IMPROVEMENTS IN MICROBE RECOVERY AND DETECTION IN WATER SAMPLES: MULTIPLE APPROACHES TO METHOD OPTIMIZATION

by

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ABSTRACT

GINA HILL KIMBLE. Improvements in microbe recovery and detection in water samples: multiple approaches to method optimization (Under the direction of DR. JAMES E. AMBURGEY)

The accurate recovery and detection of microbes from water samples is important for the protection of public health. However, not all microbes behave similarly, not all water samples have comparable physical properties, and the constituents present in samples can affect microbe properties and recoverability. This dissertation consists of four separate projects that are linked by the common theme of detection efficiency improvement for microbes in water samples.

In the first project, various modifications to USEPA Method 1623, for the detection of *Cryptosporidium* and *Giardia*, were examined. Each successful modification was incorporated into the method, and use of the final, modified method resulted in a 20% increase in *Cryptosporidium* recovery from reagent water and a 41% increase in *Cryptosporidium* from surface water samples. Additionally, the recovery of *Giardia* was improved by 37% in reagent water and 17% in surface water samples.

Similarly, the second research project aimed to improve recoveries of *Cryptosporidium* and *Giardia* from water samples. However, this study compared the effectiveness of pleated capsule filters to hollow-fiber ultrafilters for organism recovery in tap water and surface water samples. In tap water, ultrafiltration produced significantly better recoveries of *Cryptosporidium* (68%) but not *Giardia* (63%). When surface water samples were analyzed, *Cryptosporidium* recovery rates were similar for

both filter types, while *Giardia* was recovered significantly better by ultrafiltration (81%).

The third project involved the evaluation of alternative DNA extraction processes and real-time polymerase chain reaction (qPCR) for the detection of *Cryptosporidium*. After the development of the DNA extraction procedure, tap water samples were seeded and processed, then split prior to detection method. Microscopic detection had a mean recovery of 41%, while samples with qPCR detection had a mean recovery of 49%.

The fourth project focused upon the impact and effects of chemical dispersants. Chemical dispersants have been used successfully in some microbial methods, but there is limited fundamental knowledge of the dispersant effects. Both settling tests and zeta potential analyses were conducted with sodium polyphosphate and sodium metasilicate for multiple particles and microbes in varied water conditions. While settling tests were less effective in the provision of significant results, the zeta potential analyses highlighted some important considerations of for chemical dispersant selection and use. In particular, two inorganic particles and two strains of *E. coli* were tested, and the stability of the different particles in suspension was impacted differently by the chemical dispersants.

DEDICATION

I dedicate my dissertation work to my husband, Jeff, my parents, Willie and Shirley, and my sisters, Hayley, Bethany, and Cortney. Thank you all for your unconditional love and support throughout this process. Jeff, I especially thank you for the sacrifices you have made to support me and for taking care of the everyday chores of life. I love you all immeasurably.

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CT	crossing threshold
DAPI	4’, 6-diamidino-phenylindole
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FA	fluorescent antibody
IMS	immunomagnetic separation
MPC	magnetic particle concentrator
NaHMP	sodium hexametaphosphate
NaMeta	sodium metasilicate
NaPP	sodium polyphosphate
NTU	nephelometric turbidity units
PCR	polymerase chain reaction
qPCR	real-time polymerase chain reaction
TE	Tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
TOC	total organic carbon
UF	ultrafiltration
UNEX	universal nucleic acid extraction
USEPA or EPA	United States Environmental Protection Agency
WSLH	Wisconsin State Laboratory of Hygiene

CHAPTER 1: INTRODUCTION

The quality of drinking water, with respect to microbial contaminants, is an important issue for all public water suppliers and consumers. The presence of harmful microbes, particularly protozoan parasites, in drinking water poses an acute risk to public health. During a study of waterborne disease outbreaks caused by protozoan parasites, Baldursson & Karanis (2011) reported that almost two hundred outbreaks had occurred worldwide between 2004 and 2010, with recreational water as the medium in greater than thirty-three percent of the outbreaks while drinking water system contamination was identified as the source in an additional twenty percent of the outbreaks. Of these outbreaks, sixty percent were caused by *Cryptosporidium* (Baldursson & Karanis 2011). Thus, the availability of reliable detection methods for protozoans is important. However, protozoans are not the only microbes of concern as bacteria led to almost 82% of cases in drinking water outbreak-related cases in the United States during 2009-2010, while bacteria was identified as the source of 23% of recreational water outbreaks during 2011-2012 (CDC 2015; CDC 2013). Therefore, this research aims to improve detection efficiencies, provide additional analytical procedures, and explore the fundamental properties of microbe behavior in water samples of varied composition.

Currently, the United States Environmental Protection Agency (USEPA) has three methods available for the detection of *Cryptosporidium* in water samples. These include: EPA Method 1622 (*Cryptosporidium* in Water by Filtration/IMS/FA) (2005), EPA

Method 1623 (*Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA) (2005), and EPA Method 1623.1 (*Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (2012). All three methods require multiple recovery and concentration steps to be performed in order to reduce sample volume from 10 L or greater to 100 µL.

Methods 1622 and 1623 have documented limitations and shortcomings (Hu et al. 2004). Detection inefficiencies and variations in recovery rates are commonplace when either of these methods is used. However, USEPA Method 1623 is a performance-based method, meaning that the published method can be modified by a laboratory provided that required quality control criteria are met (Clancy, et al. 2003). In brief, samples are filtered to capture *Cryptosporidium* oocysts, *Giardia* cysts and other suspended matter, filters are eluted and the eluate is centrifuged prior to separation of the oocysts from debris through immunomagnetic separation (IMS), and immunofluorescence assay (FA) for microscopic identification. As a result of the many steps required to process samples, problems can occur in any stage of the analysis and can result in reduced recovery efficiency. Moreover, several hours are required to complete the steps required by any of these methods, and a microscopic examination of sample slides must occur before results can be determined. This extended time period can be problematic for a water supplier or public health official during a waterborne outbreak or a potential waterborne outbreak.

In addition to these methods, researchers have also used ultrafiltration to recover various microbes from water samples (Kimble et al. 2012; Hill et al. 2009; Polaczyk et al 2008; Hill et al. 2007; Hill et al. 2005; Morales-Morales et al. 2003). Water samples are filtered through hollow-fiber ultrafilters, and the captured organisms are then removed from the ultrafilter through a back-wash or similar procedure. Further sample processing

is based upon the organism of interest. However, chemical dispersants are often used in these methods, as filter pre-treatments and/or sample amendments, to enhance microbe recovery (Kimble et al. 2012; Hill et al. 2009; Polaczyk et al. 2008; Hill et al. 2007). Additionally, the more recent EPA Method 1623.1 incorporates the use of a chemical dispersant during the elution of organisms from pleated capsule filters. Therefore, there is value in the identification of the actual effects of chemical dispersants in order to guide the use of chemical dispersant in microbial detection methods.

The objectives of research are four-fold. The initial study, which will be discussed in Chapter 2, explored various modifications to USEPA Method 1623 in order to maximize *Cryptosporidium* recovery efficiencies and method performance, while not negatively affecting the recovery of *Giardia*. Although Method 1623 allows for the detection of both *Cryptosporidium* and *Giardia*, only *Cryptosporidium* results are currently used to generate regulatory data and are therefore the focus of this research. Furthermore, only modifications permitted within Method 1623 were selected for consideration. The method modifications studied included: a comparison of fluorescent antibody staining kits, methanol fixation, slides, filters, sample resuspension procedures and elution buffers. The expectation is that enhanced recoveries and staining procedures should yield fewer false-positive and false-negative results from various types of water samples.

The second study, discussed in Chapter 3, also focused on improved recovery of *Cryptosporidium* from water samples. However, the main objective in this study was to explore the efficacy of using an alternative filter technology. Hollow-fiber ultrafilters were tested and results were compared to results for the pleated capsule filters routinely

used to process samples with USEPA Method 1623. While pleated capsule filters are able to capture *Cryptosporidium* and *Giardia*, hollow-fiber ultrafilters may provide better capture and recovery of these microorganisms.

Whereas the first two studies aimed to improve upon the recovery and accuracy issues associated with USEPA Method 1623, the goal of the third study, detailed in Chapter 4, was to offer an alternative method and perhaps more rapid analytical results. Furthermore, any alternate method that was developed during this research phase had to produce comparable or better precision and accuracy than could be achieved with Method 1623. This study encompassed the development of a quantitative, real-time polymerase chain reaction (qPCR) based method of analysis for *Cryptosporidium* in water samples. While sample concentration was still required, the qPCR process is rapid and once concentrated; multiple samples can be analyzed concurrently. Even though PCR-based detection methodologies show promise, there are currently no standardized methods approved by the USEPA for the analysis of *Cryptosporidium*. Therefore, the need for the development of a reliable qPCR technique was crucial.

Lastly, the fourth study (discussed in Chapter 5) focused upon chemical dispersants. Chemical dispersants have previously been used sample amendments or to pre-wet filters in order to enhance the recovery of various microbes from water samples (Kimble et al. 2012; Hill et al. 2009; Polaczyk et al 2008; Hill et al. 2007; Hill et al. 2005; Morales-Morales et al. 2003). More recently, USEPA Method 1623.1 (2012), which incorporates a chemical dispersant into the elution step of the method, was developed. Even though chemical dispersants have been shown to aid in microbe recovery, the mechanisms by which this occurs are not fully understood. Therefore, the

purpose of this research was to study the resultant actions of two chemical dispersants (sodium polyphosphate and sodium metasilicate) when used with various particles under different water quality conditions in order to better understand how and when chemical dispersants could be beneficial to microbe recovery. This was accomplished by an examination of dispersant effects on particle settling and a study of zeta potential to determine whether changes in surface charge occur and the effects of any surface charge alterations.

CHAPTER 2: IMPROVEMENTS IN *CRYPTOSPORIDIUM* RECOVERY AND VARIABILITY THROUGH MODIFICATIONS TO UNITED STATES ENVIRONMENTAL PROTECTION AGENCY METHOD 1623

2.1 Abstract

The use of USEPA Method 1623 for the detection of *Cryptosporidium* in water often results in low and variable rates of recovery. According to data disseminated in Method 1623, less than a quarter of tested surface water samples had *Cryptosporidium* recoveries of 60% or greater. This research explored various method modifications in order to produce a method with higher recovery and less variability. Modifications, that increased the percent recovery, without any adverse effects, were used for the analysis of subsequent samples. Once method modifications were implemented, recoveries of *C. parvum* in reagent water increased by greater than 20% to an average recovery of 76%. In surface water, a significantly higher (P=0.049) mean recovery of 67% for *C. parvum* was achieved with the modified method. *Giardia* is simultaneously detected when using Method 1623. The method modifications significantly increased the recovery of *Giardia* in reagent water by 37% (P=0.0002), while also allowing for a 17% higher recovery in surface water samples (P=0.21). The end result was an optimized method that achieved the best percent recoveries and the least variable recoveries in various water matrices.

2.2 Introduction

At present, there are three methods that are approved by the United States Environmental Protection Agency (USEPA) for the detection of *Cryptosporidium*, which are Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA (2005), Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (2005), and Method 1623.1 *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (2012). Since both 1622 and 1623 are performance-based methods, different modifications may be applied and used to enhance recovery. Although only *Cryptosporidium* is currently regulated, any modifications tested and implemented needed to allow for acceptable recovery of *Giardia*. Various modifications were tested to examine effects on recovery and variability of recovery. Modifications tested included: stain, methanol fixation, filter, sample resuspension, and elution buffer.

Cryptosporidium is a genus that contains protozoan parasites. There are several different species of the *Cryptosporidium* organism. However, there are two main species that infect humans, *Cryptosporidium parvum* and *Cryptosporidium hominis*. *C. hominis* is almost exclusively isolated from humans, while *C. parvum* has many animal hosts as well (Rochelle et al. 2006). The organism is present in the environment, and oocysts can often be found in water (Quintero-Betancourt 2003). *Cryptosporidium* can contaminate surface water when feces from animals or infected humans enter a water body.

While in the environment, the *Cryptosporidium* sporozoites are encapsulated in an oocyst. When the oocyst reaches an intestine, the oocyst splits open, and the infectious sporozoites are released. The sporozoites then enter the lining of the intestine and cause can cryptosporidiosis. For healthy individuals, this illness can produce gastrointestinal

symptoms that last for approximately one to two weeks, while the immune system works to fight off the infection. However, cryptosporidiosis can be fatal in immune-compromised individuals whose immune systems cannot fight the infection. Therefore, it is imperative that reliable methods be available for the analysis of drinking water in order to protect the public from waterborne *Cryptosporidium*.

Outbreaks of *Cryptosporidium*, associated with drinking water, have been documented in the United States since the early 1980s. In 1993, an outbreak of *Cryptosporidium* in the municipal drinking water supply made over 400,000 people ill and killed approximately 100 individuals in Milwaukee (MacKenzie et al. 1994). It took this, the largest waterborne outbreak ever in the United States, to bring *Cryptosporidium* to prominence concerning drinking water analyses and regulations (McCuin & Clancy 2005).

Analysis of water samples for *Cryptosporidium* is not easy to perform, in part because of the size of the organism. *Cryptosporidium* oocysts are quite small, measuring only 4-6 µm (Quintero-Betancourt 2003). Currently, certifiable or approved methods for the analysis of *Cryptosporidium* in water include EPA Method 1622 (2005), EPA Method 1623 (2005), and EPA Method 1623.1 (2012). All three methods entail the filtration of a water sample to capture and concentrate oocysts, separation of the oocysts from debris through immunomagnetic separation, and immunofluorescence assay for microscopic identification. These methods both provided significant improvements over the USEPA Information Collection Rule method, which was developed in 1996 (Clancy et al. 2003). However, both Method 1622 and 1623 have limitations and shortcomings (Hu et al. 2004). Since Method 1623 is the method being used for analysis of *Cryptosporidium* in

this study, this will be the method of focus. Although not the focus of this study, results for *Giardia* will also be discussed.

The major problems encountered with USEPA Method 1623 are low and variable recoveries of oocysts (DiGiorgio et al. 2002; Hu et al. 2004). This is problematic since low recoveries can lead to false negative results for water samples, thus potentially allowing drinking water containing low levels of *Cryptosporidium* to be distributed to the public (USEPA 1999). The specific step or steps of the method leading to low recoveries have not been fully identified (Hu et al. 2004). Therefore, these problematic areas might involve the physical equipment (e.g., filter) or reagents (e.g., stain) used, or the problem could involve the way in which the analyst performs the method (e.g., sample transfer). Another factor that might play a role in low or variable recoveries is the difficulty of slide examination. Slide examination in Method 1623 requires a great deal of expertise, and confirmation of an oocyst is often difficult to achieve (USEPA 1999; Clancy et al. 2003).

Since the identification of oocysts using Method 1623 relies on microscopy, the interpretation of the results by the analyst is somewhat subjective. Safeguards, in the form of multiple microscopic identification steps are required by the method, but misinterpretations can still be made. The initial presumptive identification involves the detection of an oocyst, which looks like a small, green sphere, when using epifluorescent microscopy. Following this presumptive identification, the analyst must perform a two-stage confirmation. First, the analyst must identify appropriate staining patterns of the suspected *Cryptosporidium* oocyst with 4',6-diamidino-phenylindole (DAPI). Secondly, through the use of differential interference contrast (DIC) microscopy, the analyst must confirm that there are no abnormal or atypical structures present on the observed

organism (USEPA 1999). Nevertheless, even with these additional confirmatory steps required, false positives and false negatives are still possible (Sturbaum 2003).

In summary, *Cryptosporidium* is a protozoan parasite that can survive in water and has been identified as a source of waterborne illness. Analytical methods do exist for the detection of this organism, but there is a need to improve upon recovery efficiencies and detection accuracies. There is the potential that method modifications to USEPA Method 1623 could allow for better recovery and more precise detection. Thus, this study attempted to improve on Method 1623 so that *Cryptosporidium* could be detected more accurately and reliably in water supplies.

Previous researchers have made attempts to improve the performance of Method 1623 (or 1622) through modifications or changes to the method. In brief, researchers have changed filters, IMS procedures, stains, and seeding procedures in an attempt to improve recovery and decrease variability of results. One often-studied change involves the filter. According to Hu et al. (2004) a significant decline in *Cryptosporidium* recovery occurs during the filtration step. Thus, there is a good reason why researchers often try different filters when modifying Method 1623.

One previous study compared the use of the standard Envirochek filter to a hollow-fiber ultrafilter (Hemoflow F80A) for processing both reagent and surface water samples (turbidities ranged from 2.5 to 45 NTU) with Method 1622 (Simmons et al. 2001). The authors reported that there was no significant difference between the filters when processing reagent water; but when surface water was analyzed, the ultrafilter performed significantly better (Simmons et al. 2001). The authors of a second study that also used the same type of hollow-fiber ultrafilters reported recoveries of 83% with

ultrafiltration versus 46% for Envirochek HV filters for *C. parvum* from seeded tap water samples (Hill et al. 2009).

Two other studies performed comparisons of the standard Envirochek and the Envirochek HV filters. The first of these studies found that Envirochek HV filters performed significantly better than standard Envirochek filters when low turbidity (11 NTU) raw water was the matrix, but reported that there was no significant difference with high turbidity (88 to 99 NTU) water (DiGiorgio et al. 2002). The second study tested the standard Envirochek and the Envirochek HV filter with reagent water, tap water, and reclaimed water (turbidities less than 1.75 NTU) using both live oocysts and ColorSeed. In reagent water, the standard Envirochek filter achieved a better mean percent recovery with either type of oocysts, but the difference was not significant (Quintero-Betancourt 2003). In tap water, the results were mixed. Tap water spiked with live oocysts had a better mean percent recovery when the Envirochek HV filter was used, but the standard Envirochek produced a better mean percent recovery when ColorSeed was used as the spike source (Quintero-Betancourt 2003). In reclaimed water, the samples were only analyzed using ColorSeed, and the average mean percent recovery for the HV was 40% compared to 32% for the standard Envirochek. The study by Quintero-Betancourt et al. (2003) also compared EasyStain to Aqua-Glo. Although each was reported to be equally effective in the detection of *Cryptosporidium*, EasyStain was preferred for the lower levels of background fluorescence and nonspecific binding (Quintero-Betancourt 2003).

A third study compared the recovery efficiencies of *Cryptosporidium* and *Giardia* with five types of filters. The filters used included the standard Envirochek, the Envirochek HV, Filta-Max, Millipore flatbed membrane filter, and Sartorius flatbed

membrane filter. Initial tests were performed with distilled water samples that were seeded with EasySeed. Since the capsule design of both the Envirochek and Envirochek HV filters would allow, these filters were subject to either elution or a backwash (Wohlsen et al. 2004). For distilled water samples, mean recoveries of *Cryptosporidium* as low as 0.2% (standard Envirochek with elution) to 53% with the Envirochek HV with a backwash (Wohlsen et al. 2004). Mean recoveries for *Giardia* were similar, 4% (standard Envirochek with elution) to 59% for the Envirochek HV with a backwash (Wohlsen et al. 2004). The researchers then selected the three filters (Envirochek HV with backwash, Filta-Max, and Sartorius membrane filter) with the highest recoveries, and these were tested with surface water samples that had been seeded with ColorSeed (Wohlsen et al. 2004). The Envirochek HV filter with a backwash produced the highest mean recovery for both *Cryptosporidium* (51%) and *Giardia* (37%) (Wohlsen et al. 2004). Raw water turbidities were not specified.

In addition to the above modifications, two studies used altered immunomagnetic separation (IMS) dissociation procedures. The first of these studies involved the use of heat dissociation (80^{o}C for 30 minutes) compared to acid dissociation followed by heat. Method 1623 specifies two acid dissociations without heat (Ware et al. 2003). The researchers found that heat dissociation alone produced a significantly better mean percent recovery in both reagent and raw water, while also improving the 4',6-diamidino-2-plenylindole (DAPI) confirmation rate.

The second IMS modification study used the same heat dissociation procedure (80^{o}C for 30 minutes), while comparing the efficacy of using ColorSeed instead of live oocysts to seeded surface water samples with turbidities ranging from 1.4 to 2,700 NTU.

The study showed very similar mean percent recoveries (43% for ColorSeed compared to 45% for live oocysts) and percent relative standard deviations (22% for ColorSeed compared to 25% for live oocysts) for the two seed sources, and no significant difference was found (Francy et al. 2004).

The current project explored various modifications to United States Environmental Protection Agency Method 1623. Method 1623 is a performance-based method for the analysis of *Cryptosporidium* and *Giardia* in water. The various modifications that were tested included: filter type, slide type, methanol fixation, elution buffer, IFA kit, and sample resuspension method. These variations were assessed in terms of the mean percent recovery and percent relative standard deviation that was achieved. The first modification assessed was stain, followed by methanol fixation, and slide type. After that initial assessment, the best combination was selected and used in the processing of other samples. After a filter was selected, subsequent samples were subjected to changes in transfer method, and elution buffer. All future modifications, that increased the percent recovery, without any adverse effects, were used for the analysis of subsequent samples. The desired end result was an optimized method that achieved the best percent recoveries and the least variable recoveries in various water matrices.

2.3 Methods and Materials

The December 2005 version of EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (USEPA 2005) was used for all sample analyses. The specific procedures outlined in the method were followed, with the exceptions of the items involved in the modification study. All slide examinations and organism

determinations were made in a manner consistent with those described in Method 1623 (USEPA 2005).

2.3.1 Oocysts and Water Matrices

Oocyst stocks were obtained from three sources. Live, flow-cytometry sorted spikes were obtained from the Wisconsin State Lab of Hygiene (WSLH). These spiking suspensions were heat inactivated at 80^{o}C for 30 minutes prior to sample seeding. Additionally, preparations from Waterborne, Inc. (New Orleans, LA, USA) were obtained. These preparations included one vial containing 10^6 oocysts and a second vial containing 10^6 cysts. These preparations were enumerated by hemacytometer counts and diluted to appropriate spiking concentrations for slide and staining comparisons. Lastly, EasySeed (BTF, Sydney, Australia) gamma irradiated spiking suspensions were used for filter comparisons.

For the stain comparison, methanol fixation comparison, and slide comparison, serial dilutions of the Waterborne spiking suspension (New Orleans, LA, USA) were prepared. Aliquots (50 µL) of the diluted spiking suspension were placed directly on slides, without the entire method having been performed. This allowed for a faster determination of which stain, fixation, or slide produced better recoveries, while eliminating variances from other method procedures.

For samples processed through the entire 1623 procedure, the water was obtained from two locations. Deionized water was used for method blanks and reagent water samples. Raw or untreated water samples were collected from the raw water influent tap at the Franklin Water Treatment Plant (Charlotte, NC, USA). The source water came from Mountain Island Lake, with an average total organic carbon (TOC) concentration of

less than 2.0 mg/L and turbidities that ranged from 2.5 to 4.6 nephelometric turbidity units. Source water samples were used for matrix and matrix spike samples. Matrix samples were unseeded source water samples that were analyzed to determine whether or not oocysts and/or cysts were naturally present in the untreated water. Matrix spike samples were seeded with either EasySeed or the WSLH viable organisms.

2.3.2 Filtration

Samples were filtered and the filters were eluted as specified in Method 1623. Two capsule filters, the standard Envirochek (Pall Corporation, catalog no. 12110) and the Envirochek HV (Pall Corporation, catalog no. 12107), were compared in this study. Three ten-liter raw water samples, seeded with EasySeed, were filtered through each type of capsule filter. After filtration was complete, each filter was removed from the filtration assembly and the outlet end was capped.

2.3.3 Elution

The Laureth-12 based elution buffer specified in Method 1623 was used for elution of all samples except those used for elution buffer comparisons. For the elution buffer modifications, the standard elution buffer was compared to two different modified elution buffer solutions; the first with an addition of 0.10% Tween-80 and the second with an addition of 0.01% sodium polyphosphate. The elution buffer comparison between the standard elution buffer and the Tween-80 addition involved the analysis of five seeded reagent water samples eluted with each buffer type. For the comparison of the standard elution buffer to the elution buffer with sodium polyphosphate, three spiked raw water samples were analyzed using each buffer type. In order to begin the elution procedure, each capsule was filled to the top of the filter element with elution buffer, the

inlet end was capped, and the filter was placed horizontally on a wrist-action shaker. The filter was shaken at 900 rpm with the filter bleed valve in the twelve o'clock position. After five minutes, the capsule was removed, and the liquid contents were poured into a 250 mL centrifuge tube. This procedure was repeated with the bleed valve in the four o'clock position and the eight o'clock position. However, the eluate was not poured into the centrifuge tube after the shake in the four o'clock position. After the final shake, the eluate was added to the centrifuge tube.

