AP-	7	5	12
Total	71	9	80

Table 22: Contingency Table for AP and SF Reactions of TSC Presumptive Negative *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	39	9	48
AP-	9	16	25
Total	48	25	73

Table 23: Contingency Table for AP and SF Reactions of CP ChromoSelect Presumptive Positive *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	64	5	69
AP-	10	1	11
Total	74	6	80

Table 24: Contingency Table for AP and SF Reactions of CP ChromoSelect Presumptive Negative *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	6	6	12
AP-	8	37	45
Total	14	43	57

Table 25: Contingency Table for AP and SF Reactions of m-CP Presumptive Positive *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	31	7	38
AP-	7	4	11
Total	11	38	49

Table 26: Contingency Table for AP and SF Reactions of m-CP Presumptive Negative *C. perfringens* Colonies of Pasteurized Samples

	SF+	SF-	Total
AP+	0	1	1
AP-	0	51	51
Total	0	52	52

Table 27: Contingency Table for AP and SF Reactions of total combined presumptive positive *C. perfringens* colonies in pasteurized and unpasteurized samples

	SF+	SF-	Total
AP+	345	36	381
AP-	50	15	65
Total	395	51	446

Table 28: Contingency Table for AP and SF Reactions of total combined presumptive negative *C. perfringens* colonies in pasteurized and unpasteurized samples

	SF+	SF-	Total
AP+	91	22	113
AP-	35	242	277
Total	126	264	390

APPENDIX 2: *Clostridium perfringens* bench sheet

Clostridium perfringens Membrane Filtration Procedure
CP ChromoSelect, TSC and m-CP 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 *C. 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 *C. perfringens* colonies, and it is hazardous to handle. The red color of the *C. 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 *C. perfringens* enumeration in water, such as the widely used Tryptose–Sulphite-Cycloserine (TSC) agar medium. The enumeration of *C. 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 *C. perfringens* by membrane filtration. It gives distinctive green colonies of *C. 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 *C. perfringens*.

General

Agar	**Detectable Bacteria**	**Supplements**
CP ChromoSelect	*C. perfringens*	D-cycloserine
TSC	*C. perfringens*	D-cycloserine
m-CP	*C. perfringens*	D-cycloserine, polymyxin B sulfate, ferric chloride, phenolphthalein diphosphate, Indoxyl-β-D-glucoside, m-CP 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
- m-CP agar base
- D-cycloserine
- Deionized water
- 4.5% ferric chloride
- 0.5% phenolphthalein diphosphate
- Indoxyl β-D-glucoside
- m-CP 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
-

m-CP

- Weigh out 7.11 g/100mL m-CP 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 m-CP 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 *C. 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

<u>Read results of membrane filtration</u>

- 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.
- m-CP 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 *C. perfringens* colonies based on distinctive colony color. In the case of one of the media, m-CP agar, there is an added step of treating the colonies with ammonium hydroxide fumes to elicit a distinctive color change that is diagnostic for *C. perfringens*. The compositions of the three different media are shown in the table below.

Component	Tryptose Sulfite Cycloserine Agar	CP *ChromoSelect* Agar	Modified m-CP 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 (per liter)			
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 + $6H_2O$	none	none	0.09
Indoxyl-ß-D-glucoside	none	none	0.06

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 m-CP 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 m-CP agar because it has been found that the presence of magnesium ions enhances growth of *C. perfringens*.

Ferric ammonium citrate and ferric chloride are sources of iron, which is another enhancer of *C. perfringens* growth. Ferric ammonium citrate plays a major role in TSC agar. *C. 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 *C. 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* spp. and enteric bacteria, except for *Proteus* spp.. 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 m-CP 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 m-CP 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 m-CP agar to act as a substrate for the enzyme acid phosphatase, which is possessed by *C. perfringens*, but not by certain other *Clostridia* species. When yellow presumptive *C. 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 *C. perfringens*. Colonies which remain yellow are other *Clostridia* species.

To summarize, *Clostridium perfringens* colonies grown on m-CP 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
C. 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

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