2.3.4 Sample Resuspension

Samples were further processed through concentration by centrifugation consistent with Method 1623. After centrifugation, the liquid was carefully aspirated to approximately five milliliters above the packed pellet, using a vacuum pump and a Pasteur pipet. The centrifuge tube was then either vortexed for thirty seconds or the sample was triturated (by repeatedly pipetting a 5 mL volume into and out of a 10 mL serological pipet) for thirty seconds prior to transfer to a Leighton tube. Five spiked reagent water samples were vortexed prior to sample transfer, while five additional spiked reagent water samples were triturated prior to sample transfer. After transfer, the centrifuge tube was rinsed with approximately five milliliters of reagent water and the rinse was transferred to the same Leighton tube.

2.3.5 Immunomagnetic Separation

Immunomagnetic separation (IMS) for all samples followed the procedure in Method 1623 with the use of the Dynal Dynabeads-GC Combo kit (Invitrogen, Dynal, Oslo, Norway). After addition of the buffers and beads, the sample was rotated for one hour. After rotation, the sample tube was removed and placed in a magnetic particle

concentrator (MPC), which was rocked by hand for two minutes. The cap was removed and the supernatant was poured into a fifty milliliter centrifuge tube. The supernatant was placed in the refrigerator in case a sample had to be reanalyzed due to poor recoveries. The sample tube was then rinsed three times with 500 microliter (μL) aliquots of 1X SL-buffer A. Each rinse was placed in a 1.5 mL conical microcentrifuge tube, leaving some headspace.

The microcentrifuge tube was placed in a second magnetic particle concentrator, and rocked for one minute. After the minute had passed, the liquid was aspirated. This left a small, brown dot at the back of the microcentrifuge tube. Finally, the magnet was removed from the MPC, and 100 μL of 0.1 N HCl was added to the tube. The 100 μL volume was a slight modification from the standard method. Method 1623 uses 50 μL acid volumes, but this study used 100 μL volumes so that one-half of the acid dissociation could be used for real-time polymerase chain reaction (qPCR) analysis (data shown in Chapter 4). This variation was previously used by Quintero-Betancourt (2003) with no adverse effects. The tube was vortexed for thirty seconds and then allowed to stand at room temperature for ten minutes. After ten minutes, the tube was vortexed for fifteen seconds, placed back in the MPC, and the magnet was replaced. The sample stood undisturbed for fifteen seconds. Next, 10 μL of 1.0 N NaOH was added to the center of a well slide. Without removing the tube from the MPC, 50 μL of the sample was quantitatively placed onto the well slide and 50 μL was placed in another microcentrifuge tube for PCR analysis. Following this procedure, the magnet was again removed from the MPC and another dissociation using 100 μL of 0.1 N HCl was performed.

2.3.6 Sample Staining and Slide Examination

The 100 μL sample that had been placed on the well slide was dried on a slide warmer (approximately 40 minutes) at 39°C. In order to do another optimization comparison, five dried samples were fixed with methanol, while five samples were not. Methanol fixation involved placing one drop of absolute methanol on the dried sample and waiting approximately five minutes for the methanol to dry.

Additionally, six slides were stained with Aqua-Glo (Waterborne) and six slides were stained with EasyStain (BTF). Procedures recommended by the manufacturer were followed. Slides used were either Dynal or Waterborne. After incubation, the stain was aspirated with a vacuum pump and Pasteur pipet, and the sample well was washed according to the manufacturer's recommendations. After one minute, the rinse was aspirated; and 50 μL of DAPI staining solution was added to the slide well. The DAPI was allowed to stand on the slide well for two minutes before being aspirated. This was followed by another rinse and aspiration. Finally, a mounting medium was added to the slide well, a cover slip was added, and the cover slip was sealed with clear fingernail polish.

Each slide was scanned for apple-green fluorescing circles (*Cryptosporidium*) or ovals (*Giardia*). When observed, the microscope UV filter block was used to analyze the DAPI staining pattern. If the proper pattern existed, the organism was counted. In addition, a minimum of three cysts and oocysts and any questionable organisms were also subjected to differential interference contrast (DIC) microscopy using 1000X total magnification to look for any atypical characteristics.

2.4 Statistics

Recovery efficiency for each sample was calculated by dividing the number of recovered organisms by the number of organisms seeded into the ten-liter sample. The resulting fraction was then multiplied by 100 to obtain a percent recovery. Statistical comparisons were made using analysis of variance (ANOVA), with statistical significance set at 0.05 (Minitab 15, Minitab, Inc., State College, PA, USA).

2.5 Results and Discussion

2.5.1 Comparison of Staining Procedures

The first tests involved placing aliquots of an oocyst/cyst suspension on either Dynal (9 mm) or Waterborne (15 mm) slides, fixing the sample with methanol, and staining the sample with EasyStain or Aqua-Glo. The mean recovery for *Cryptosporidium* using EasyStain was 94% (SD=12%), while slides stained with Aqua-Glo produced a mean recovery of 82% (SD=24%). The difference was not statistically significant (P=0.410). For *Giardia*, the mean recovery with EasyStain was 97% (SD=11%) and 103% (SD=11%) for Aqua-Glo. As with *Cryptosporidium*, the difference for *Giardia* was not statistically significant (P=0.494).

With regard to the elimination of methanol fixation, slides stained with EasyStain yielded a percent recovery of 102% for *Cryptosporidiu*m based on hemacytometer counts. However, when Aqua-Glo was used without methanol fixation, the *Cryptosporidium* recovery fell dramatically to only 28%. This difference was determined to be statistically significant (P=0.000). Figure 1 shows a summary of this information for *Cryptosporidium*. When slides were examined for *Giardia*, there was a 100% recovery with EasyStain, while Aqua-Glo recovery was 78% (P=0.081).

Lastly, tests were done to determine whether or not the slide that was used made a difference in recovery. When EasyStain was used with Dynal slides, the percent recovery for *Cryptosporidium* was 95% compared to 94% for Waterborne slides (P=0.95). Using Aqua-Glo on Dynal slides produced a percent recovery of 90%, while the percent recovery was determined to be 78% for Waterborne slides (P=0.28). For *Giardia*, results were similar but somewhat more variable. When EasyStain was used on Dynal slides, the *Giardia* recovery was 93%, compared to a recovery of 100% for Waterborne slides (P=0.43). Dynal slides stained with Aqua-Glo yielded a 90% recovery for *Giardia*, while Waterborne slides had a recovery of 109% (P=0.19).

For *Cryptosporidium*, EasyStain provided better percent recoveries whether methanol fixation was used or not. In addition, EasyStain provided better percent recoveries of *Cryptosporidium* on both types of slides, but the difference for Dynal slides was very small. These findings are consistent with those of Quintero-Betancourt (2003) who reported that although the stains were equally effective, EasyStain provided improved enumeration and differentiation. The likely reason for better percent recoveries with EasyStain was the specificity of the stain. The samples stained with EasyStain were easier to examine because there was less background fluorescence.

When EasyStain was used, methanol fixation did not significantly change recoveries, but the DAPI staining virtually disappeared without it. This was an important issue since DAPI staining patterns aid the analyst in making a positive identification. Without the DAPI staining, confirmatory identification was much more difficult. Methanol fixation was thus determined to be beneficial and used throughout the remainder of the study.

With regard to slide selection, the use of either slide appeared to yield acceptable recoveries. Waterborne slides allowed for a larger aliquot of sample to be added to the slide with less risk of overflowing the well. However, the examination of the Dynal slides was faster because of the smaller sample well. In addition, no sample in this study ever overflowed the well of a Dynal slide. Even though both slides produced acceptable results, the Dynal slide was preferred for the reduced slide examination time.

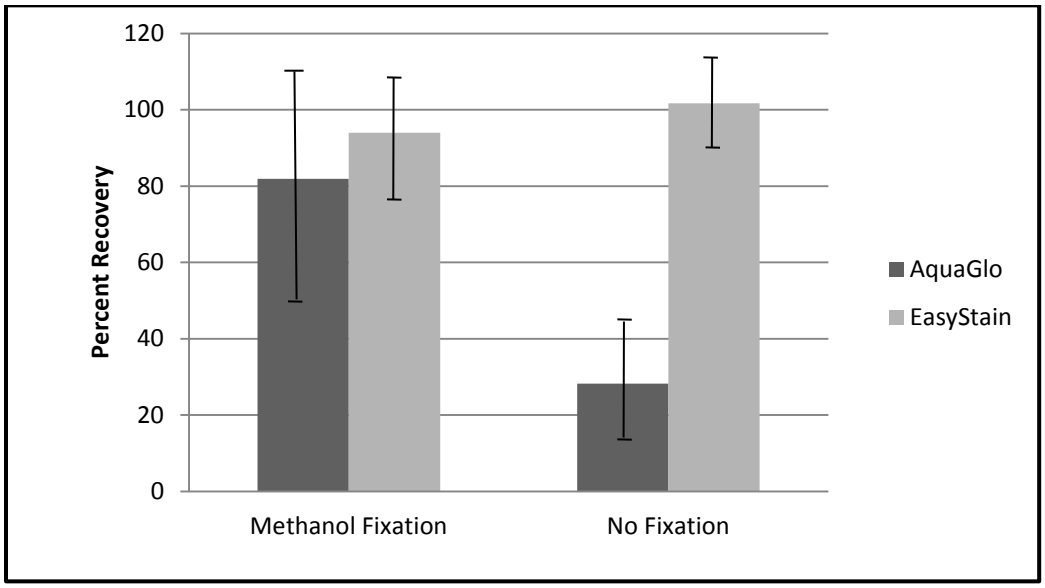

FIGURE 1: Summary of results for positive control comparisons - *Cryptosporidium*

2.5.2 Comparison of Filters

The second set of modifications involved testing standard Envirochek and Envirochek HV filters. For seeded source water samples, the mean recovery of *Cryptosporidium* using the standard Envirochek filters was 29%. The Envirochek HV filters produced a higher mean recovery of 40% for *Cryptosporidium*. However, this difference was not statistically significant (P=0.407). The Envirochek HV also produced better results with regard to percent relative standard deviation. The Envirochek HV had

a standard deviation of 14% versus a 15% standard deviation for the standard Envirochek filter. Figure 2 shows the mean percent recoveries and percent relative standard deviations for source water samples using each type of filter. Again, the results for *Giardia* were similar to those for *Cryptosporidium*. For *Giardia*, the standard Envirochek filter yielded a 51% recovery compared to a 64% recovery for the Envirochek HV filter (P=0.519). The Envirochek HV also allowed for an improvement in the standard deviation between samples. The samples analyzed with the standard Envirochek had a standard deviation of 28% compared to 16% for the Envirochek HV.

Since better mean percent recoveries and a lower relative standard deviation were achieved using the Envirochek HV filters, these filters were used for the remainder of the study. Quintero-Betancourt (2003) also found that the Envirochek HV filters performed better. The Envirochek HV filters have a slightly higher cost per filter, but the increased cost was deemed acceptable since the recovery efficiency was considerably better, and the results were much less variable.

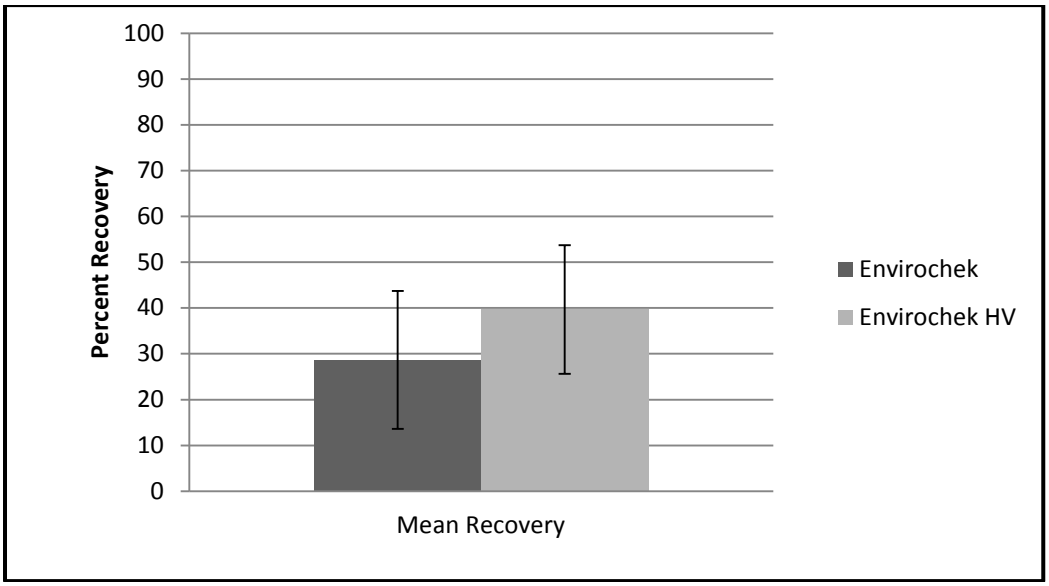

FIGURE 2: Summary of results for filters – *Cryptosporidium*

2.5.3 Comparison of Sample Resuspension Methods

The next modification involved the comparison of using either vortexing or trituration of the sample prior to transfer from the centrifuge tube to a Leighton tube. For this modification, seeded reagent water samples were used. For the vortexed samples, a mean percent recovery of 72% for *Cryptosporidium* was achieved, while this resuspension method involved a standard deviation of 15%. The mean percent recovery for samples subjected to trituration was 76%, and a standard deviation of 15% was again attained. The difference in mean percent recovery between sample resuspension methods was not found to be significant (P=0.735). When the recovery for *Giardia* was considered, vortexed samples had a mean recovery of 86% (SD=11%), while samples subjected to trituration had a mean recovery of 78% (SD=6%). As with *Cryptosporidium*, the difference in recovery of *Giardia* for each resuspension method was not statistically significant (P=0.199). There was no significant difference between the methods, but samples that were resuspended using trituration did have better *Cryptosporidium* recovery (and the relative standard deviation was not adversely affected). So, trituration replaced vortexing. Figure 3 shows the results of trituration and vortex.

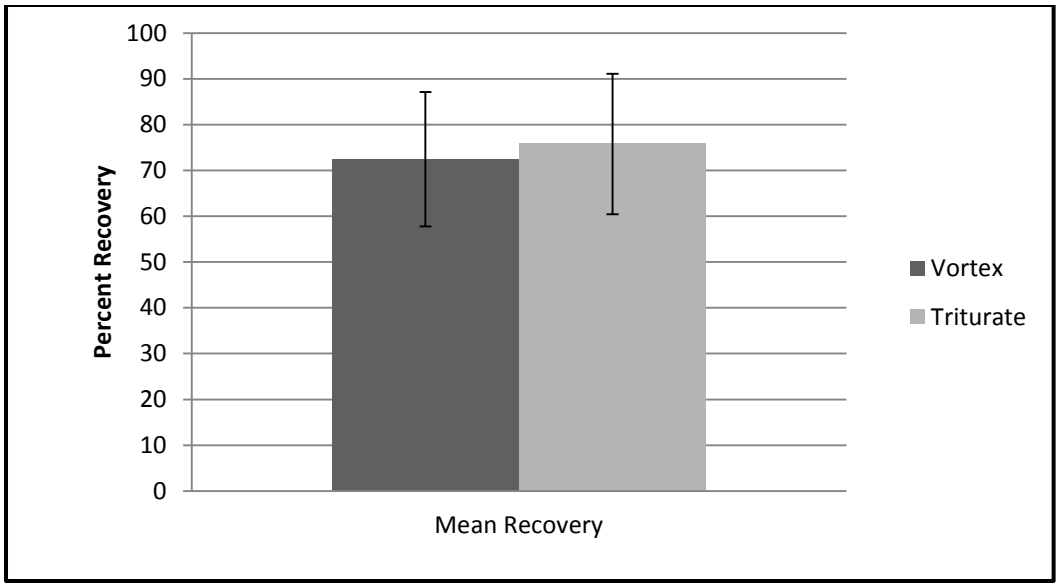

FIGURE 3: Summary of results for sample resuspension – *Cryptosporidium*

2.5.4 Comparison of Elution Buffers

The fourth modification was that of altering the elution buffer. Two different elution buffer modifications were used. For the first elution buffer modification (the addition of 0.10% Tween-80), five reagent water spikes were eluted with the standard elution buffer, while five reagent water spikes were eluted with the modified elution buffer. The mean percent recovery of *Cryptosporidium* for the five samples analyzed using the standard elution buffer was determined to be 76%, and the standard deviation was 15%. When using the modified elution buffer the mean percent recovery achieved was 71%, and the standard deviation was 11%. The difference in the mean percent recoveries between the two elution buffers was not statistically significant (P=0.566). These results are further shown in Figure 4. As for *Giardia* recovery, samples processed with the standard elution buffer had a mean recovery of 78% (SD=6%), while samples processed with the modified elution buffer had a mean recovery of 86% (SD=6%). The difference in Giardia recoveries was not statistically significant (P=0.082).

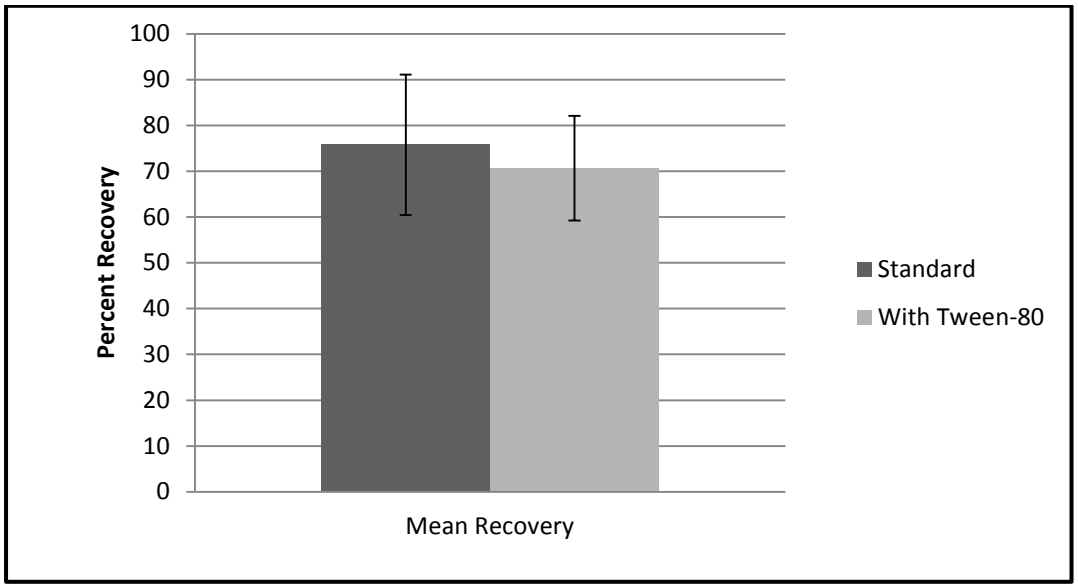

FIGURE 4: Summary of results for elution buffer with 0.10% Tween-80 - *Cryptosporidium*

The second elution buffer modification (the addition of 0.01% sodium polyphosphate) was performed using spiked source water samples. Three spiked source water samples were eluted from the filter using the standard elution buffer preparation, while three additional spiked reagent water samples were eluted using the elution buffer with the added sodium polyphosphate. Using the standard elution buffer, the mean percent recovery achieved was 67% with a standard deviation of 16%. The samples eluted with the elution buffer containing sodium polyphosphate had an average mean percent recovery of 65% and a standard deviation of 11%. While a small decrease in the mean percent recovery was found when sodium polyphosphate was added, this decline was not significant (P=0.865), although the variability was lower for the second elution buffer formulation. These results are summarized in Figure 5.

When *Giardia* recovery was considered, the use of the standard elution buffer produced a mean recovery of 21% (SD=15%) and the modified elution buffer allowed for

a recovery of 25% (SD=12%). As with *Cryptosporidium*, the difference in *Giardia* recovery was not statistically significant (P=0.313).

To summarize the elution buffer results, the standard elution buffer described in Method 1623 produced a better mean recovery for *Cryptosporidium* than either modified elution buffer with Tween-80 or sodium polyphosphate added, but the variability was slightly greater in samples processed with the standard elution buffer. Since neither modification of the standard elution buffer improved recovery efficiency, the standard elution buffer was retained and used for future sample processing.

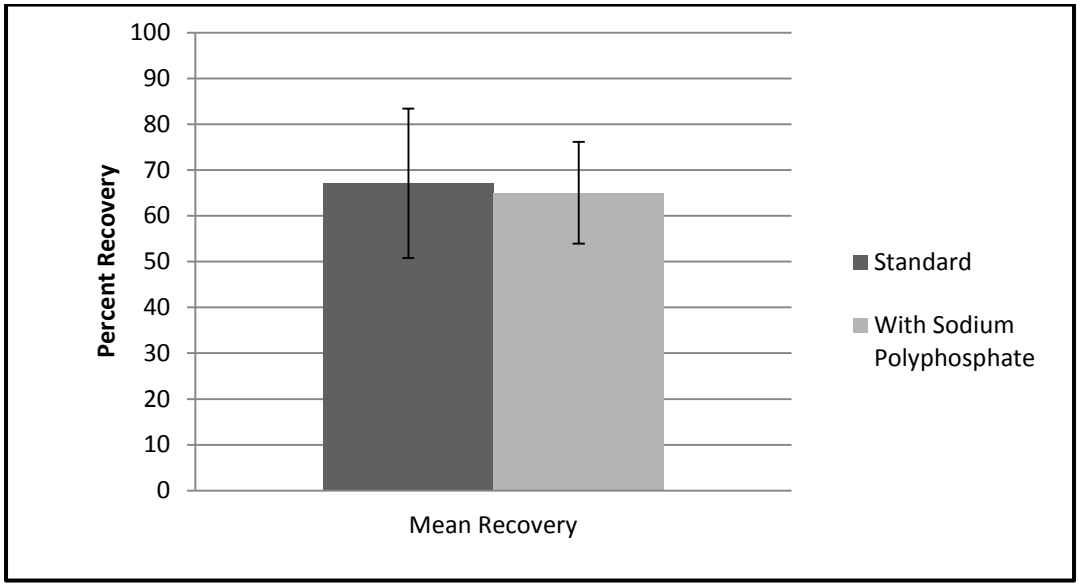

FIGURE 5: Summary of results for elution buffer with 0.01% Sodium Polyphosphate - *Cryptosporidium*

2.6 Conclusions

The final method modifications that were implemented or maintained for use included: EasyStain, methanol fixation, Envirochek HV filters, and standard elution buffer. Use of the optimized method has produced a method with a mean percent recovery of 76% for *Cryptosporidium* and a standard deviation of 15% in reagent water.

In source water, the optimized method has produced a mean percent recovery of 67% for *Cryptosporidium* with a standard deviation of 16%.

Without the use of the method modifications, the average *Cryptosporidium* recovery that could be achieved in reagent water was 55%. However, when source water samples were analyzed, the mean *Cryptosporidium* recovery was typically less than 30%. Therefore, the optimized method, has allowed for a greater than 20% increase in mean recovery of *Cryptosporidium* from reagent water and a statistically significant increase (P=0.049) in recovery from raw surface water. Results for the modified method versus the unmodified method are summarized in Figures 6 and 7.

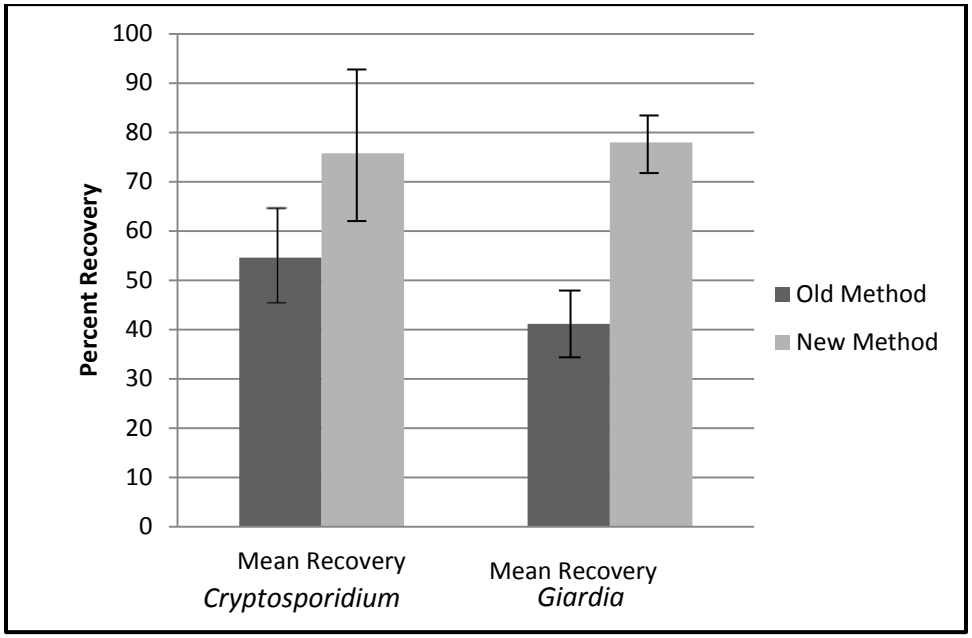

FIGURE 6: Summary for the modified method for *Cryptosporidium* and *Giardia* in reagent water samples

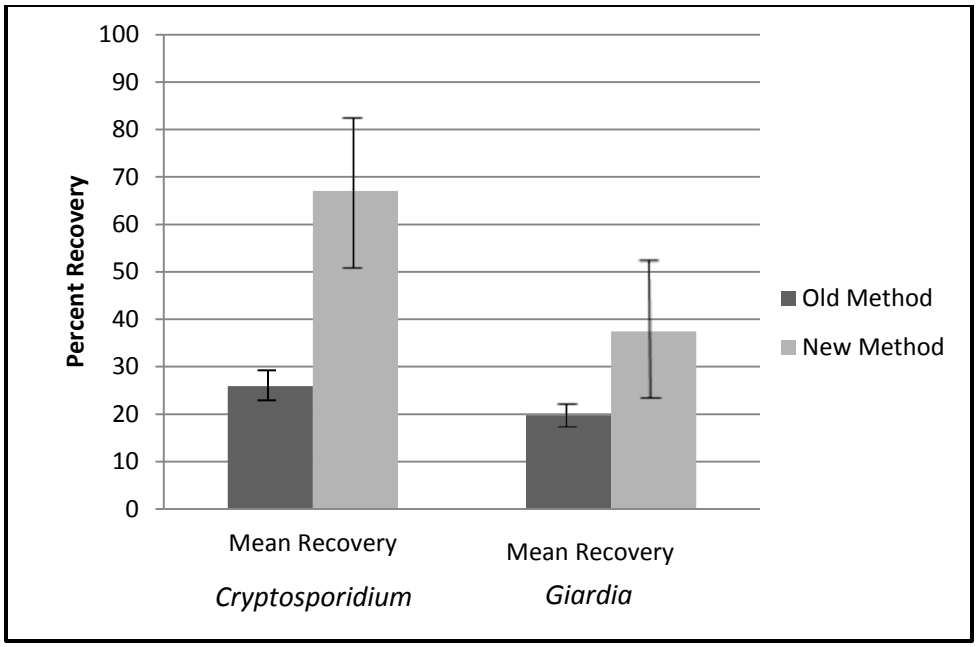

FIGURE 7: Summary for the modified method for *Cryptosporidium* and *Giardia* in source water samples

Since Method 1623 allows for the simultaneous detection of both *Cryptosporidium* and *Giardia*, the desired goal of this study was to improve *Cryptosporidium* recovery while not negatively impacting the recovery of *Giardia*. To this end, the modified method has resulted in a significantly better mean recovery of *Giardia* in reagent water samples. The unmodified method produced a *Giardia* recovery of 41% in reagent water samples, while the modified method has produced a mean recovery of 78% (P=0.0002). As for source water samples, the recovery of *Giardia* has improved from 20% to 37% (P=0.21). Therefore, the study has produced a method that allows for better recoveries of both *Cryptosporidium* and *Giardia* in both reagent and source water samples.

CHAPTER 3: COMPARISON OF HOLLOW-FIBER ULTRAFILTERS WITH PLEATED CAPSULE FILTERS FOR SURFACE AND TAP WATER SAMPLES USING UNITED STATES ENVIRONMENTAL PROTECTION AGENCY METHOD 1623

3.1. Abstract

The USEPA Method 1623 is designed specifically for the detection of *Cryptosporidium* and *Giardia*, but the method has some issues with low and variable recoveries. Ultrafiltration has been used effectively for microorganism recovery from water samples but is not approved by USEPA. In order to determine the efficacy of using ultrafiltration, ten-liter tap water and surface water samples were seeded with *Cryptosporidium parvum* and *Giardia lamblia* and concentrated with either a pleated capsule filter or a single-use hollow-fiber ultrafilter. For *Cryptosporidium*, oocyst recovery in tap water was significantly higher for ultrafiltration (68%) versus the capsule filter (37%), while ultrafiltration recovered 65% of oocysts in surface water versus 61% for the capsule filter. However, *Giardia* cyst recovery was mixed. In tap water, the capsule filter produced significantly better recovery (85%) of *Giardia* compared to ultrafiltration (63%), but the surface water ultrafiltration recovery (81%) was significantly better than the capsule filtration recovery (40%). Overall, ultrafiltration recoveries were equal to or better for *Cryptosporidium*, but recoveries of *Giardia* varied based upon the filter used and the type of water analyzed.

3.2. Introduction

Cryptosporidium and *Giardia* are protozoa that can be present in surface water and can remain after conventional drinking water treatment processes. Outbreaks of *Cryptosporidium*, associated with drinking water, have been documented in the United States since the early 1980s (Solo-Gabriele & Neumeister 1996). In 1993, an outbreak of *Cryptosporidium* in the municipal drinking water supply made over 400,000 people ill and killed approximately 100 individuals in Milwaukee (MacKenzie et al. 1994). When the Milwaukee outbreak occurred in 1993, there were no *Cryptosporidium* testing requirements for public water suppliers. However, with the promulgation of the Long Term 2 Enhanced Surface Water Treatment Rule (LT2), surface water monitoring for *Cryptosporidium* became a requirement for water suppliers with a population of 10,000 or greater (USEPA 2006). Public water suppliers that were required to sample under the LT2 rule had to perform monthly monitoring for 24 months.

Currently, the approved methods for the detection and analysis of these two microorganisms are EPA Method 1623 (USEPA 2005) and EPA Method 1623.1 (2012). There can be problems with recovery and variability when Method 1623 is used (Hu et al. 2004; DiGiorgio et al 2002). Thus, researchers have tried different modifications in order to improve the performance of Method 1623. One method alteration that has been tested involves the type of filter used. The most commonly used filter in Method 1623 is a capsule filter, the Envirochek HV, but some researchers have tested the efficacy of hollow- fiber ultrafilters (Hill et al. 2009; Hill et al. 2005; Morales-Morales et al. 2003; Simmons et al. 2001).

One study that compared the use of the standard Envirochek filter to a hollow-fiber ultrafilter (Fresenius Hemoflow F80A) for processing both reagent and surface water samples with Method 1622 reported no significant difference between the filters when processing reagent water; but the ultrafilter performed significantly better when surface water was analyzed (Simmons et al. 2001). A second study using the same type of hollow-fiber ultrafilter reported recoveries of greater than 80% for *C. parvum* from seeded tap water samples (Hill et al. 2005). A more recent study that compared ultrafiltration to Method 1623, found that ultrafiltration (UF) produced significantly better recoveries of *Cryptosporidium*, but not *Giardia*, in tap water samples (Hill et al. 2009).

The objective of this study was to determine the recovery of *Cryptosporidium* and *Giardia* from both surface water and tap water using the Envirochek HV (Pall Corporation, Ann Arbor, MI, USA) and Fresenius Optiflux 200NR filters (Fresenius Medical Care, Lexington, MA, USA). Fresenius Optiflux 200NR filters are high-flux, hollow-fiber, polysulfone dialysis filters with a surface area of 2.0 m^2, a fiber inner diameter of 200 µm, and a molecular weight cutoff of approximately 30 kDa; these filters were operated in the cross-flow mode for this study.

3.3. Methods and Materials

The December 2005 version of EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (USEPA 2005) was used for all sample analyses. The specific procedures outlined in the method were followed, with the exception of the ultrafiltration procedure. All slide examinations and organism determinations were made in a manner consistent with those described in Method 1623 (USEPA 2005).

3.3.1. Oocyst Stocks and Water Matrices

Ten-liter treated water (n=5) and raw water (n=5) samples were obtained from the Franklin Water Treatment Plant (Charlotte, NC, USA), whose surface water comes from Mountain Island Lake. Turbidity for the tap water is generally in the range of 0.1-0.3 nephelometric turbidity units (NTUs), while the source water generally has turbidity values of less than 5 NTUs. Total organic carbon (TOC) averages approximately 1 mg/L in the tap water and is less than 2 mg/L in the source water. Flow-cytometry sorted oocyst/cyst suspensions obtained from the Wisconsin State Laboratory of Hygiene (WSLH) were seeded into each water sample. Different sets of spiking suspensions were used during analyses, but each suspension contained a specified number of cysts/oocysts in the range of 149-172, according to the specification sheets provided with the suspensions.

3.3.2. Sample Processing

Five tap water samples were processed with the pleated capsule filter, and five tap water samples were processed with the follow-fiber ultrafilter. Additionally, five surface water samples were processed with each type of filter. Unseeded control samples of each water type were also processed, and neither *Cryptosporidium* nor *Giardia* was detected in any of the control samples. Filtration of samples through the pleated capsule filters was performed with a diaphragm pump (Shurflo, Cypress, CA, USA), and UF was performed with a peristaltic pump (Cole Parmer Instrument Company, Vernon Hills, IL, USA). After filtration, samples were processed using Method 1623 techniques with the exception of the elution procedure. The pleated capsule filters were eluted as specified in Method 1623, but the ultrafilters were backwashed according to the procedure used by

Hill et al. (2005), with a solution that contained 0.2% Tween-80, 0.01% sodium polyphosphate, and 0.01% Antifoam A. Following concentration by centrifugation at 1,500 x g and aspiration of the supernatant, each sample was further processed using Immunomagnetic separation (Dynabeads GC-Combo, Invitrogen, Dynal, Oslo, Norway), and slides were stained (EasyStain, BTF, Sydney, Australia) according to the procedures in Method 1623.

The two filtration methods are similar in the amount of time required for completion. The pleated capsule filtration required approximately ten minutes, while the time required for the ultrafiltration procedure was approximately fifteen to twenty minutes. Since elution/backwash procedures were also different for the two types of filters, the time required for this step also varied slightly between the filter types. The elution procedure performed on the pleated capsule filter can be completed in twenty to twenty-five minutes, while the backwash procedure performed on the hollow-fiber filter can be completed in five to ten minutes. Overall, each method can be completed in approximately five to six hours.

3.3.3. Statistics

Recovery efficiency for each sample was calculated by dividing the number of recovered organisms by the number of organisms seeded into the 10-liter sample. The resulting fraction was then multiplied by 100 to obtain a percent recovery. Statistical comparisons were made using one-way ANOVA with statistical significance set at 0.05 (Minitab 15, Minitab Inc., State College, PA, USA).

3.4. Results and Discussion

For the tap water samples (n=5 per filter type), the mean recovery of *C. parvum* for the pleated capsule filters was 37% (SD=17), whereas the use of the ultrafilters achieved a significantly higher (P=0.007) mean recovery of 68% (SD=10) for *C. parvum*. For *Giardia*, the pleated capsule filters produced a mean recovery of 85% with a (SD=6), while UF achieved a mean recovery of 63% with a (SD=8; P=0.001).

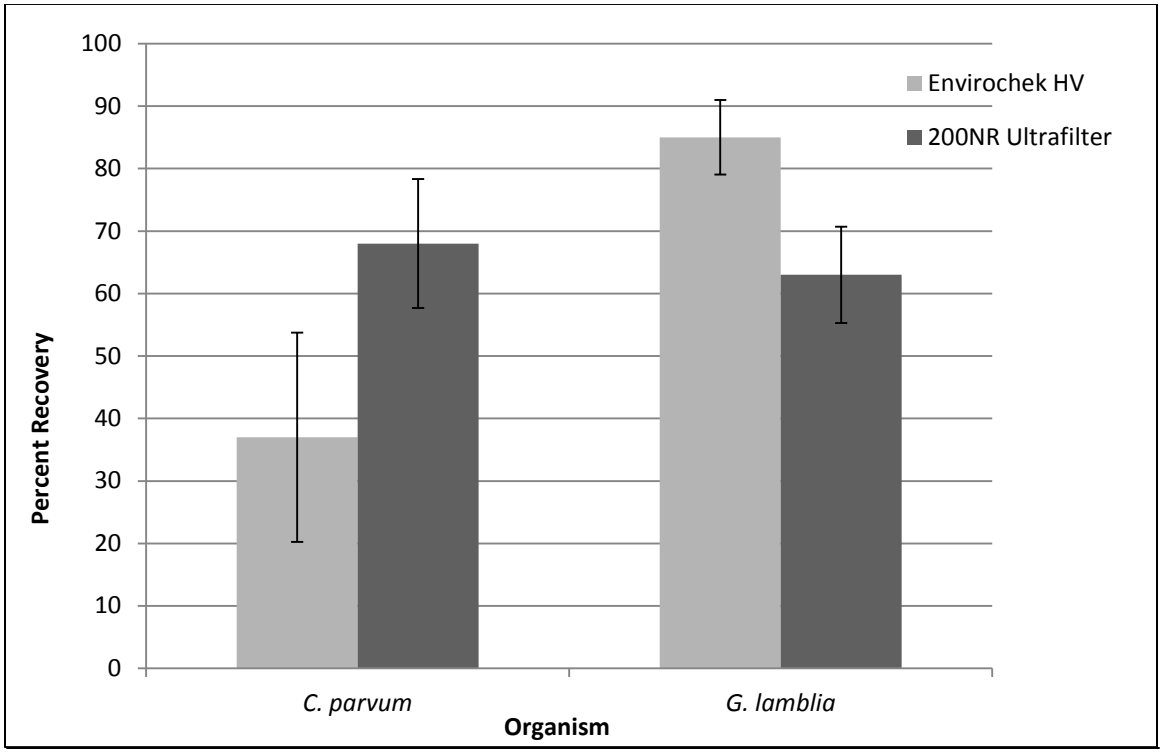

FIGURE 8: Mean recovery of *Cryptosporidium* and *Giardia* in treated drinking water samples for each filter type

Figure 2 shows the results each filter type by organism in surface water samples. For the surface water samples (n=5 per filter type), the mean recovery of *C. parvum* was 61% (SD=14) when using pleated capsule filters and 65% (SD=7) when using UF. No statistically significant difference was found between the recoveries (P=0.63). For *G. intestinalis*, recoveries in surface water for the pleated capsule filters averaged 40%

(SD=12). Whereas, UF achieved a significantly higher mean recovery of 81% (SD=5) for *Giardia* in surface water (P=0.00009).

When compared to previous research, this study has produced similar and dissimilar results. As found in the current study, Hill et al. (2009) reported that ultrafiltration produced significantly better recoveries of *Cryptosporidium* but not *Giardia* in tap water samples. Conversely, while the current study did not find a difference in recoveries of *Cryptosporidium* in surface water, Simmons et al. (2001) reported significantly better recoveries of *Cryptosporidium* with ultrafiltration. However, these two studies used different models of ultrafilters and pleated-capsule filters.

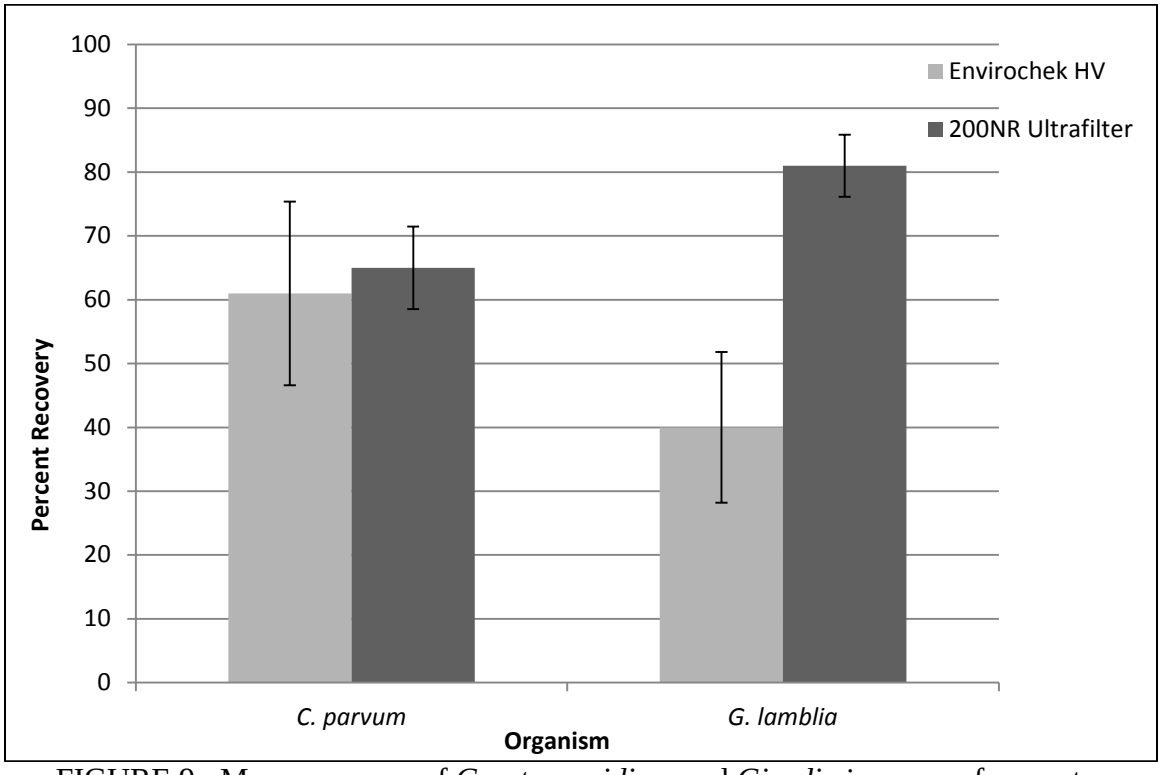

FIGURE 9: Mean recovery of *Cryptosporidium* and *Giardia* in raw surface water samples for each filter type

The results from this study demonstrate that ultrafiltration can provide similar or better recoveries of *Cryptosporidium* and *Giardia* than recoveries from pleated capsule filters when applied to surface water. When applied to tap water samples, ultrafiltration recoveries were significantly better than Envirochek HV filters for *Cryptosporidium*, but *Giardia* recoveries were better with the Envirochek HV (although overall method recoveries with UF were still greater than 60%). Based solely on the results of this study with one surface water source, ultrafiltration may be a viable option to improve *Cryptosporidium* and *Giardia* recoveries from both surface and tap water samples using USEPA Method 1623, but more samples of these and other types and sources of water need to be examined.

CHAPTER 4: DETECTION AND QUANTITATION OF *CRYPTOSPORIDIUM PARVUM* THROUGH ULTRAFILTRATION OF 10-LITER WATER SAMPLES AND REAL-TIME PCR

4.1. Abstract

While USEPA Method 1623 is the gold-standard for the detection of *Cryptosporidium* in water samples, quantitative real-time polymerase chain reaction (qPCR) has been successfully used to detect *Cryptosporidium* in aqueous matrices. This study examined various modifications to a basic commercial nucleic acid extraction procedure in order to enhance detection sensitivity and recovery of *Cryptosporidium*, while also examining the comparability of qPCR and microscopic detection of *Cryptosporidium* in water samples. Ten-liter seeded tap water samples were concentrated through ultrafiltration, backwashing and centrifugation prior to purification by immunomagnetic separation (IMS). After IMS, samples were split and detection was performed by microscopy and qPCR. Mean recovery for microscopy was 41%, while mean recovery for qPCR was 49% (P=0.013). Therefore, ultrafiltration followed by qPCR could provide an alternative means of detection for *Cryptosporidium* in water samples.

4.2. Introduction

Cryptosporidium is a protozoan parasite found in the feces of infected hosts. The organism can contaminate surface water when fecal material is transported into a water

body. A study of source water from sixty-six surface water treatment plants conducted by LeChevallier et al. (1991) found that *Cryptosporidium* was present in 87% of the locations sampled. If the contaminated surface water is consumed without proper treatment, illness can occur. *Cryptosporidium* can survive conventional water treatment and chlorine disinfection. Outbreaks of *Cryptosporidium*, associated with drinking water, have been documented in the United Kingdom and the United States since the early 1980s. Since that time, over 150 outbreaks of *Cryptosporidium* have occurred worldwide (Baldursson & Karanis 2011; Karanis et al. 2007). In 1993, an outbreak of *Cryptosporidium* in the municipal drinking water supply infected more than 400,000 people and killed approximately 100 individuals in Milwaukee (MacKenzie et al. 1994). Thus, effective detection technologies are imperative in order to accurately detect these organisms and prevent outbreaks.

At present, there are three methods that are approved by the United States Environmental Protection Agency (USEPA) for the detection of *Cryptosporidium* in water. These include: Method 1622 (*Cryptosporidium* in Water by Filtration/Immunomagnetic Separation (IMS)/Immunofluorescence Assay (IFA)) (2005), EPA Method 1623 (*Cryptosporidium* and *Giardia* in Water by Filtration/Immunomagnetic Separation (IMS)/Immunofluorescence Assay (IFA)) (2005), and EPA Method 1623.1 *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (2012). Detection inefficiencies and variations in recovery rates are commonplace when either Method 1622 or 1623 is used (Hu et al. 2004; DiGiorgio et al. 2002).

Another option for the analysis of *Cryptosporidium* in water is real-time polymerase chain reaction (qPCR). PCR is a molecular-based method of detection,

which relies on the extraction of deoxyribonucleic acid (DNA) from the organism. If the organism in question is present in the sample, the extracted DNA can be amplified, and the organism can be detected. Numerous qualitative and quantitative PCR methods have been used for aqueous matrices, but there are no standardized methods for the analysis of *Cryptosporidium* with qPCR (Yang et al., 2013; Polaczyk et al. 2008; Hill et al. 2007; Sturbaum et al. 2002; LeChevallier et al. 2000; Di Giovanni et al. 1999; Kostrzynska et al. 1999). Each of these methods varied in the specific procedure and analytical tools, but there are some common elements. First, DNA must be extracted or released from an oocyst. Then, the DNA must be collected and cleaned (or purified). Finally, the DNA must be amplified or copied in order to be detected. In order to amplify DNA, the double-stranded DNA must be denatured (or separated into two individual strands). This creates binding sites on each strand of DNA. Primers, which are specific to a fragment (or section) of the organism's DNA, anneal (or attach) to these binding sites. Following this step, a polymerase adds nucleotide bases to the primer, and the copies can then be copied creating exponential growth. Since DNA can be amplified (or exponentially copied), PCR methods provide a high level of sensitivity.

The intent of the current study was to implement a quantitative PCR method to detect *Cryptosporidium* and to compare the results with those achieved through microscopy using split samples. In order to accomplish this, filtration, concentration, and DNA extraction/purification steps had to be developed. Modifications were performed for various DNA extraction steps, while a qPCR amplification program was used to detect DNA. Since EPA Method 1623 is an approved method for the detection and enumeration of *Cryptosporidium* by microscopy, samples were split prior to the detection

method in order to compare the quantitative PCR results to the oocyst counts recorded during microscopic examination of slides.

4.3. Materials and Methods

4.3.1. Water Samples

Treated tap water samples were obtained from a laboratory faucet at a water treatment plant in Charlotte, NC. Ten-liter water samples were collected in cubitainers, seeded with oocysts and filtered on the same day as collection. No dechlorination of the samples was performed.

4.3.2. Microorganisms

Flow-cytometry sorted spiking suspensions containing *Cryptosporidium parvum* were obtained from the Wisconsin State Laboratory of Hygiene. Each suspension contained between 150-180 oocysts and was less than six weeks old when seeded into a water sample.

4.3.3. Filtration Set-up

The filtration set-up used was the same as reported by Hill et al (2005). New size #24 (6.4 mm ID) and #73 (8 mm ID) silicone tubing (Nalgene, Rochester, NY) was used for the filtration experiments. All tubing connections and fittings were cleaned after each use. The hollow-fiber ultrafilters (high-flux Fresenius Optiflux 200NR polysulfone dialysis filters) had a surface area of 2.0 m^2, a fiber inner diameter of 200 µm, and a molecular weight cutoff of approximately 30 kDa (Fresenius Medical Care, Lexington, MA). A new ultrafilter was used for each water sample. A peristaltic pump (Watson-Marlow Model 505S Wilmington, MA) was used for all experiments.

4.3.4. Ultrafiltration Procedure

Ultrafilters were wetted immediately before use with 0.1% sodium polyphosphate (NaPP) as described by Hill et al. (2005). The cross flow rate varied between 2.0-2.2 L/min, and the pressure was operated between 6-9 psi in order to keep the permeate rate between 0.6-0.8 L/min for each experiment. Samples were filtered until approximately 150 mL was left in the retentate (sample) reservoir. The volume that was held in the ultrafilter and tubing was also pumped back into the retentate reservoir for a total sample volume of approximately 250 mL. The ultrafilter was then backflushed. The backflushing solution contained 0.2% Tween-80 (Sigma-Aldrich catalog #P-1754), 0.01% NaPP (Sigma-Aldrich catalog #305553), and 0.01% Antifoam A (Sigma-Aldrich catalog #A-5758). As described by Polaczyk et al. (2008), a 150 mL backwash sample was collected in a beaker at a rate of 600 mL/min. The retentate and backwash were added to a 500 mL centrifuge tube for secondary concentration at 1,200 x *g*. Figure 1 shows all of the steps used in the analysis of the water samples.

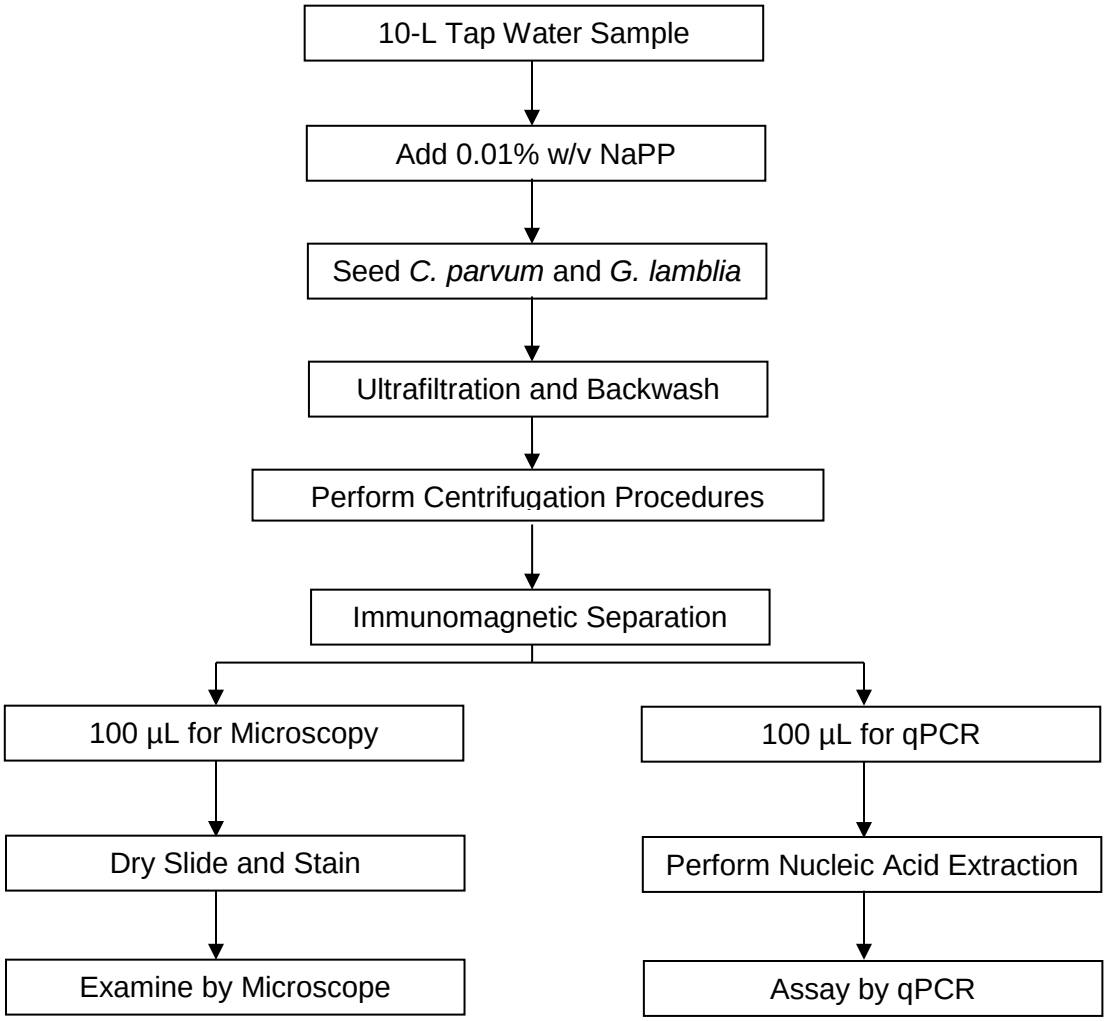

FIGURE 10: Flow diagram showing concentration and analysis steps used

4.3.5. Secondary Concentration

Centrifugation was used as a secondary concentration method. The contents of the 500 mL centrifuge tube were vortexed for 30 seconds and poured evenly into two 250 mL centrifuge tubes that were balanced and then centrifuged for 30 minutes at 1,200 x *g*. Centrifuge tubes were allowed to coast to a stop. Following the initial centrifugation, the supernatant in each centrifuge tube was aspirated to 35 mL. Centrifuge tubes were vortexed for 30 seconds and the contents were further mixed by 30 seconds of trituration with a 10 mL pipet. Finally, the contents of one centrifuge tube were added to the second

centrifuge tube. The centrifuge tube was rinsed twice with deionized water and the rinsate was added to the second centrifuge tube. The sample was then centrifuged a second time for 15 minutes at 1,200 x *g*. After the second centrifugation, the supernatant was vacuum-aspirated to 5 mL at 2 in. Hg. The contents of the centrifuge tube were vortexed for 30 seconds and then triturated for 30 seconds. The sample concentrate was then further purified with immunomagnetic separation.

4.3.6. Immunomagnetic Concentration

Immunomagnetic separation (IMS) was performed with a Dynabeads® GC-Combo kit (Invitrogen Dynal, Oslo, Norway). The IMS procedure was performed in accordance with USEPA Method 1623 (2005) with the exception of one modification. During the IMS procedure, 100 µL of 0.1 N HCl was added to the microcentrifuge tube, which contained the magnetic beads and attached oocysts. Method 1623 specifies 50 µL acid volumes, but this study used 100 µL volumes so that one-half of each sample could be applied to a slide and stained, while the second half of the sample could be analyzed by real-time polymerase chain reaction (qPCR). After the first acid dissociation, 50 µL of the sample was quantitatively placed onto a well slide and 50 µL was placed into a microcentrifuge tube for qPCR analysis. A second acid dissociation was also performed using 100 µL of 0.1 N HCl, and the acid was divided as in the previous dissociation. The sample designated for qPCR had 100 µL of 1 N NaOH added prior to nucleic acid extraction.

4.3.7. Monoclonal Antibody Staining

After sample slides were dried, samples were stained as specified in USEPA Method 1623 (2005). The monoclonal antibody stain used was EasyStain (BTF, Sydney,

Australia). Dynal Spot-on slides (Invitrogen Dynal, Oslo, Norway) were examined within seven days of staining by epifluoresence/differential interference contrast microscopy (Olympus BX61, Center Valley, PA).

4.3.8. Basic Nucleic Acid Extraction Procedure

The (Biofire Diagnostics 1-2-3 SWIPE Sample Purification) kit served as the basic extraction/purification mechanism for the DNA preparation, although several modifications to the prescribed protocol (discussed in the following sections) were tested and implemented to increase method sensitivity.

The basic nucleic acid extraction procedure that was used to extract DNA began with the addition of an extraction buffer to a bead tube, which contained 0.10-0.25 mm glass beads. After the extraction buffer was added, either a known number of oocysts or a concentrated sample was added to the bead tube. After sample addition, the bead-beating procedure was performed. The bead-beating consisted of the bead tube being shaken for 5 minutes at maximum speed in a Vortex Genie 2 with the Turbo Mix attachment (Scientific Industries, Bohemia, NY). After bead-beating, the sample was transferred to a spin column (Biofire Diagnostics, Salt Lake City, UT) and centrifuged. The spin column was washed once with Buffer 2 (Biofire Diagnostics, Salt Lake City, UT) and centrifuged twice to ensure the complete removal of Buffer 2. Lastly, the nucleic acid was eluted from the spin column to a final volume of 55 µL through the addition of Buffer 3, (i.e., Tris-EDTA (TE) buffer) (Biofire Diagnostics, Salt Lake City, UT), room temperature incubation, and centrifugation.

The various modifications that were investigated included: extraction buffer type, extraction buffer addition point, number of spin column/extraction buffer cycles,

extraction buffer volume, and TE buffer volume. Extraction comparison experiments were performed using small volume, serially diluted oocyst stock suspensions.

4.3.9. Extraction Buffer Type

In order to develop a more sensitive extraction method, two types of lysis/extraction buffer were tested. The lysis/extraction buffer (Buffer 1) provided in the Biofire Diagnostics kit was compared to the UNEX lysis/extraction buffer (Microbiologics, Catalog # MR0501) used in previous studies (Hill et al., 2015; Shields et al. 2013; Water Research Foundation 2010). In order to assess the efficacy of each buffer, oocysts were added to bead tubes at a concentration that would yield approximately 125 oocysts per reaction. Six qPCR reactions were tested for each extraction buffer.

4.3.10. Extraction Buffer Addition Point

Once an extraction buffer was selected, the time of addition or application point of the extraction buffer was tested. In order to test this, extraction buffer was either added to the bead tube (seeded with oocysts) before bead beating or after bead beating. Again, six reactions were tested for each addition time.

4.3.11. Extraction Buffer Volume

The volume of extraction buffer used was also tested to determine the optimal volume for method sensitivity. Bead tubes were seeded with small volumes of oocyst stocks, calculated to contain approximately sixteen to twenty-four oocysts per reaction, and processed through DNA extraction and purification. Twelve capillaries were analyzed for samples processed through both spin column cycles with the same 450 µL aliquot of extraction buffer. Alternatively, twelve capillaries were analyzed for samples

that received 200 µL of extraction buffer during the first spin column cycle, followed by a second aliquot (250 µL) of fresh extraction buffer that was added during the second spin column cycle.

4.3.12. Spin Column Cycles

After the volume of extraction buffer was optimized, the number of spin column cycles was examined to determine if an increase in DNA recovery could be achieved (as indicated by an associated decrease in qPCR CT values). Bead tubes were seeded at four different levels ranging from eight to thirty-three oocysts per reaction, and the DNA extraction/purification procedure was performed. Thirty-one total reactions were performed with one spin column cycle, and thirty-one total reactions were performed after recovering DNA using two spin column cycles.

4.3.13. Buffer Volume

The final DNA extraction modification that was tested involved the volume of Tris-EDTA (TE) buffer that was used to extract the DNA template from the spin column. Three volumes (100 µL, 55 µL, and 30 µL) were tested. Eighteen reactions were performed for the 100 µL volume, while sixteen reactions were performed for the 55 µL volume, and twelve reactions were performed for the 30 µL volume.

4.3.14. Real-time PCR Analysis

Amplification of *Cryptosporidium* DNA was performed using glass capillaries on a RAPID Real-time PCR System (Idaho Technology, Salt Lake City, UT). A TaqMan® assay, reported by Hill et al. (2007) and Jothikumar et al. (2008), was used to detect *Cryptosporidium parvum*. The optimal amount of DNA template to be added to each capillary was also assessed. These tests were completed after the extraction buffer was

selected and the time of addition of the extraction buffer to the bead tube had been determined (but prior to other method development testing). Three different volumes of DNA template (1 µL, 5 µL, and 8 µL) were tested in order to determine which volume enhanced detection and recovery. The amplification protocol used was as follows: denaturation at 95^{o}C for 15 min, followed by 45 cycles of denaturation at 95^{o}C for 10 s, annealing at 55^{o}C for 20 s, and extension at 72^{o}C for 15 s. Each 20 µL reaction contained 10 µL of 2x PCR master mix (QuantiTect Probe PCR Kit, Qiagen, Valencia, CA), 8 µL of DNA, and primers and probe as described by Hill et al. (2007). Six reactions per sample were processed by qPCR, and the mean crossing threshold (CT) was calculated for each sample. The intercept of the standard curve was then subtracted from the mean CT, and the resulting number was divided by the slope of the standard curve. This resulted in a calculated log value that was converted to a number.

4.3.15. Statistics

Since samples were split into two final detection methods, the total number of organisms seeded was divided by two to calculate recovery efficiency. For slides, the recovery efficiency was calculated by dividing the number of counted organisms by half the number of organisms seeded into the 10-liter sample. For qPCR, the recovery efficiency was calculated by dividing the calculated number of oocysts, based on the standard curve, by half the number of organisms seeded into the 10-liter sample. Paired t-tests were performed, using statistical analysis software (Minitab 15), to determine if qPCR recovery/calculation was significantly different from that achieved with slide examination. In addition, CT values and standard deviations were calculated for the extraction modifications. Any samples that were non-detects, (i.e., CT value >44) were

discarded from the dataset to avoid skewing results in the calculations of these values. Extraction modifications were tested for statistical differences using ANOVA (Minitab 15). For each statistical analysis, the significance level (α) was set at 0.05.

4.4. Results and Discussion

4.4.1. Comparison of Buffer Types

The UNEX extraction buffer, used in conjunction with the Biofire Diagnostics 1-2-3 SWIPE Sample Purification kit (but replacing the Biofire Diagnostics kit's lysis buffer) yielded better qPCR results than when the Biofire Diagnostics kit was used without modification. While none of the capillaries containing oocyst DNA that had been extracted with Buffer 1 tested positive, 83% of the capillaries from the UNEX buffer were positive. Capillaries that produced non-detects as a result were not included in calculations of mean CT values and standard deviation values. Since all capillaries where Buffer 1 was used produced non-detects, no mean CT could be calculated. For the UNEX buffer, the mean CT was calculated to be 39.3 (SD=1.78).

4.4.2. Extraction Buffer Addition Point

When the UNEX buffer was added after bead beating, only two of six (33%) capillaries had detections, while six of six (100%) capillaries had detections when the buffer was added prior to bead beating. Although there was no statistically significant reduction in the mean CT value for samples processed with the buffer added before bead beating (P=0.894), the percentage of positive samples increased. Therefore, for the remainder of testing, UNEX extraction buffer was added prior to bead beating.

4.4.3. Extraction Buffer Volume

When 450 µL of UNEX buffer was added in a single application, the mean CT value for all capillaries seeded with sixteen to twenty-four oocyst equivalents per capillary was 37.3 (SD=2.72), while the mean CT increased slightly to 37.6 (SD=1.15) when the extraction buffer was added as two aliquots of 200 µL and 250 µL, respectively. The slight increase in CT produced by using two smaller volume aliquots was not statistically significant (P=0.686). However, more positive capillaries were achieved through all seed levels, as summarized in Table 1, when the single addition of 450 µL of extraction buffer was used, so this was maintained throughout the remainder of processing.

TABLE 1: Summary of percent positive data and crossing threshold data for extraction buffer volume and TE buffer volume modifications

	Percent Positive	Mean CT
Extraction Buffer Volume		
200+250 µL	83% (10/12)[a]	37.6 (SD=1.15)
450 µL	100% (12/12)	37.3 (SD=2.72)
TE Buffer Volume		
100 µL	72% (13/18)	36.8 (SD=1.87)
55 µL	94% (15/16)	36.7 (SD=1.51)
30 µL	50% (6/12)	40.3 (SD=2.77)

[a] Number of positive reactions out of total number of reactions performed

4.4.4. Spin Column Cycles

When a seed value of eight oocysts per reaction was used, forty percent of samples that passed through the spin column once were positive (mean CT=40.6; SD=2.85) and eighty-three percent were positive (mean CT=39.3; SD=1.40) when passed through twice (P=0.307). At seed doses of thirteen (mean CT=39.6; SD=1.30), sixteen (mean CT=39.2; SD=1.52), and thirty-three (mean CT=37.5; SD=1.66) oocysts per

reaction, all reactions were positive when the spin column procedure was performed twice. Alternatively, when one spin column cycle was used with thirteen oocysts per reaction, only fifty percent of the reactions were positive (mean CT=37.2; SD=1.11) (P=0.031). However, with higher seed doses of sixteen (mean CT=37.7; SD=1.69) (P=0.051) and 33 (mean CT=35.2; SD=3.29) (P=0.124) oocysts per reaction, all reactions were positive when one spin column cycle was performed. Overall, seventy-one percent of the samples passed through the spin column once were positive while ninety-seven percent of samples were passed through the spin column twice tested positive for *Cryptosporidium*. Thus, this modification was continued throughout the remainder of the study.

4.4.5. Buffer Volume

Once an extraction procedure had been determined, different volumes of TE buffer were tested in order to determine the optimum volume for removal of DNA template from the spin column filter. Three volumes ranging from 100 µL to 30 µL were tested. Samples contained in 100 µL of TE buffer had a mean CT value of 36.8 (SD=1.87) but were positive for only 72% of the samples. When a 30 µL TE buffer volume was used, the mean CT was higher (40.3, SD=2.77), and the number of positive samples also decreased. In between these two volumes, a 55 µL volume was tested. For samples contained in 55 µL of TE buffer, the mean CT value was 36.7 (SD=1.51). The use of 55 µL of TE buffer allowed for more positive results, as shown in Table 1. The change in mean CT value between all TE buffer volumes was statistically significant (P=0.001). More specifically, the CT values for 30 µL were significantly higher than both the 100 µL volume (P=0.006) and 55 µL volume (P=0.001). The 55 µL TE buffer

volume was retained for use in order to optimize recovery and sensitivity of *Cryptosporidium* since the mean CT value was lower, the standard deviation was lower, and the number of samples with detections was higher.

4.4.6. DNA Template Volume

Once the addition point of the extraction buffer was determined, tests were conducted to determine the amount of extracted DNA template to be used. Three volumes (i.e., 1µL, 5 µL and 8 µL) were tested. The mean crossing threshold for the 1 µL template volume was 37.6 (SD=0.324), while use of the 5 µL template yielded a mean CT of 38.0 (SD=2.14). Finally, when 8 µL of template was used, the mean crossing threshold was 37.8 (SD=4.33). There was no significant difference in the mean crossing threshold, at 120 oocysts per capillary, based upon the volume of template used (P=0.751), but the use of an 8 µL template was selected for use in future tests since it produced the greatest sensitivity as shown in Table 2.

TABLE 2: Percentage of positive reactions for multiple seed doses at each template volume tested

	Seed Dose (oocysts per capillary)			
Extracted Template Volume (µL)	4	8	16	120
1	0% (0/4)[a]	0% (0/4)	0% (0/4)	50% (1/2)
5	50% (2/4)	25% (1/4)	50% (2/4)	50% (1/2)
8	50% (2/4)	33% (1/4)	75% (6/8)	100% (4/4)

[a] Number of positive reactions out of total number of reactions performed

4.4.7. qPCR Results

The detection limit for this assay was determined to be 2 oocysts per capillary. A standard curve, shown in Figure 2, was developed and had an R^2 value of 0.996, a slope of -3.39, and an intercept of 41.3.

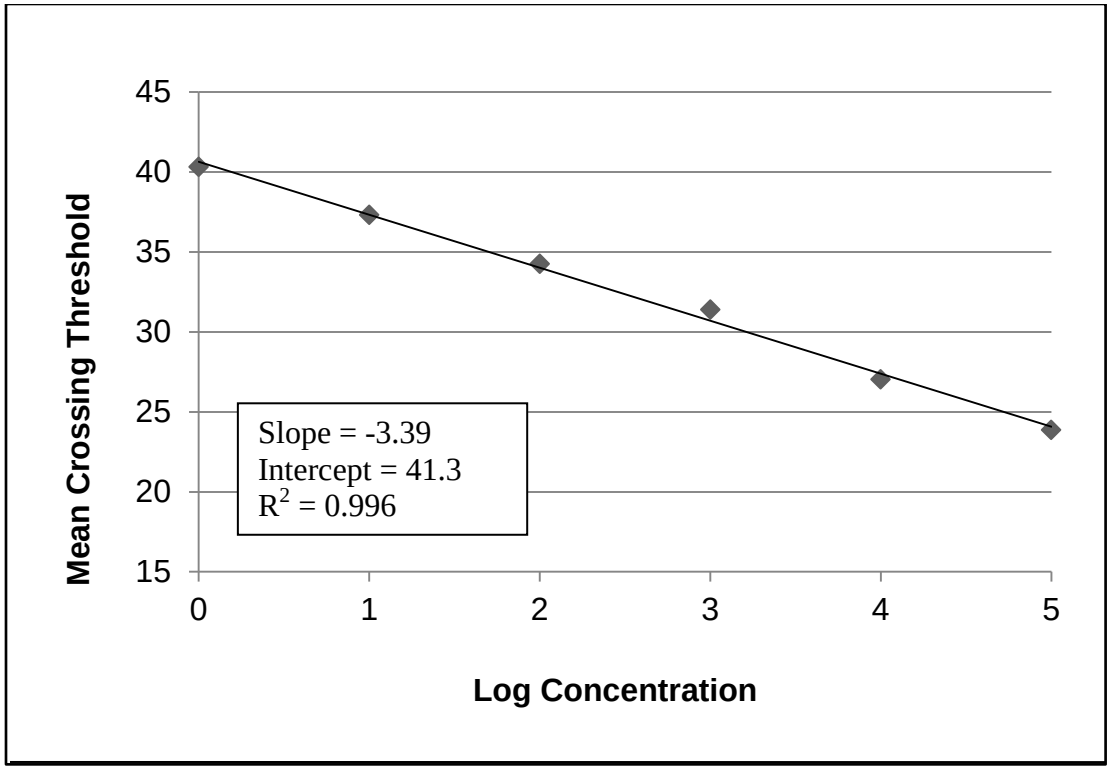

FIGURE 11: Real-time PCR standard curve for *Cryptosporidium parvum*

4.4.8. Comparison of Oocyst Recoveries for Microscopy Versus qPCR

In order to determine the effectiveness of qPCR for the detection of oocysts, tap water samples were spiked with known concentrations of oocysts and processed with the ultrafiltration procedure described in Sections 2.4-2.6. As shown in Table 3, the number of oocysts calculated by qPCR was higher than the number counted with microscopy for each separate experiment. When compared, the percent recovery of oocysts calculated by

qPCR was significantly higher than the percent recovery when microscopy was used (P=0.013).

TABLE 3: Percentage of *Cryptosporidium parvum* oocysts recovered by microscopy versus qPCR

Experiment number	Percent recovery by microscopy	Percent recovery by qPCR
1	50%	64%
2	23%	29%
3	53%	59%
4	59%	68%
5	20%	27%
Mean	41%	49%
Std. Deviation	18%	20%

4.5. Conclusions

Based upon the results of the various modifications that were tested, a final nucleic acid extraction procedure was developed and used for all ultrafiltration samples. Prior to addition of the concentrated sample, 450 µL of the lysis buffer was added to a bead tube. After sample addition, the bead-beating procedure was performed. After bead-beating, the sample was transferred to a spin column and centrifuged at 7800 x *g* for 2 minutes. The spin column was then reloaded with the same sample and centrifuged a second time at 7800 x *g* for 2 minutes. The spin column was washed once with Buffer 2 and centrifuged for 2 minutes at 7800 x *g*. To ensure the complete removal of Buffer 2, the spin column was transferred to a new microcentrifuge tube and centrifuged at 7800 x *g* for 3 minutes. Lastly, the nucleic acid was eluted from the spin column through the addition of 55 µL of TE buffer, a 2 minute room temperature incubation, and subsequent

centrifugation for 2 minutes at 7800 x *g*. Two incubation and centrifugation cycles were performed for nucleic acid elution.

As shown by the results of qPCR and microscopy (Table 3), both methods produce recoveries within the EPA acceptable range of 13-111% for matrix spike samples. In previous research performed with tap water, samples seeded with a low number of organisms (i.e., 150 oocysts) produced recoveries of 51% (SD=18) (Hill et al. 2009). Additionally, in a study of reagent and surface water samples, ultrafiltration recoveries of 42% for *Cryptosporidium* were reported (Simmons et al. 2001). Ultrafiltration performed on nineteen surface water samples resulted in a mean recovery of 47.9% (Kuhn & Oshima 2002). Finally, in a high seed (i.e., >500,000 oocysts) tap water study of both ultrafiltration and qPCR, oocysts were recovered at 91% (SD=8.8%), and qPCR detection occurred at a mean crossing threshold of 27.0 (SD=2.30) (Hill et al. 2007).

Since fluorescence microscopy is time-consuming and relatively expensive, qPCR represents a rapid and cost-effective option for the detection of oocysts from water samples. While qPCR did provide a significantly higher average recovery and detection efficiency in this research when compared to microscopy, there are still areas of needed improvement. As reported in this study, there is some variability in the ultrafiltration and concentration methods. While recovery, overall, was encouraging, there were two samples that were affected by much lower recovery for both microscopy and qPCR. Therefore, this was not a byproduct of the detection method (i.e., qPCR or microscopy) but was instead consistent for those samples regardless of the detection method.

While this study showed that qPCR could allow for the recovery of *Cryptosporidium* from water samples, there are still some limitations that were not addressed. The qPCR assay used in this study is not species-specific, and the method performed in this study does not allow for viability to be assessed.

CHAPTER 5: THE IMPACT OF CHEMICAL DISPERSANTS AND WATER QUALITY PARAMETERS ON PARTICLE STABILITY

5.1 Abstract

The interactions between particles and other constituents in water can affect the stability of the particles. These interactions can be altered by the use of chemical dispersants. However, the mechanisms by which dispersants can aid in particle stability are not completely understood. Further, particle stability can also be affected by physical water quality parameters. The focus of this study was to identify how two chemical dispersants (sodium polyphosphate and sodium metasilicate) influence particle stability when used in water samples with different chemical properties. The initial phase of this study was on settling tests to determine if the two chemical dispersants could increase the stability of suspensions of five types of particles in 10 water formulations representative of US surface water samples (varying in TOC, turbidity, and hardness). For the vast majority of test conditions, suspensions of inorganic particles (alumina, silica, kaolin, etc.) were not significantly increased when a chemical dispersant was added. During the final phase of this research, zeta potential was measured to determine how the surface charge of four types of particles (alumina, kaolin, *E. coli* (ATCC 25922), and *E. coli* (ATCC 11775) was affected by water sample conditions and chemical dispersants. Two strains of *E. coli* were tested and showed marked differences in response to the presence of a chemical dispersant. The zeta potential of *E. coli* (ATCC 25922) became more

negative with 0.05% sodium metasilicate (P=0.000) or 0.05% sodium polyphosphate use (P=0.000). However, the zeta potential of *E. coli* (ATCC 11775) was not significantly affected by 0.05% sodium metasilicate (P=0.535) or 0.05% sodium polyphosphate (P=0.370). Overall, dispersants were not generally effective at increasing the stability of inorganic particle suspensions under the conditions studied, but dispersants did impact the zeta potential of kaolin, alumina, and *E. coli*, although results significantly varied with the bacterial strain.

5.2 Introduction

Currently, the theory that is widely used to explain the actions of colloids in water is the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Dahirel & Jardat 2010). As shown in Figure 12, DLVO theory is a theory of colloid stability that includes electrostatic repulsion between particles and attractive Van der Waals forces.

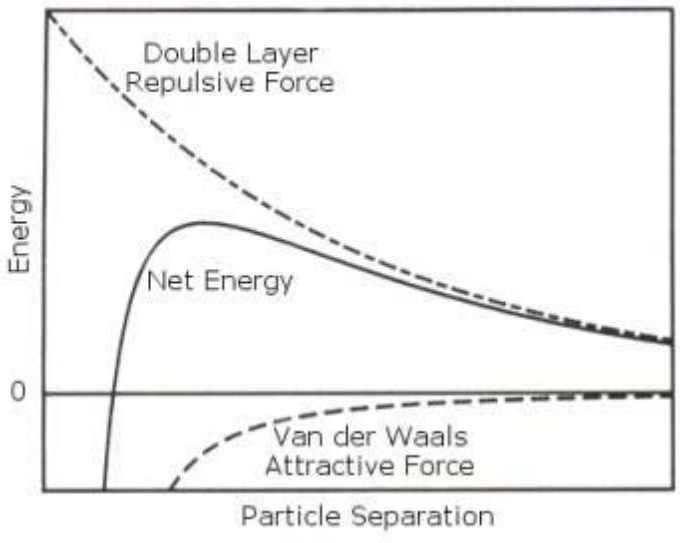

FIGURE 12: Diagram of double layer repulsion and Van der Waals attractive forces (Malvern Instruments Ltd., 2012)

When colloids are not charged, agglomeration can occur due to Van der Waals forces, which can cause the colloids to more quickly settle out of an aqueous suspension. However, when similarly charged colloids are present in a suspension, the charged colloids repel each other and remain dispersed in water. Based upon the principle of the Schulze-Hardy Rule, electrolytes in solution affect the stability of microorganisms, and coagulation of suspended particles is controlled by the charge (or valence) of the electrolytes (Lytle et al. 2002). The charge of particles in suspension can be altered by the addition of chemicals such as sodium polyphosphate (NaPP) or by the alteration of other physical water parameters, such as pH or ionic strength (Hill et al. 2005; Lytle et al. 2002; Sharma et al. 1985). For example, in low ionic strength solutions, suspensions have been reported to remain stable for several days (Czigany et al. 2005). Dispersion of microbes (instead of agglomeration) is desirable during analytical processing to aid in recovery and to reduce variability of results from sample to sample.

5.2.1 Zeta Potential – Phosphate Effects

Sodium pyrophosphate (similar to other polyphosphates) contains a negatively charged anion that when added to aqueous solutions, can alter bacterial surface charge (i.e., make surfaces more negative), reduce agglomeration of bacteria, and aid in dispersion of bacteria (Sharma et al. 1985). Sodium polyphosphate has been used with ultrafiltration of water samples to alter surface charge and reduce the adhesion of microbes to the filter, sample collection vessel, or other particulate in the sample (Hill et al. 2009; Polaczyk et al. 2008; Hill et al. 2007; Hill et al. 2005). These resulting effects of the addition of phosphates to aqueous solutions may be the result of negative charge being increased on surface sites (Sharma et al. 1985). Some of the physical

characteristics of water samples that might alter the effectiveness of NaPP or other chemical dispersants include: pH, turbidity, sediment type, and ionic strength. Ionic strength and pH have been found by previous researchers to affect surface charges of particles (Keck et al. 2006; Lytle et al. 2002; Sharma et al. 1985). Moreover, Lytle et al. (2002) reported that the surface charge of microorganisms affects the processes and interactions, including attachment to particles and other media, related to the microorganisms.

5.2.2 Zeta Potential – pH Effects

As shown in Figure 13, zeta potential is the electrical potential of a particle at the plane of shear. Zeta potential cannot be directly measured but can be calculated from electrophoretic mobility. Electrophoretic mobility is defined as the velocity of a particle moving in an electrical field, such as a negatively charged particle moving toward a positive charged electrode. However, electrophoretic mobility can vary even for the same organism when factors such as pH and ionic strength are varied (Lytle et al. 2002). In a study of two different strains of *E. coli*, electrophoretic mobility was found to be different for each strain of *E. coli* under the same conditions, and the two strains responded differently to changes in ionic strength and pH (Lytle et al. 1999). Moreover, in water samples with an ionic strength of 10.5 mM, conductivity of 838 µS/cm, and pH of 8.4, the same strain of *E. coli* was reported to have different zeta potential values depending upon the state of the organism (i.e., dead or starved) and the culture media used (Soni et al. 2008). Prior reports suggest that the zeta potential of the same species of organism could vary from water sample to water sample if different conditions exist.

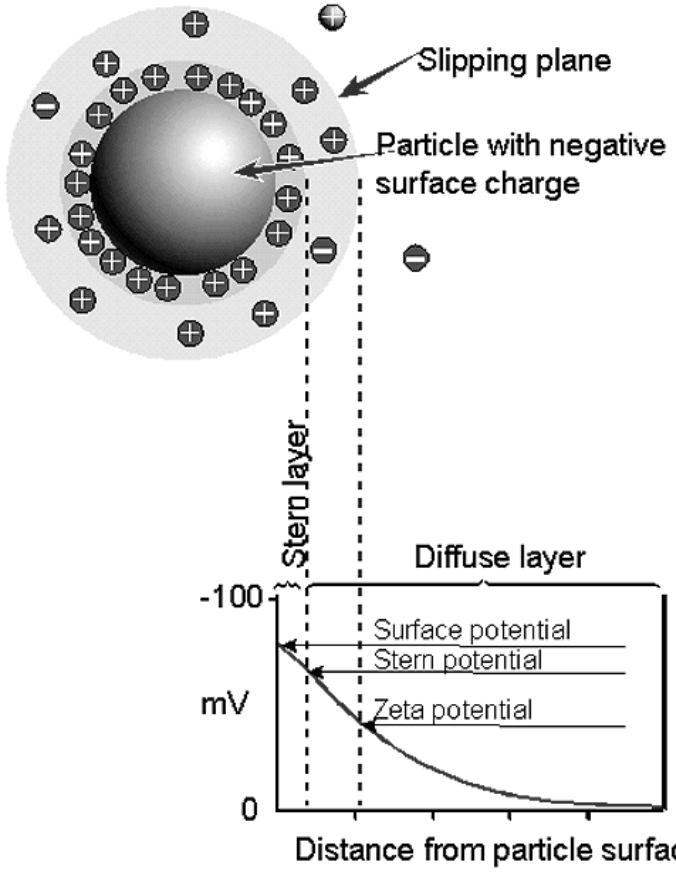

FIGURE 13: Zeta potential diagram (Malvern Instruments Ltd., 2015)

The activity of hydrogen ions in a solution is the pH, which is a measure of the acidity or basicity of a solution. The pH of a solution assists in the determination of surface charge and affects the dissociation of various functional groups present on bacteria surfaces (Sharma et al. 1985). For example, when phosphates are added to a solution with bacteria, phosphate ion bonding to hydrogen atoms of bacterial surface sites is typical and leads to a more negative bacterial charge (Sharma et al. 1985). A negative surface charge is common for microbes when pH values are close to neutral (Keck et al. 2006). For example, in a study that included viruses, bacteria, and protozoans in a solution designed to simulate natural or treated drinking water, Polaczyk (2010) reported negative zeta potentials for all organisms tested. However, an alteration of the pH of a

solution containing bacteria can lead to different surface charge values as indicated by zeta potential readings (Sharma et al. 1985). This occurs because zeta potentials typically become more negative as pH values increase or become more basic, while zeta potentials become more positive as pH values decrease or become more acidic. In spite of this, the effect of pH on zeta potential can also vary for different organisms. For example, for wild-type *E. coli* (point of zero charge=2.1), electrophoretic mobility was independent of pH above a pH value of 5 while a decrease in pH resulted in more positive electrophoretic mobility readings for *E. coli* O157:H7 (point of zero charge=4.3) over the entire pH range tested (Lytle et al. 1999). For *Cryptosporidium*, zeta potential is also influenced by pH (Lytle et al. 2002). The point of zero charge (zeta potential of 0 mV) for *Cryptosporidium* was found to occur at a pH of 2.5, while the zeta potential was reported as approximately -20 mV at a pH of 7.19 (Lytle et al. 2002). Moreover, the cation concentration necessary to reduce zeta potential decreases as the cation increases in valence (Lytle et al. 2002).

5.2.3 Zeta Potential – Ionic Strength Effects

In addition to pH, the ionic strength of a solution can affect the actions of chemical dispersants and can lead to differences in zeta potential values. Ionic strength is a measure of the ions in a solution and is weighted by the valence of the ions. According to Sharma and colleagues (1985), the ionic strength assists in the determination of surface charge though changes in the electrical double layer. Specifically, as available surface sites become saturated by negatively charged phosphates, additional phosphates only cause an increase in ionic strength and result in a less negative zeta potential through compaction of the electrical double layer on affected surfaces (Sharma et al. 1985).

Therefore, the addition of phosphates to a solution make zeta potentials more negative up to a certain point, but this action reverses after all surface sites are filled and ionic strength increases (Sharma et al. 1985). For example, as ionic strength increases, the electrophoretic mobility of *Cryptosporidium* and wild-type *E. coli* increases or becomes less negative (Lytle et al. 2002; Lytle et al. 1999). However, in a previous study with *E. coli* O157:H7, ionic strength had very little effect on electrophoretic mobility (Lytle et al. 1999). Additionally, electrophoretic mobility was found to increase or become less negative as salt concentration increased in solutions and decrease or become more negative as pH increased (Lytle et al. 2002). There are several different equations that can be used to calculate zeta potential from electrophoretic mobility, but the Helmholtz-Smoluchowski equation has effectively been used in the calculation of zeta potential for *Cryptosporidium parvum* and *E. coli* in water with low ionic strength (e.g., 1 mM) (Polaczyk 2010).

5.2.4 Zeta Potential – Microbial Recovery

When water samples are processed and analyzed for microbial presence, the physical composition of the water samples vary from source to source and vary within the same sampling source over time. The characteristics of water samples (i.e., matrix interferences) can influence the recovery rates and detection of the microbes of concern. Therefore, there is value in understanding how water quality parameters can affect recovery and detection of microbes. Moreover, this knowledge can lead to informed decision-making with respect to methods or procedures to reduce matrix interferences.

Microbes, as with non-microbial particles, have physical characteristics that affect recovery and interact with water sample components. Two ways that previous

researchers have used to enhance recovery of microbes from water samples have been through the use of sample additives or filter pre-treatments (Kimble et al. 2013; Kimble et al. 2012; USEPA 2012; Hill et al. 2009; Polaczyk et al. 2008; Hill et al. 2005; Morales-Morales et al. 2003). Chemical dispersants and filter pre-treatments have been used in conjunction with hollow-fiber polysulfone ultrafilters and, more recently, pleated capsule filters.

As a part of the ultrafiltration process for microbial methods, the filter is usually wetted or blocked prior to filtration (Kimble et al. 2012; Hill et al. 2009; Polaczyk et al. 2008; Hill et al. 2005; Morales-Morales et al. 2003). Blocking has most often been performed with calf serum, which normally occurs during an overnight period (Hill et al. 2009; Hill et al. 2007; Morales-Morales et al. 2003). However, a chemical dispersant, sodium polyphosphate (NaPP), has been used to wet filters immediately prior to use (Kimble et al. 2012; Polaczyk et al. 2008; Hill et al. 2005). Moreover, sodium polyphosphate has also been used as a sample amendment either in conjunction with calf serum or sodium polyphosphate wetting (Kimble et al. 2012; Hill et al. 2009; Hill et al. 2007).

Additionally, chemical dispersants have been tested with pleated capsule filters in conjunction with the filter elution procedure in USEPA Method 1623, for the improved recovery of *Cryptosporidium* and *Giardia* from water samples (Kimble et al. 2013; USEPA 2012). In brief, water samples are filtered through a 1 µm pleated capsule filter in order to capture *Cryptosporidium* oocysts, which are 4-6 µm in diameter, and *Giardia* cysts, which range from 5-15 µm in width by 8-18 µm in length (USEPA 2005). After filtration, elution is performed to release any cysts/oocysts from the filter prior to

centrifugation. Although both of these modifications use sodium polyphosphate or sodium hexametaphosphate as the chemical dispersant during the elution process, the procedures used are different. USEPA Method 1623.1 (2012) was developed to improve upon previous methods and includes the use of a 5% sodium hexametaphosphate solution as a filter pre-treatment prior to elution but after sample filtration. According to USEPA (2015), recoveries of *Cryptosporidium* improved more than 20% when this procedure was added. Alternatively, when 0.01% sodium polyphosphate was added to elution buffer, but no other procedures were altered, there was no difference in the recovery of *Cryptosporidium* (Kimble et al. 2013).

5.2.5 Research Objectives

Sodium polyphosphate alters surface charge, thereby increasing surface charge repulsion in samples (Hill et al. 2009; Polaczyk et al. 2008; Hill et al. 2007; Hill et al. 2005). Although sodium polyphosphate has been used to aid in the recovery of microorganisms, there is a lack of fundamental knowledge about how sodium polyphosphate alters surface charge under different physical conditions and at different concentrations. Aside from sodium polyphosphate, there are other chemicals (e.g., sodium metasilicate) that could be used as dispersants in water samples. This research aimed to identify how two chemical dispersants (sodium polyphosphate and sodium metasilicate) influenced particle stability when used in water samples with different chemical properties.

5.3 Methods and Materials

5.3.1 Water Samples

In order to test chemical dispersants under various conditions, eight "model" waters were made. The chemical make-up each model water solution is shown in Table 4. In order to make the model water solutions, two components were added to deionized water. Instant coffee was used to adjust total organic carbon (TOC) concentration and calcium carbonate (Sigma-Aldrich, Saint Louis, MO) dissolved in HCl was used to adjust hardness levels.

TABLE 4: Chemical properties of model water solutions

	Total Organic Carbon	Hardness	Ionic Strength	pH
Model Water 1	1 mg/L as coffee	20 mg/L as $CaCO_3$	1.76 mM	3.70
Model Water 2	1 mg/L as coffee	200 mg/L as $CaCO_3$	12.2 mM	2.66
Model Water 3	1 mg/L as coffee	20 mg/L as $CaCO_3$	4.16 mM	6.46
Model Water 4	1 mg/L as coffee	200 mg/L as $CaCO_3$	14.6 mM	4.08
Model Water 5	10 mg/L as coffee	20 mg/L as $CaCO_3$	1.76 mM	3.49
Model Water 6	10 mg/L as coffee	200 mg/L as $CaCO_3$	12.2 mM	2.70
Model Water 7	10 mg/L as coffee	20 mg/L as $CaCO_3$	4.16 mM	6.58
Model Water 8	10 mg/L as coffee	200 mg/L as $CaCO_3$	14.6 mM	7.02

5.3.2 Particles

Once the chemical properties of the model waters were adjusted as specified in Table 4, turbidities of the model water samples were adjusted. Turbidity, measured in nephelometric turbidity units (NTU), can be defined as the cloudiness of a liquid, such as water, caused by particulate matter. In order to adjust turbidity, four sediment types (i.e., kaolin, silica, diatomaceous earth and alumina) and microspheres were used. Kaolin

(Sigma-Aldrich, Saint Louis, MO) is a clay mineral, while silica (Sigma-Aldrich, Saint Louis, MO) is fine sand. Diatomaceous earth (Sigma-Aldrich, Saint Louis, MO), comes from sedimentary rock, and alumina (Sigma-Aldrich, Saint Louis, MO) is aluminum oxide. Microspheres (Polysciences, Warrington, PA) are manufactured polystyrene beads or spheres that are available in different sizes. During this study, 1 µm carboxylated microspheres and 3 µm non-carboxylated microspheres were used. The kaolin, silica, diatomaceous earth and microspheres used in the test samples were used as received from the manufacturer. However, due to the relatively large particle size of the alumina, this compound was ground with a mortar and pestle for fifteen minutes to reduce the particle size from 50 µm to approximately 1 µm. The sediment types were selected in order to determine whether dispersants behaved differently based upon particulate type since predominant sediment types vary in different locations, and particulate matter in a water sample could play a role in the ability to recover microorganisms from that water. Microspheres were selected for use as a surrogate for *Cryptosporidium* as previous researchers have done (Hill et al. 2005). Additionally, Stokes' Law was used to calculate the setting velocity of each particulate material. The settling velocity was then used to calculate the distance each particle would fall within twenty-four hours. The properties of each particle are described in Table 5.

TABLE 5: Properties of particles

	Approximate Size	Density[2]	Calculated Depth Fallen in 24 Hours[3]	Point of Zero Charge
Kaolin	1-3 µm[1]	2630 kg/m^3	12-108 in.	3–4.8
Silica	0.25 µm	2300 kg/m^3	0.60 in.	2.8-3.0
Diatomaceous Earth	2 µm[1]	2300 kg/m^3	38 in.	-
Alumina	1 µm[1]	3950 kg/m^3	22 in.	7.8-9.1
Microspheres	1 µm	1050 kg/m^3	0.38 in.	-
Microspheres	3 µm	1050 kg/m^3	3.5 in.	-
E. coli	0.5 µm	1100 kg/m^3	0.19 in.	-

[1]Particle size estimated by microscopic examination

[2]Density – average value for particle

[3]Calculations performed with Stokes' Law (assumes spherical particles and no aggregation/interaction

5.3.3 Microbes

For zeta potential tests and initial growth tests, a loop of *E. coli* from a stock culture slant was added to test suspensions and maintained at room temperature overnight prior to analyses. Stock cultures were grown overnight on brain heart infusion agar at 35±0.5^{o}C and refrigerated post-incubation. Two separate *E. coli* strains (ATCC 25922 - FDA strain Seattle 1946 and ATCC 11775 – strain NTCC 9001) were used for the zeta potential tests. For final growth tests, *E. coli* (ATCC 11775) was obtained in a freeze-dried pellet (NSI Solutions, Raleigh, NC) that had a certified quantification value. Prior to use, pellets were brought to room temperature and added to deionized water to make a stock suspensions. Colilert-18 (Idexx Laboratories, Westbrook, ME) was used with the Quanti-tray 2000 in order to obtain quantified results in the most probable number (MPN) format. Growth tests were conducted in sterile deionized water, model water one,

model water one adjusted to a pH of 6, and model water one adjusted to a pH of 11.5. In order to assess growth, one set of samples was analyzed immediately following pH adjustment while a second set of samples was allowed to sit undisturbed at room temperature for 24±2 hours after pH adjustment prior to analysis. All microbes were used within 24 of seeding with the exception of the *E. coli* used to test zeta potential to determine if there was a change in zeta potential after 7 days in the water sample.

5.3.4 Chemical Dispersants

Two chemical dispersants (sodium polyphosphate ((Sigma-Aldrich, Saint Louis, MO) and sodium metasilicate (Sigma-Aldrich, Saint Louis, MO)) were tested at a concentration of 0.1% in each of the model water solutions. The concentration of 0.1% was selected because this concentration has been tested successfully with sodium polyphosphate (but not other chemical dispersants) (Hill et al. 2005). For microbe tests, dispersant concentrations of 0.01% and 0.001% were tested.

5.3.5 Settling Tests - Phase 1

In order to conduct initial tests to determine the effectiveness of the chemical dispersants with sediment or microspheres, the following procedure was used. Fifty milliliter conical centrifuge tubes were filled to approximately 40 mL (an approximate height of 3.5 inches) with a model water solution and one sediment or microspheres. A chemical dispersant was then added and an initial turbidity was recorded. In addition to the samples tested with chemical dispersants, control samples (no dispersant added) were also tested for each condition and initial turbidity readings were recorded. Separate sample cells were used for each dispersant, and sample cells were rinsed three times with tap water followed by three rinses with deionized water between replicates. Turbidity

readings were taken using either a Hach 2100Q or a Hach 2100P turbidimeter. Both instruments measure turbidity by making a comparison of the light scattered by the sample versus light scattered by standards or reference solutions. Following the initial turbidity reading, samples were allowed to sit undisturbed for 24±2 hours. After settling, the turbidity of the supernatant (unsettled) portion of each sample was measured and a post-settled turbidity reading was recorded for each sample. Samples were performed in triplicate.

5.3.6 Settling Tests - Phase 2

In addition to the initial tests performed, a second round of testing was conducted with model water solution one. For these samples, pH was controlled at a minimum of two separate values for each particle type. The pH controlled tests were performed with kaolin, silica, and alumina. The pH values selected for each particle were based upon the point of zero charge value of each particle. For kaolin, the point of zero charge has been reported from 3 to 4.8 (Schroth & Sposito 1997; Ferris & Jepson 1975; Tschapek et al 1974). Alumina has had reported values of 7.8 to 9.1 for the point of zero charge, while the point of zero charge for silica has been reported as 2.8 to 3.0 (Tschapek et al 1974; Parks 1965). Adjustments to pH were made prior to the initial turbidity reading and 0.1 N HCl (Fisher Scientific, Pittsburg, PA), 6 N HCl, and 1 N NaOH (Fisher Scientific, Pittsburg, PA) were used to adjust pH values. Unless noted otherwise, three replicates were performed for each condition tested.

5.3.7 Settling Tests - Phase 3

Finally, tests were performed for alumina and kaolin that involved a pre-settling period of two hours. This was done to allow larger particles to settle, while smaller

particles would remain in suspension. After the initial two hour settling period, the supernatant was removed and transferred to a second conical centrifuge tube. Sodium polyphosphate, sodium metasilicate, or no dispersant was added to each test vessel. These tests were conducted at three pH values for each particle. Kaolin tests were performed at pH values of 3, 5, and 8. One pH value near the isoelectric point was selected, while pH values above and below the isoelectric point were also selected. Therefore, alumina was also tested at three similarly selected pH values (5, 8, and 11). After pH adjustment, turbidity readings were obtained, and samples were allowed to sit undisturbed for 24±2 hours. After settling, the turbidity of the supernatant (unsettled) portion of each sample was measured and a post-settled turbidity reading was recorded for each sample. Each sample condition was performed in triplicate.

5.3.8 Zeta Potential - Phase 4

Initially, titrations were performed in order to determine the zeta potential of various particles at multiple dispersant concentrations. Titrations were performed for kaolin, alumina, and *E. coli* in model water one (ionic strength of 1.76 mM and approximate conductivity of 188 µS/cm). Kaolin and alumina samples were allowed to pre-settle for 24±2 hours prior to zeta potential analysis of the supernatant. Dispersants were auto-titrated in 0.01% increments from an initial concentration of 0.00% to a final concentration of 0.10%. Zeta potential of the resultant suspension was measured in triplicate initially and in triplicate at each 0.1% increment. Additionally, the zeta potential of *E. coli* in suspension was also tested with sodium chloride (NaCl) as an additive. This was done to determine whether any change in zeta potential was the result of a change in ionic strength due to the sodium in each dispersant or if the polyphosphate

or metasilicate was the source of a change in charge. The addition of sodium chloride to a concentration of 0.10% resulted in a change in ionic strength from 1.76 mM to 3.93 mM. Finally, pH titrations, from pH 3 to pH 12, in increments of 0.5 pH units $\pm$ 0.2 pH units, were also performed for *E. coli* in order to determine the effect of pH on zeta potential. pH was adjusted with 0.1 N NaOH and 0.1 N HCl. A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) with a Malvern MPT-2 automated titrator was used for all zeta potential readings. Once sample cells were placed into the instrument, the temperature was allowed to equilibrate for 120 seconds to 25^{o}C as specified in the software standard operating procedure (SOP). Additionally, the SOP was set to perform 10-20 readings per zeta potential value. This setting allowed the instrument to determine the exact number of readings per zeta potential measurement. Three to six samples were analyzed for each test condition, while one titration was completed for alkalinity, TOC, and hardness.

5.3.9 Zeta Potential - Phase 5

Based upon the results collected during titration studies, additional, standardized zeta potential tests were conducted for two subspecies of *E. coli* (ATCC 25922 and ATCC 11775), alumina, and kaolin in model water one. Prior to zeta potential analysis, all new folded capillary cells were rinsed with deionized water. This was performed by attaching two 10 mL syringes, one filled with 10 mL of deionized water and syringing the water back and forth six times. Samples were inverted six times prior to being loaded into a 5 mL syringe. Each of the three replicates per test condition came from the same syringe, and the syringe was inverted three times before separately loading each sample into the folded capillary cell. Once the cell was loaded and cleared of air bubbles, two

drops of sample were allowed to exit the cell to ensure the cell was completely filled with sample. Sample cells were then capped and placed into the instrument. Once in the instrument, all samples were equilibrated to 25^{o}C (120 seconds), as specified in the instrument SOP, prior to zeta potential measurements being taken. Thirty zeta potential measurements were collected for each zeta potential reading, and three readings were obtained for each of the three samples per test condition. A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used for all zeta potential readings. Test conditions included are shown in Table 6.

TABLE 6: Zeta potential grab sample test conditions

Kaolin	Alumina	*E. coli* (ATCC 25922)	*E. coli* (ATCC 11775)
Control	Control	Control	Control
pH 6	pH 6	pH 6	pH 6
-	-	pH 7	-
-	-	pH 8	-
pH 11.5	pH 11.5	pH 11.5	pH 11.5
-	-	0.02% NaCl	-
-	-	0.05% NaCl	0.05% NaCl
-	-	0.08% NaCl	-
0.05% NaPP	0.05% NaPP	0.05% NaPP	0.05% NaPP
0.01% NaMeta	0.01% NaMeta	-	-
-	-	0.05% NaMeta	0.05% NaMeta
0.09% NaMeta	0.09% NaMeta	-	-
-	-	Sample Age	-

Specific test conditions were selected to test the effect of pH and ionic strength while other test conditions were selected to determine the general variability of zeta potential data under similar test conditions. The pH values of 6 and 11.5 were specifically selected because the addition of sodium polyphosphate raised sample pH to approximately 6, while the addition of sodium metasilicate raised sample pH to

approximately 11.5. All pH specific samples were adjusted to within ± 0.2 pH unit with 0.1 N NaOH.

5.3.10 Statistics

In order to obtain a percent retention value for turbidity, the final (post-settled) turbidity was divided by the initial turbidity reading for each test sample. The resulting fraction was then multiplied by 100 to obtain a percent retention of turbidity or particulate. Statistical comparisons of settling tests and *E. coli* toxicity/growth tests were made using analysis of variance (ANOVA) and t-tests, with statistical significance set at 0.05 (Minitab 17, Minitab, Inc., State College, PA, USA). For zeta potential, a mean zeta potential and a standard deviation for each test condition was calculated. Zeta potentials for each test condition were compared using t-tests with statistical significance set at 0.05, while multiple conditions were compared using ANOVA with statistical significance set at 0.05 (Minitab 17, Minitab, Inc., State College, PA, USA).

5.4 Results and Discussion

5.4.1 Kaolin

Kaolin was tested with 0.1% of each chemical dispersant and with each of the model water solutions. Overall, the addition of 0.1% sodium polyphosphate resulted in an increased retention of kaolin in suspension. However, in all cases, the total retention was less than 20%. Additionally, the use of 0.1% sodium metasilicate also increased the retention of kaolin particles in suspension when hardness was at 20 mg/L as $CaCO_3$ but not when hardness was 200 mg/L as $CaCO_3$. The results for all samples are summarized in Table 7.

TABLE 7: Summary results for kaolin

	Initial Turbidity Range (NTU)	% Turbidity Retained	P value
Model Water 1	48.1-54.6		
0.1% NaPP		18%	0.063
0.1% NaMeta		12%	0.018
No Dispersant		6%	
Model Water 2	45.4-55.5 & 127-167		
0.1% NaPP		7%	0.015
0.1% NaMeta		2%	0.826
No Dispersant		2%	
Model Water 3	46.7-80.6		
0.1% NaPP		12%	0.108
0.1% NaMeta		8%	0.370
No Dispersant		4%	
Model Water 4[1]	33.3-53.1 & 122-129		
0.1% NaPP		8%	0.195
0.1% NaMeta		4%	0.732
No Dispersant		3%	
Model Water 5	46.7-67.3		
0.1% NaPP		12%	0.005
0.1% NaMeta		11%	0.132
No Dispersant		5%	
Model Water 6[1]	54.3-56.6 & 124-132		
0.1% NaPP		6%	0.168
0.1% NaMeta		4%	0.553
No Dispersant		3%	
Model Water 7	49.1-66.0		
0.1% NaPP		13%	0.140
0.1% NaMeta		10%	0.312
No Dispersant		7%	
Model Water 8[1]	51.1-52.0 & 112-118		
0.1% NaPP		7%	0.245
0.1% NaMeta		5%	0.465
No Dispersant		5%	

[1]n=2

Even though retention of kaolin particles in suspension was low even with dispersants added, there were some conditions where dispersants improved retention. Therefore, kaolin was selected for the phase 2 supplementary pH controlled tests. Turbidity values for the pH-controlled tests were similar to those in the previous tests. At both pH values tested, kaolin was retained in suspension significantly better when 0.1% sodium polyphosphate was used. Table 8 summarizes the results for all test conditions.

TABLE 8: Summary results for kaolin in pH adjusted model water one

	Actual pH Range	% Turbidity Retained	P value
pH 3	3.17-3.28		
0.1% NaPP		8%	0.000
0.1% NaMeta		4%	0.138
No Dispersant		1%	
pH 5	4.33-4.90		
0.1% NaPP		9%	0.000
0.1% NaMeta		2%	1.000
No Dispersant		2%	

Due to the fact that the majority of kaolin particles were still settling for any condition, the pre-settling procedure described in phase 3 of the settling tests was implemented (see Section 5.3.7). In this phase, additional pH-controlled tests were performed for pre-settled kaolin in model water one. After two hours of settling, turbidity values for the pre-settled tests ranged from 13.4 NTU to 21.9 NTU. With 0.1% sodium polyphosphate, the retention of kaolin particles in suspension was significantly improved at the three pH values tested. Table 9 summarizes the data for all phase 3 tests with kaolin.

TABLE 9: Summary results for kaolin phase 3 tests

	Actual pH Range	% Turbidity Retained	P value
pH 3	2.89-3.21		
0.1% NaPP		18%	0.015
0.1% NaMeta		10%	0.381
No Dispersant		8%	
pH 5	4.85-5.09		
0.1% NaPP		14%	0.009
0.1% NaMeta		8%	0.047
No Dispersant		6%	
pH 8	7.87-8.12		
0.1% NaPP		22%	0.015
0.1% NaMeta		13%	0.160
No Dispersant		9%	

Upon conclusion of the settling tests, zeta potential analyses were performed for kaolin particles in model water one. These analyses involved the titration of each chemical dispersant from an initial concentration of 0.00% to a final concentration of 0.10%. When sodium polyphosphate was titrated into kaolin samples (n=4), the most negative mean zeta potential (-37.3 mV; SD=3.30) occurred at a sodium polyphosphate concentration of 0.05% (P=0.001). This was more negative by 15.2 mV from the initial mean zeta potential of -22.0 mV (SD=11.5). Grab samples (n=3) performed for kaolin suspensions had a mean zeta potential result of -10.2 mV (SD=1.75), while grab samples with 0.05% sodium polyphosphate added to the suspension produced a mean zeta potential of -26.5 mV (SD=11.7) (P=0.003). A difference in zeta potential was noted

between the initial titration condition and the initial condition for grab samples. While there was variability in the zeta potential values, there was also the possibility that sodium polyphosphate leaked from the titrant tube and into the sample prior to the collection of initial condition zeta potential measurements. Zeta potential results for the entire titration range are shown in Figure 14.

However, at a concentration of 0.05% sodium polyphosphate the mean pH of all titration samples was 6.41. Since pH also affects zeta potential, there was a need to determine the extent of this affect. Therefore, grab samples (n=3) were analyzed for zeta potential at a pH value of 6 (actual value of 6.03). At a pH of 6, the mean zeta potential of kaolin in model water one was -16.4 mV (SD=4.24), which is significantly less negative than the zeta potential at a 0.05% concentration of sodium polyphosphate (P=0.000). Similarly, when the results for grab samples at a pH of 6 were compared to grab samples with 0.05% sodium polyphosphate (pH of 6.12) the mean zeta potential was significantly more negative when 0.05% sodium polyphosphate was used (P=0.036). Moreover, the titration samples actually reached a mean pH value of 6 (actual value of 6.08) at a sodium polyphosphate concentration of 0.02%. In order to further assess pH versus sodium polyphosphate affects, the mean zeta potential of kaolin at a sodium polyphosphate concentration of 0.02% (-30.2 mV; SD=7.76) was compared to the mean zeta potential of kaolin at pH 6 and found to be significantly more negative (P=0.000). Based upon this result, zeta potential data for 0.02% sodium polyphosphate and 0.05% sodium polyphosphate were compared and the zeta potential was significantly more negative at 0.05% with only a 0.33 unit pH change from 6.08 to 6.41 (P=0.012).

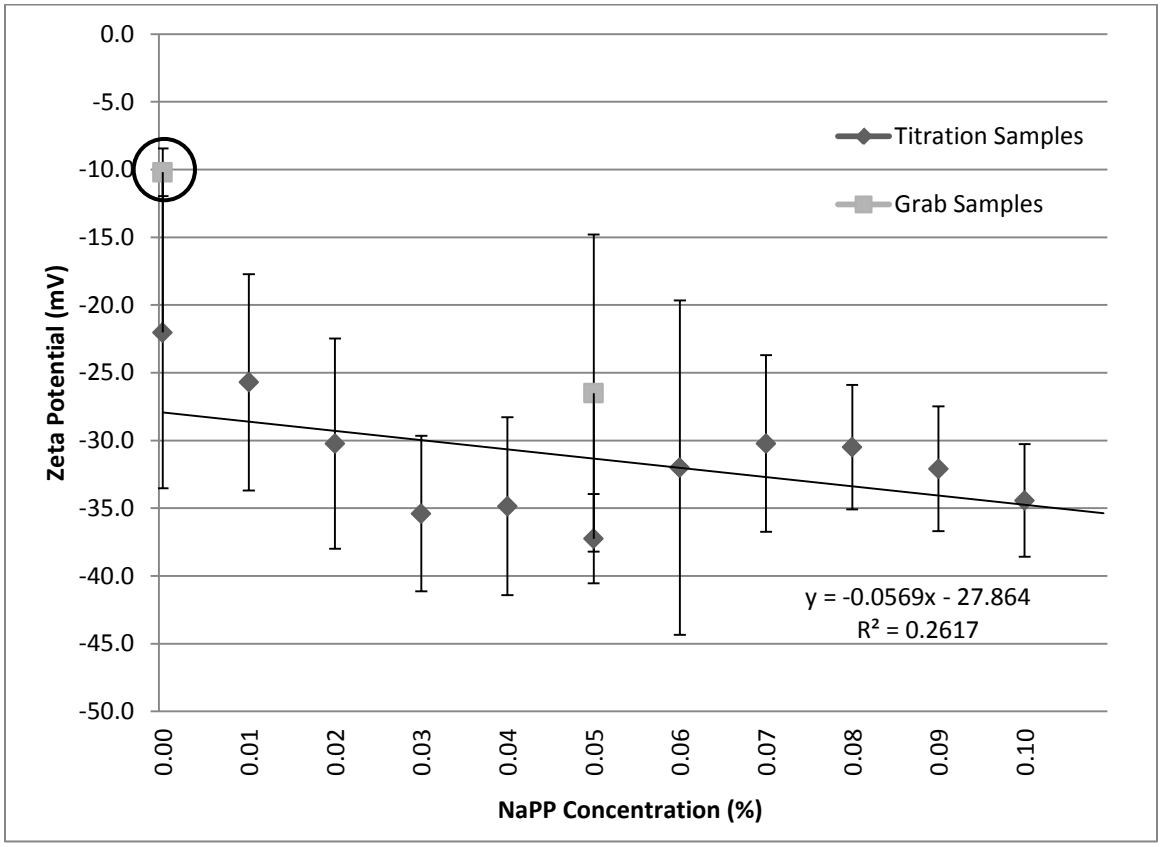

FIGURE 14: Mean zeta potential of kaolin particles at increasing concentrations of sodium polyphosphate

Prior to titration with sodium metasilicate, the mean initial zeta potential for kaolin particles in this condition was -22.2 mV (SD=4.32). When grab samples (n=3) were analyzed, the mean zeta potential for kaolin was -10.2 mV (SD=1.75). The probable reason for this difference, as recognized through initial condition pH values, was leakage of sodium metasilicate from the titrant tube prior to sample analyses. However, at a concentration of 0.01% sodium metasilicate, all test samples were at pH 10 or higher and grab samples showed no significant difference in zeta potential after a 0.01% concentration of sodium metasilicate was added (P=0.263).

Based upon the leakage of sodium metasilicate from the titrant tube, initial condition zeta potential values were discarded and grab sample zeta potential values were

used for statistical analyses. Since the addition of sodium metasilicate resulted in a substantial pH increase, grab samples (n=3) were analyzed for zeta potential at a pH value 11.5. At a pH of 11.5, the mean zeta potential of kaolin (-40.9 mV; SD=7.44) was significantly more negative than the mean zeta potential at a pH of 6 (P=0.000). At a concentration of 0.09% sodium metasilicate, the mean pH of titration samples was 11.5. Therefore, the mean zeta potential of kaolin at a 0.09% concentration of sodium metasilicate was compared to the mean zeta potential of kaolin at a pH of 11.5. At a pH of 11.68, the grab samples with 0.09% sodium metasilicate (-26.6 mV; SD=19.9) had less negative zeta potential readings than those conducted with pH adjustment to 11.5 with NaOH (P=0.072). Therefore, any significant decrease in zeta potential for kaolin at the sodium metasilicate concentrations tested was likely a function of pH increase and not the result of charge alteration of the kaolin through sodium metasilicate addition. A summary of zeta potential values for pH 6 and pH 11.5 is shown in Table 10, while Figure 15 shows the zeta potential of kaolin in suspension when sodium metasilicate is added.

TABLE 10: Summary results for kaolin and alumina at pH 6 and pH 11.5

	Kaolin	Alumina
Mean Zeta Potential (pH 6)	-16.4 mV (SD=4.24)	-15.8 mV (SD=2.03)
Mean Zeta Potential (pH 11.5)	-40.9 mV (SD=7.44)	-35.3 mV (SD=13.8)

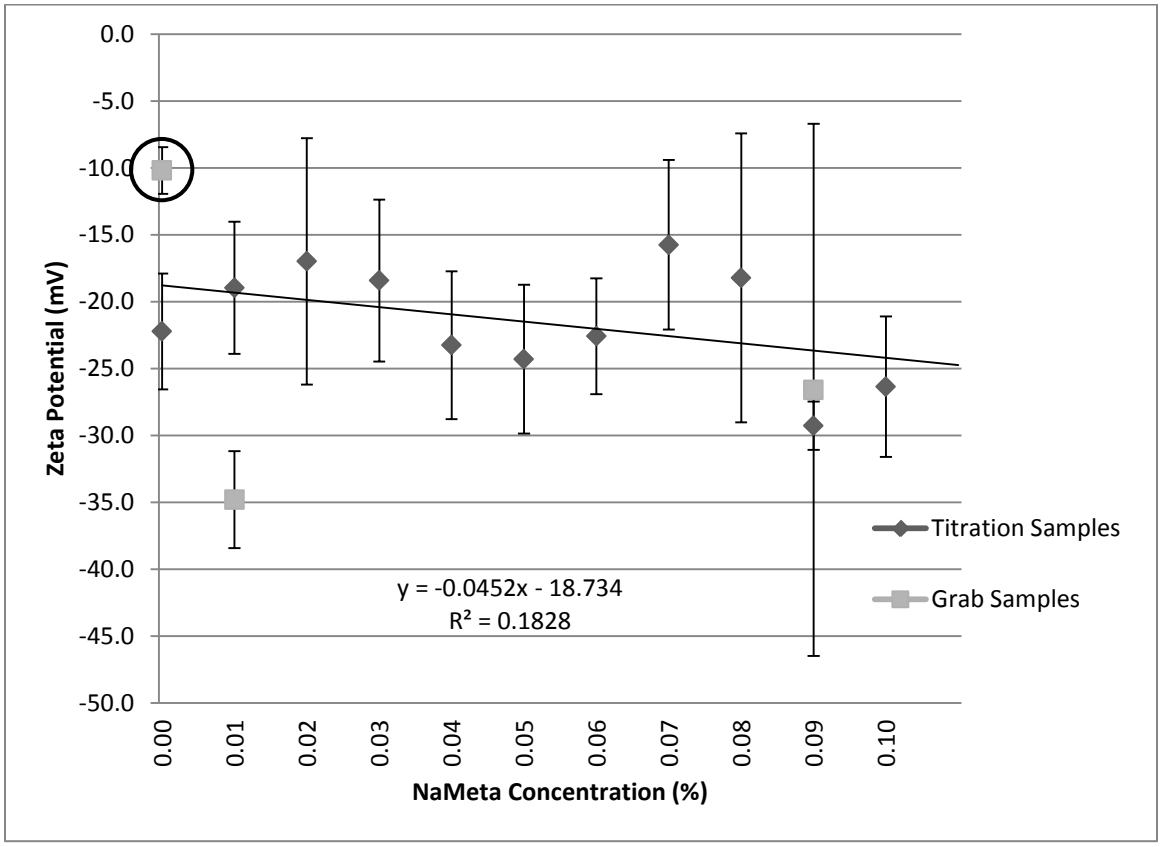

FIGURE 15: Mean zeta potential of kaolin particles at increasing concentrations of sodium metasilicate

5.4.2 Silica

Silica was also tested using 0.1% of each chemical dispersant in each of the model water solutions. When hardness was increased to 200 mg/L as $CaCO_3$ in model waters two, four, six, and eight, the use of sodium metasilicate resulted in a general decrease in the retention of silica particles in suspension. This suggests that high hardness could lead to poorer retention with sodium metasilicate. The results for all silica tests are summarized in Table 11 below. None of the differences were statically significant for silica for either dispersant versus the control samples.

TABLE 11: Summary results for silica

	Initial Turbidity Range (NTU)	% Turbidity Retained	P value
Model Water 1	18.4-20.5 & 42.4-48.7		
0.1% NaPP		13%	0.840
0.1% NaMeta		21%	0.548
No Dispersant		14%	
Model Water 2	37.0-88.5		
0.1% NaPP		15%	0.812
0.1% NaMeta		2%	0.275
No Dispersant		19%	
Model Water 3	18.1-19.9 & 42.3-78.2		
0.1% NaPP		20%	0.602
0.1% NaMeta		17%	0.822
No Dispersant		20%	
Model Water 4[1]	33.5-90.6		
0.1% NaPP		9%	0.273
0.1% NaMeta		2%	0.106
No Dispersant		5%	
Model Water 5	15.5-19.6 & 39.5-64.4		
0.1% NaPP		21%	0.905
0.1% NaMeta		17%	0.647
No Dispersant		22%	
Model Water 6[1]	48.4-50.7 & 78.9-88.2		
0.1% NaPP		7%	0.535
0.1% NaMeta		3%	0.268
No Dispersant		5%	
Model Water 7	15.8-21.4 & 47.9-61.4		
0.1% NaPP		21%	0.958
0.1% NaMeta		16%	0.611
No Dispersant		21%	
Model Water 8[1]	43.9-44.8 & 81.5-93.4		
0.1% NaPP		6%	1.000
0.1% NaMeta		3%	0.398
No Dispersant		6%	

[1]n=2

After the initial tests were conducted without pH being controlled, a second set of tests were performed at specific pH values. For silica, pH values of 2, 3, and 7 were used. These values were selected because a pH value of is near the point of zero charge for silica, while a pH of 2 is below this point and a pH value of 7 is above this point.

When pH was controlled at 2-3, initial turbidity values ranged from 5.02 NTU to 11.6 NTU. At a pH of 7, the initial turbidity ranged from 19.4 NTU to 43.1 NTU. As shown in Table 12, at a pH of 3, near the PZC, the use of either dispersant resulted in significantly worse retention of silica in suspension. This suggests a negative impact for dispersants near the PZC of the target particle. Due to the consistently small size of silica particles used for the study (refer to Table 5), pre-settling tests were not necessary.

TABLE 12: Summary results for silica in pH adjusted model water one

	Actual pH Range	% Turbidity Retained	P value
pH 2	1.70-1.83		
0.1% NaPP		45%	0.687
0.1% NaMeta		50%	0.959
No Dispersant		51%	
pH 3	2.98-3.13		
0.1% NaPP		35%	0.021
0.1% NaMeta		41%	0.041
No Dispersant		54%	
pH 7	6.78-7.25		
0.1% NaPP		35%	0.839
0.1% NaMeta		49%	0.341
No Dispersant		37%	

5.4.3 Diatomaceous Earth

Diatomaceous earth was tested as a particulate in 5 of the model water solutions (#1, #2, #3, #5, and #7). Under no test condition, was more than 5% of diatomaceous earth retained in suspension. This lack of retention of diatomaceous earth was likely due to the settling velocity of the particle (1.60 in/hr) as shown in Table 5. A summary of diatomaceous earth results is shown in Table 13. Due to the high sedimentation potential of this particle type, no further experiments were performed with diatomaceous earth.

TABLE 13: Summary results for diatomaceous earth

	Initial Turbidity Range (NTU)	% Turbidity Retained	P value
Model Water 1	63.2-97.2		
0.1% NaPP		3%	0.230
0.1% NaMeta		1%	0.013
No Dispersant		3%	
Model Water 2	39.8-75.9		
0.1% NaPP		4%	0.757
0.1% NaMeta		1%	0.020
No Dispersant		3%	
Model Water 3	52.1-84.1		
0.1% NaPP		4%	1.000
0.1% NaMeta		1%	0.022
No Dispersant		3%	
Model Water 5	54.9-62.5		
0.1% NaPP		5%	0.519
0.1% NaMeta		5%	0.246
No Dispersant		4%	
Model Water 7	54.0-61.2		
0.1% NaPP		4%	0.519
0.1% NaMeta		5%	0.265
No Dispersant		5%	

5.4.4 Alumina

Aluminum oxide or alumina was also tested as a particle of interest. When tests were performed in model waters one, two, and three, initial turbidity varied from 70.3 NTU to 130 NTU. However, the average retention of particulate was two percent or less for each test condition including control samples. Due to the low retention of alumina in these tests, pH controlled tests were conducted. When pH was controlled for alumina, three pH values were selected, and all tests were conducted in model water one. A pH value of 8 was selected since this is near the point of zero charge for alumina, while pH values above (pH 11) and below the point of zero charge (pH 5) were also selected for test samples. When pH was controlled at 5 (actual range of 4.84 to 5.44), a mean

retention of 3% or less was achieved for each test condition (initial turbidity range of 35 NTU to 81 NTU). Similarly, at pH values of 8 and 11, all test conditions had a mean retention of 2%, while initial turbidity readings ranged from 31.6 NTU to 104 NTU).

Based upon Stokes Law calculations (as shown in Table 5), the determination was made that particles larger (up to 5 µm) than the average 1 µm particles were present in the alumina, and the rapid settling was likely due to gravity. Therefore, alumina was allowed to settle out of suspension for two hours, and the supernatant, which should have contained smaller particles, was removed and used for pH adjusted test samples. Again, pH values of 5, 8, and 11 were tested with pre-settled alumina. Retention of alumina in suspension was similar for all conditions tested, and all phase 3 alumina results are summarized in Table 14.

TABLE 14: Summary results for alumina phase 3 tests

	Actual pH Range	% Turbidity Retained	P value
pH 5	4.84-5.06		
0.1% NaPP		19%	0.461
0.1% NaMeta		20%	0.051
No Dispersant		17%	
pH 8	8.03-8.28		
0.1% NaPP		22%	0.826
0.1% NaMeta		27%	0.073
No Dispersant		22%	
pH 11	10.94-11.38		
0.1% NaPP		21%	0.804
0.1% NaMeta		21%	0.627
No Dispersant		20%	

After the completion of settling tests, the zeta potential analyses described in phase 4, initial dispersant titrations for pre-settled alumina samples (n=4), were performed. When sodium polyphosphate was titrated from an initial concentration of

0.00% to 0.10%, the mean initial zeta potential was -16.3 mV (SD=8.77), and the zeta potential steadily increased to a mean of -26.1 mV (SD=8.31) at a sodium polyphosphate concentration of 0.04% (P=0.010). When grab samples were analyzed for alumina (n=3), the mean zeta potential was -2.99 mV (SD=1.48). Grab samples (n=3) were also analyzed at a sodium polyphosphate concentration of 0.05% and compared to the titration results (P=0.720). Again, a difference in the initial condition zeta potential values was noted between titration and grab samples. As previously discussed, while there was variability in the zeta potential values, there was also the possibility that titrant (i.e., sodium polyphosphate) leaked into samples prior to the initiation of sodium polyphosphate titration. Mean zeta potential values for each sodium polyphosphate concentration are shown in Figure 16.

As with kaolin, use of either chemical dispersant caused a change in sample pH. Thus, three grab samples with alumina particles in model water one were pH adjusted to a 6 (actual value of 6.02) in order to compare the effect of pH versus sodium polyphosphate on the zeta potential readings for alumina particles. At a pH of 6, the mean zeta potential for alumina was -15.8 mV (SD=2.03), which was less negative than the mean zeta potential of alumina grab samples (n=3) with 0.05% sodium polyphosphate (actual pH of 6.12). However, this difference in zeta potential was not significant (P=0.108). Refer to Table 10 for a summary of pH data for alumina.

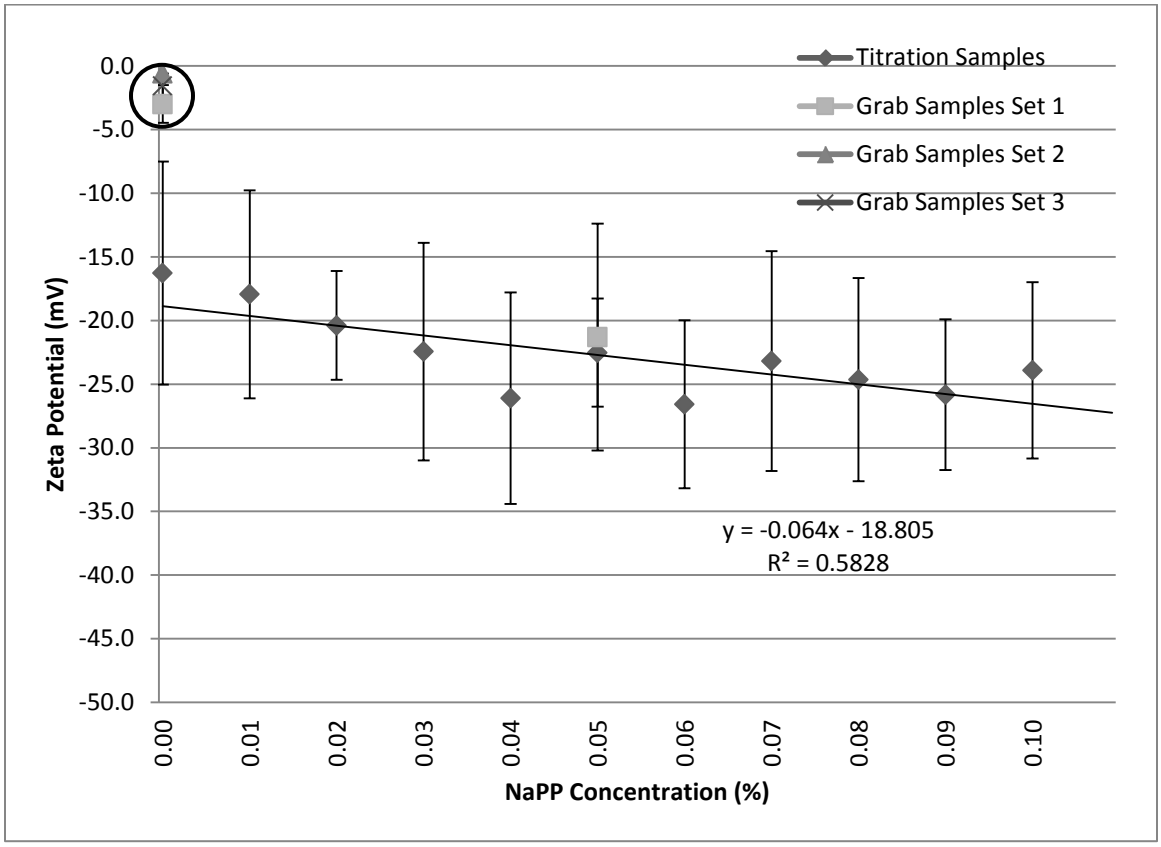

FIGURE 16: Mean zeta potential of alumina particles at increasing concentrations of sodium polyphosphate

Similar titration samples (n=3) were analyzed for zeta potential with sodium metasilicate titrated from an initial concentration of 0.00% to 0.10%. For the initial condition of no dispersant, the mean zeta potential was -18.8 mV (SD=5.72), and, as shown in Figure 17, the zeta potential showed no significant trend as sodium metasilicate concentration increased. When grab samples were performed with alumina in suspension in model water one, the zeta potential was significantly less negative than reported for the initial condition of titration samples (P=0.000). This difference was likely the function of sodium metasilicate leaking from the titrant tube and into the test sample prior to data collection. As with kaolin, the addition of sodium metasilicate, to model water one with alumina in suspension, rapidly raised pH values. At a concentration of 0.01%, the pH

had already increased to 10 or higher for all test samples and the analysis of grab samples showed no significant change in zeta potential over the range of concentrations tested (P=0.124). In order to test the effect of pH on the zeta potential readings for alumina particles, grab samples were also analyzed for zeta potential at a pH value 11.5 (refer to Table 10). At a pH value of 11.5, the mean zeta potential for alumina (-35.3 mV; SD=13.8) was significantly more negative than when tested at pH 6 (P=0.003). During titration, no tested concentration of sodium metasilicate resulted in a zeta potential value that was more negative than those attained by pH adjustment to 11.5. Additionally, when the mean zeta potential of alumina grab samples with 0.09% sodium metasilicate (-27.7 mV; SD=10.4) was compared to alumina grab samples at a pH of 11.5, there was no difference in zeta potential (P=0.208). Since the mechanism of action for alumina was pH change and pH change occurred at the lowest concentration tested, this is a probable reason why increased concentrations of sodium metasilicate did not consistently produce more negative zeta potential values.

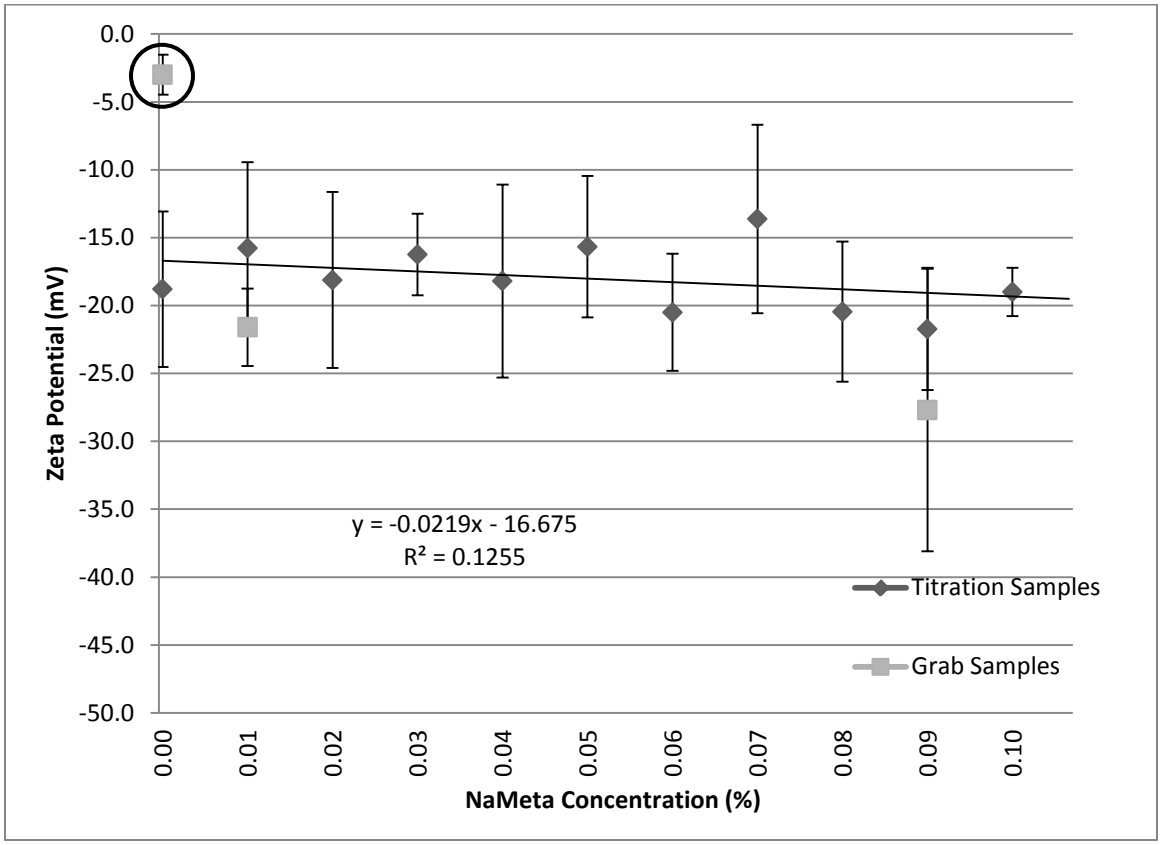

FIGURE 17: Mean zeta potential of alumina particles at increasing concentrations of sodium metasilicate

5.4.5 Microspheres

Carboxylated microspheres (1 µm) were tested in model waters one, two, three, five, and seven. When 0.1% sodium metasilicate was used in model water one and two, the initial turbidity of the samples increased due to the formation of a precipitate. A complete summary of results for tests performed with 1 µm carboxylated microspheres can be reviewed in Table 15.

TABLE 15: Summary results for 1 µm carboxylated microspheres

	Initial Turbidity Range (NTU)	% Turbidity Retained	P value
Model Water 1	3.61-25.6		
0.1% NaPP		93%	0.667
0.1% NaMeta		3%	0.000
No Dispersant		92%	
Model Water 2	8.32-63.3		
0.1% NaPP		94%	0.375
0.1% NaMeta		16%	0.006
No Dispersant		88%	
Model Water 3	3.21-8.30		
0.1% NaPP		76%	0.590
0.1% NaMeta		69%	0.980
No Dispersant		69%	
Model Water 5	16.5-29.7 & 3.94-5.03		
0.1% NaPP		94%	0.774
0.1% NaMeta		96%	0.877
No Dispersant		95%	
Model Water 7	3.86-6.02		
0.1% NaPP		84%	0.024
0.1% NaMeta		85%	0.213
No Dispersant		94%	

In addition to the 1 µm carboxylated microspheres, 3 µm non-carboxylated microspheres were also tested with model water solutions one, two, three, five, and seven. When 0.1% sodium metasilicate was added to samples with model water one and model water two a precipitate formed in the samples. Complete results for each test condition can be found in Table 16.

TABLE 16: Summary results for 3 µm non-carboxylated microspheres

	Initial Turbidity Range (NTU)	% Turbidity Retained	P value
Model Water 1	39.1-73.5		
0.1% NaPP		73%	0.174
0.1% NaMeta		17%	0.000
No Dispersant		71%	
Model Water 2	37.7-57.3		
0.1% NaPP		73%	0.562
0.1% NaMeta		40%	0.010
No Dispersant		75%	
Model Water 3	39.1-68.5		
0.1% NaPP		72%	0.606
0.1% NaMeta		42%	0.202
No Dispersant		73%	
Model Water 5	44.5-49.5		
0.1% NaPP		73%	0.020
0.1% NaMeta		74%	0.047
No Dispersant		75%	
Model Water 7	37.8-52.2		
0.1% NaPP		73%	0.255
0.1% NaMeta		73%	0.210
No Dispersant		69%	

Finally, pH adjusted tests were also performed with the 3 µm non-carboxylated microspheres in model water one. These tests were conducted at pH values of 3 and 11. As shown in Table 17, results for all test conditions were similar.

TABLE 17: Summary results for 3 µm non-carboxylated microspheres in pH adjusted model water one

	Actual pH Range	% Turbidity Retained	P value
pH 3	3.13-3.27		
0.1% NaPP		63%	0.033
0.1% NaMeta		69%	0.052
No Dispersant		60%	
pH 11	10.95-11.38		
0.1% NaPP		61%	0.033
0.1% NaMeta		63%	0.490
No Dispersant		65%	

5.4.6 *E. coli*

In order to determine whether pH adjustment had an effect on *E. coli* growth, tests were performed with sterile deionized water, model water one, model water one adjusted to a pH of 6.0 (actual pH of 5.45), and model water one adjusted to a pH value of 11.5 (actual pH of 11.31). The purpose of these samples was to determine if pH adjustment would result in dead *E. coli* either immediately or after 24 hours. All four samples in set one were analyzed immediately after pH adjustment had MPN values of >2420/100 mL. Samples in set two were allowed to sit at room temperature for 24±2 hours after pH adjustment and microbe seeding prior to analysis. For these samples, the sterile deionized water, model water one and model water one adjusted to a pH of 6, the MPN value was still >2420/100 mL. However, for the pH 11.5 sample, the MPN dropped to 866/100 mL. Therefore, an additional set of pH 11.5 samples were tested. A certified standard containing a low *E. coli* seed dose (159 to 3570 MPN/100 mL) was added to a sample of model water one that had been pH adjusted to 11.5 (actual pH of 11.34) and analyzed immediately. The result was an MPN value of 14.8/100 mL. A second set of samples were analyzed 24±2 hours after pH adjustment and seeding. The model water one sample yielded an MPN value of 120/100 mL, while the pH adjusted sample had an MPN value of <1/100 mL. This finding suggests that adjustment to a pH value of 11.5 with 0.1 N NaOH can alter the viability of some *E. coli* in model water one immediately after pH adjustment and can result in the lack of viability for all *E. coli* in suspension if held for 24±2 hours after pH adjustment. This could be important for zeta potential readings since physiological state can impact surface charge (Soni et al. 2008).

Although all zeta potential readings with *E. coli* were taken within 24 hours of seeding, zeta potential analyses were performed on *E. coli* suspensions that had been seeded 7 days prior to analysis in order to determine if this amount of age would alter the zeta potential readings. Three samples were analyzed for zeta potential at <24 hours post-seeding, while three additional samples of the same *E. coli* (ATCC 25922) suspension were analyzed for zeta potential after 7 days. Samples analyzed within 24 hours of seeding into model water one had a mean zeta potential of -10.0 mV (SD=2.07), while the mean zeta potential of the 7-day old *E. coli* (ATCC 25922) was also -10.0 mV (SD=1.16) (P=0.997). Therefore, no difference in zeta potential was found for *E. coli* (ATCC 25922) that had been prepared for 7 days. Data is shown in Figure 18 below.

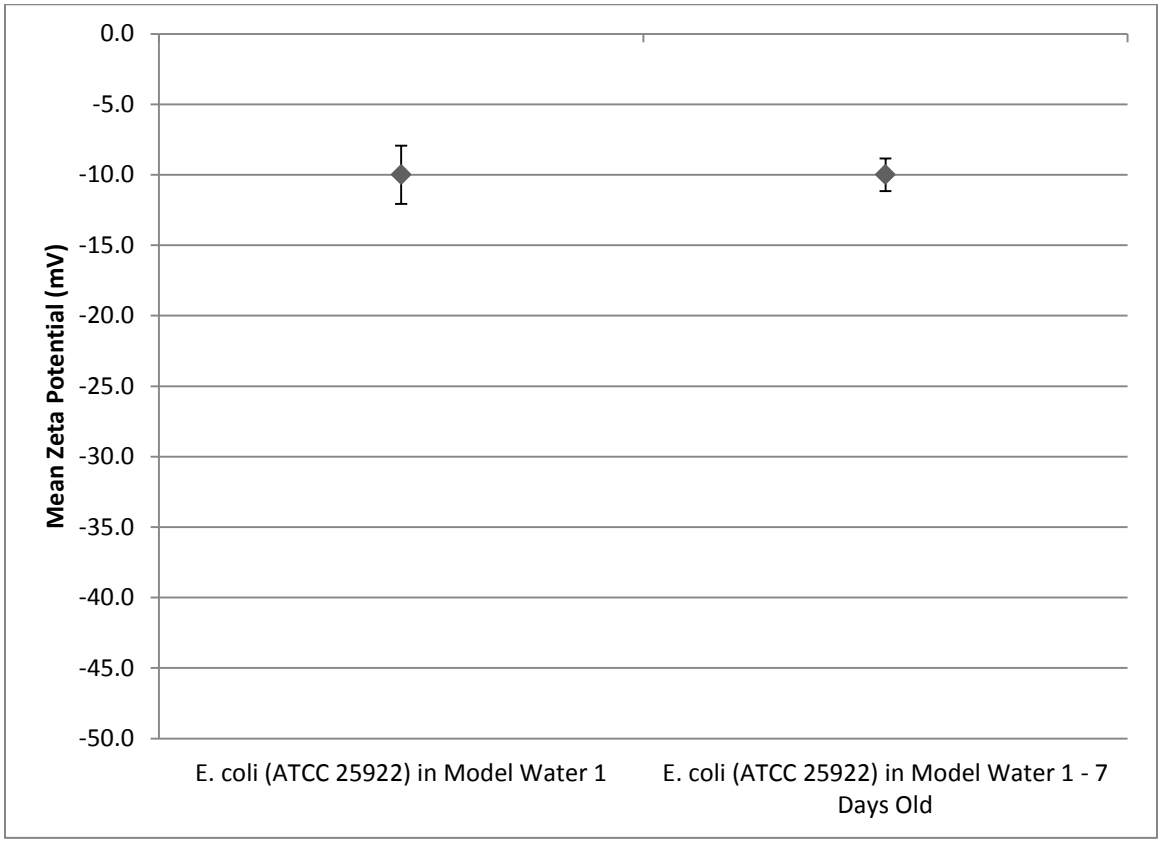

FIGURE 18: Mean zeta potential of *E. coli* (ATCC 25922) for age effect

Since zeta potential can vary based upon the pH of the sample in which the particle is located, three titration samples of *E. coli* (ATCC 25922) in model water one were performed in the pH range of 3 to 12. In addition to the pH titrations, grab samples (n=3) were also analyzed for zeta potential at pH values of 6, 7, 8, and 11.5. Figure 19 shows the mean zeta potential readings with error bars at ± 1 standard deviation for the pH titration and grab samples. There was good general agreement between grab samples and titration samples and within each group.

While an increase in pH typically results in more negative zeta potential values, this was not the case for *E. coli* (ATCC 25922) during either titration or grab sample analysis. Whereas there was a trend in more negative zeta potential values from pH 3.0 (mean zeta potential=-4.77; SD=3.04) to pH 5.0 (mean zeta potential=-11.5; SD=1.72) during pH titrations (P=0.000), from pH 5.0 to pH 11.0 (mean zeta potential=-14.4; SD=4.28), there was no negative trend in zeta potential values (P=0.070). Although this overall trend in zeta potential is atypical, Lytle and colleagues (1999) reported that electrophoretic mobility values became more negative from a pH of 2 to a pH of 5 for a wild-type *E. coli* strain but were independent of pH above a pH value of 5 to a pH value of 9. Unlike the current research, the previous study did not test pH values above 9, so a comparison with pH values of 9.5 to 12.0 cannot be made.

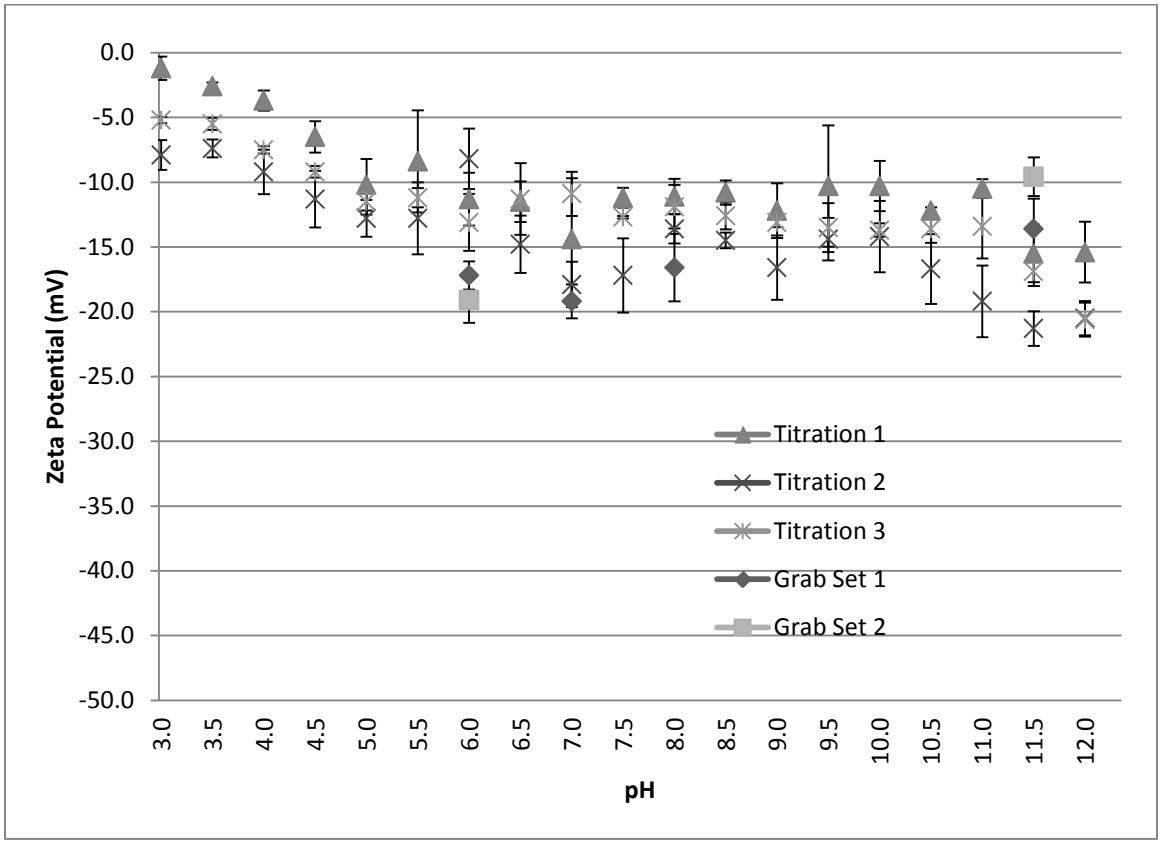

FIGURE 19: Mean zeta potential of *E. coli* (ATCC 25922) at different pH values

In addition to the variability for pH with a single strain of *E. coli*, the zeta potential was also found to vary at the same pH values for the two different *E. coli* strains. Both *E. coli* (ATCC 25922) and *E. coli* (ATCC 11775) were tested as grab samples at pH values of 6 (n=3) and 11.5 (n=3). Table 18 highlights the zeta potential differences between the two *E. coli* strains tested.

TABLE 18: Summary results for *E. coli* strains at pH 6 and pH 11.5

	E. coli ATCC 25922	*E. coli* ATCC 11775	P-value
Mean Zeta Potential (pH 6)	-17.2 mV (SD=1.09)	-6.78 mV (SD=0.948)	0.000
Mean Zeta Potential (pH 11.5)	-16.6 mV (SD=2.34)	-10.8 mV (SD=1.45)	0.009

It is important to note that while results for *E. coli* (ATCC 25922) did not follow the typical pattern of more negative zeta potential readings at increased pH values, the *E.*

coli (ATCC 11775) did. At a pH of 3.7, the mean zeta potential for *E. coli* (ATCC 11775) was -3.53 (SD=0.679), while the zeta potential increased to -6.78 (SD=0.948) when the pH was increased to 6 (P=0.000). The zeta potential (-10.8; SD=1.45) further increased when a pH value of 11.5 was tested (P=0.000). This suggests that zeta potential values cannot be predicted to increase or decrease in a specific way for general classes of microbes, as differences can exist even within a species of bacteria. Zeta potential values for each test condition can be seen in Figure 20.

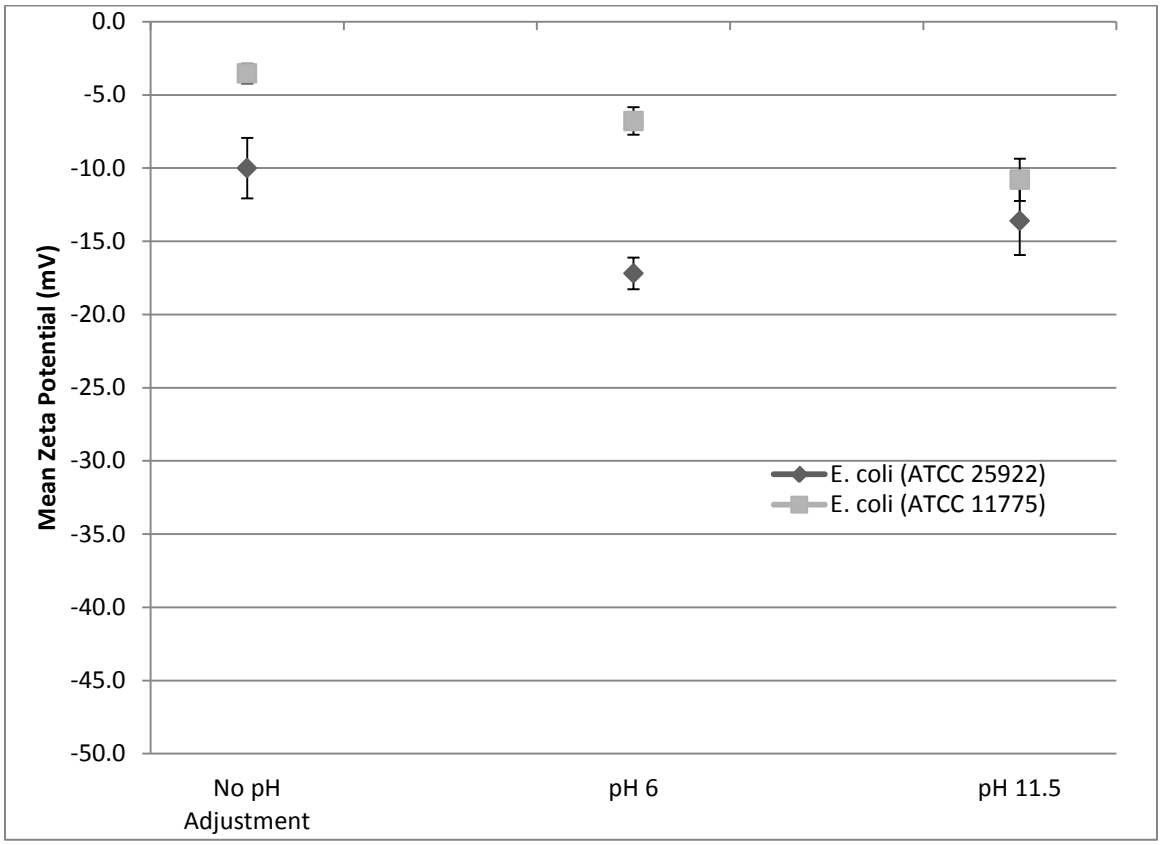

FIGURE 20: Mean zeta potential of each strain of *E. coli* at pH 6 and pH 11.5

One reason for the difference in zeta potential behavior between the two types of *E. coli* could be the result of the surface functional group variations of the different strains. Since a greater understanding of how chemical dispersants work to improve

microbe recovery was the main goal of this study, several individual conditions were tested as grab samples with both types of *E. coli* as the particle. This was done to determine if zeta potential differences consistently existed between the two strains of *E. coli*. Grab samples (n=3) were analyzed for *E. coli* (ATCC 25922) in model water one, and this resulted in a mean zeta potential of -10.0 mV (SD=2.07), while the zeta potential for *E. coli* (ATCC 11775) (n=3) was significantly different at a mean value of -3.54 mV (SD=0.679) (P=0.000). The data, shown in Figure 21, indicates a more negative surface charge for *E. coli* (ATCC 25922) than for *E. coli* (ATCC 11775).

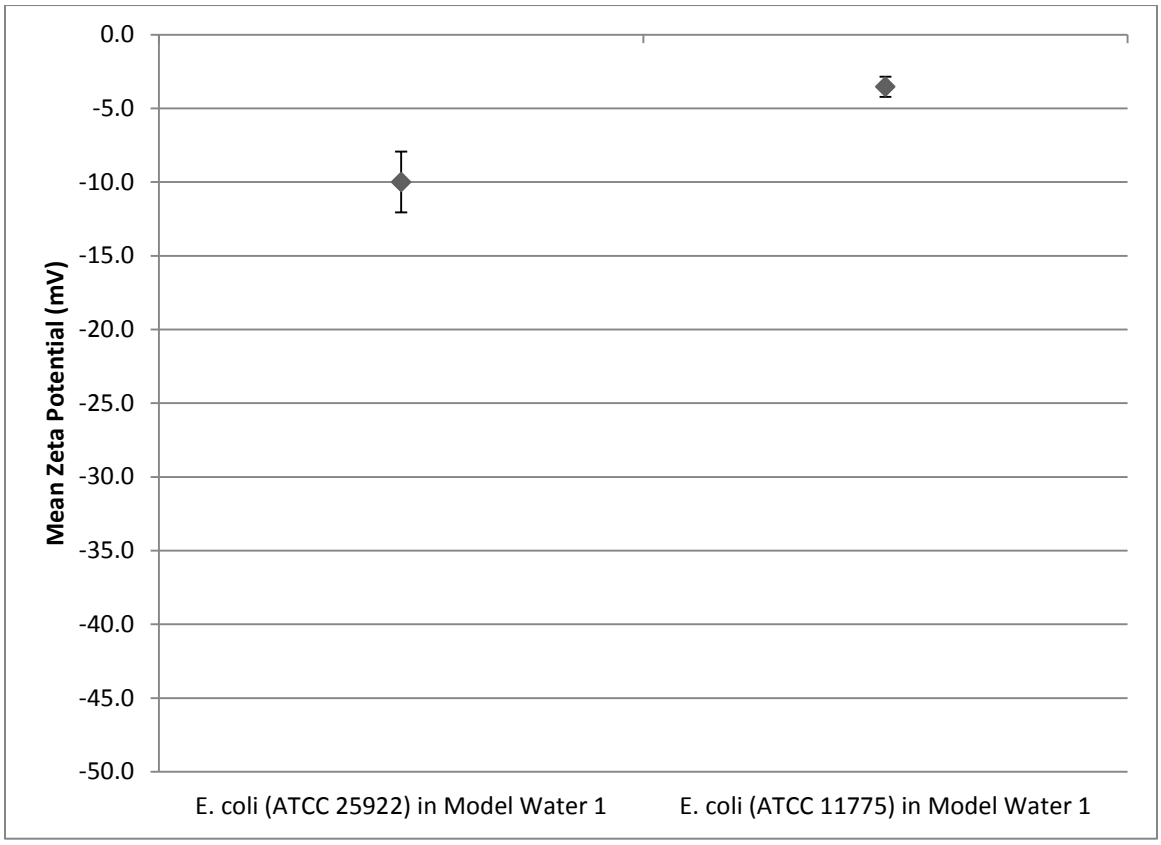

FIGURE 21: Zeta potential readings of two different strains of *E. coli*

Separate titrations of sodium polyphosphate, sodium metasilicate, and sodium chloride were performed for *E. coli* (ATCC 25922) samples in model water one. As

shown in Figure 22, for sodium polyphosphate titrations (n=5), the initial mean zeta potential was -12.1 mV (SD=2.65), while the most negative mean zeta potential reading occurred at a concentration of 0.05% sodium polyphosphate (-27.0 mV; SD=5.75) (P=0.000). Moreover, the mean zeta potential became significantly more negative with the addition of 0.01% sodium polyphosphate and stayed significantly more negative than the initial zeta potential through all sodium polyphosphate concentrations. Since the most negative zeta potential value occurred at 0.05% sodium polyphosphate during sample titrations, grab samples of *E. coli* (ATCC 25922) in model water one (n=3) were also analyzed. With no sodium polyphosphate, the mean zeta potential of this test condition was -10.0 mV (SD=2.07), while the mean zeta potential increased to -26.8 mV (SD=4.73) at a sodium polyphosphate concentration of 0.05% (P=0.000). However, the addition of 0.05% sodium polyphosphate to the grab samples also adjusted the pH of the model water to 6.07. Since pH also affects zeta potential, there is value in making a determination of this affect. When the zeta potential at pH 6 was compared to the mean zeta potential of *E. coli* (ATCC 25922) with 0.05% sodium polyphosphate, the zeta potential was still significantly more negative in the presence of 0.05% sodium polyphosphate (P=0.000).

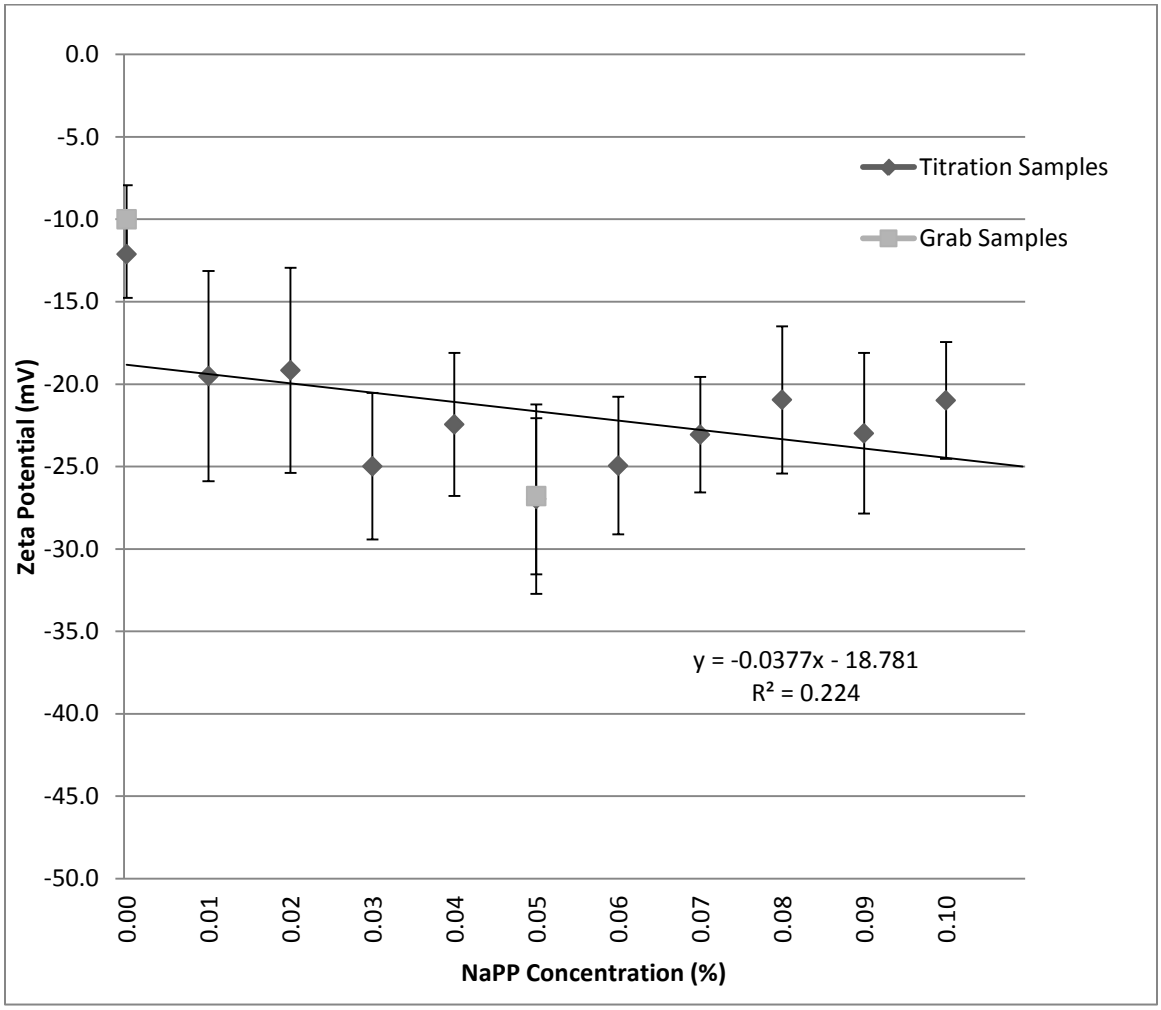

FIGURE 22: Mean zeta potential of *E. coli* (ATCC 25922) at increasing concentrations of sodium polyphosphate

In addition to grab samples performed with *E. coli* (ATCC 25922), a second set of grab samples (n=3) was analyzed with *E. coli* (ATCC 11775). At an initial sodium polyphosphate concentration of 0.00%, the mean zeta potential for *E. coli* (ATCC 11775) was determined as -3.5 mV (SD=0.679), while the mean zeta potential (-7.11; SD=0.490) was significantly more negative at a sodium polyphosphate concentration of 0.05% (P=0.000). While both *E. coli* strains showed significantly more negative zeta potential readings when 0.05% sodium polyphosphate was present in the samples, the zeta potential values at this sodium polyphosphate concentration are significantly different

based upon *E. coli* strain (P=0.000). These results are highlighted in Figure 23. Just as occurred with *E. coli* (ATCC 25922), the addition of 0.05% sodium polyphosphate adjusted the pH of the samples to approximately pH 6 (actual pH=5.99). When compared to the mean zeta potential of grab samples performed with *E. coli* (ATCC 11775) at a pH of 6, there is no significant difference in zeta potential for samples with 0.05% sodium polyphosphate (P=0.370). This is in contrast to *E. coli* (ATCC 25922), where a significant difference in zeta potential was noted for samples with 0.05% sodium polyphosphate.

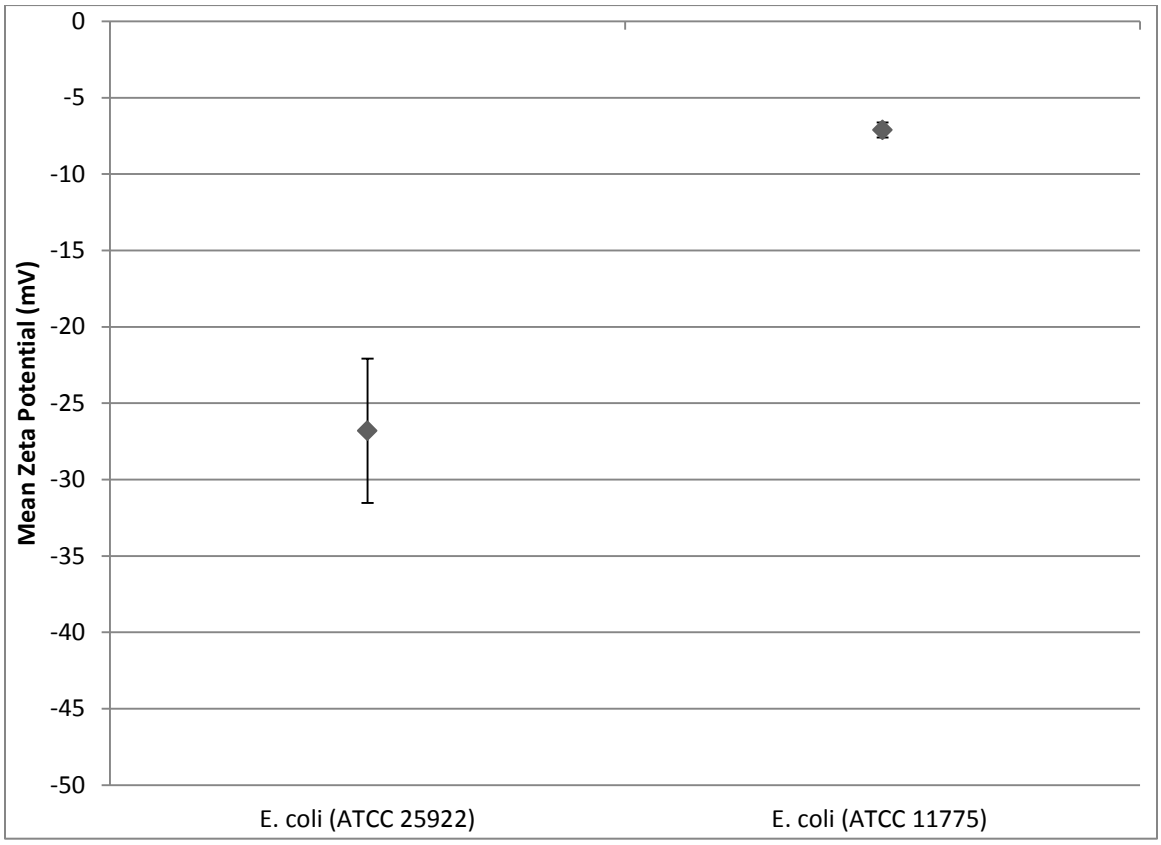

FIGURE 23: Comparison of zeta potential for two strains of *E. coli* in model water one with 0.05% sodium polyphosphate

Prior to the titration of sodium metasilicate into an *E. coli* (ATCC 25922) sample, the initial mean zeta potential (n=3) was -14.0 mV (SD=3.02). Significant differences were observed above a sodium metasilicate concentration of 0.02% or higher. Figure 24 shows zeta potential data collected through the range of sodium metasilicate concentrations tested. When grab samples were analyzed, the initial mean zeta potential was -10.0 mV (SD=2.07), while the addition of 0.05% sodium metasilicate produced a mean zeta potential of -26.0 mV (SD=2.64) (P=0.000).

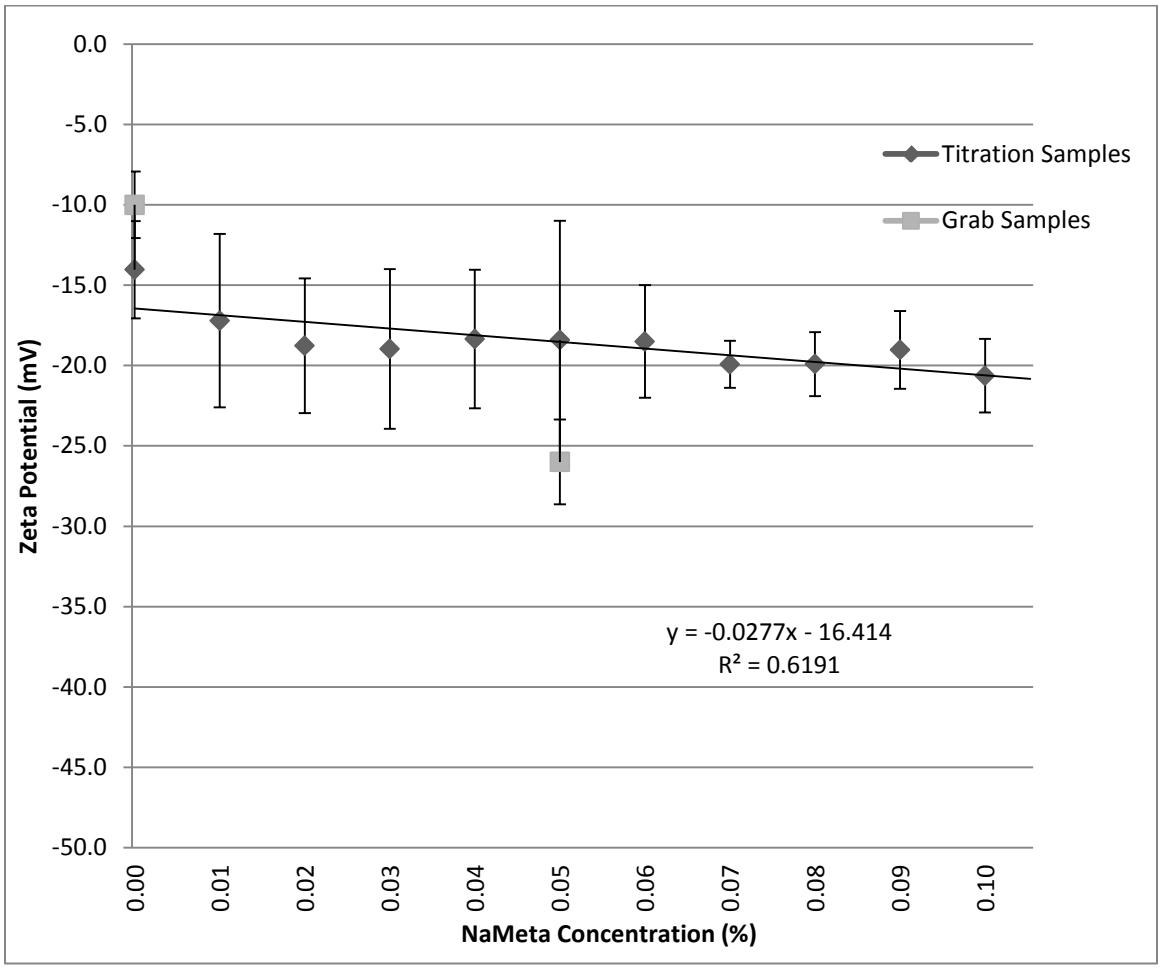

FIGURE 24: Mean zeta potential of *E. coli* (ATCC 25922) at increasing concentrations of sodium metasilicate

As with sodium polyphosphate, additional grab samples were analyzed with a second strain of *E. coli* (ATCC 11775) (n=3) to determine if a differences in zeta potential existed at a 0.05% concentration of sodium metasilicate. At an initial sodium metasilicate concentration of 0.00%, the mean zeta potential for *E. coli* (ATCC 11775) was determined as -3.54 mV (SD=0.679), while the mean zeta potential (-10.4 mV; SD=0.698) was significantly more negative at a sodium metasilicate concentration of 0.05% (P=0.000). Again, both *E. coli* strains showed significantly more negative zeta potential readings when 0.05% sodium metasilicate was present in the samples, and the zeta potential values at this sodium metasilicate concentration varied significantly based upon the *E. coli* strain present in suspension (P=0.000). These results are highlighted in Figure 25.

However, as with sodium polyphosphate, the addition of sodium metasilicate resulted in a pH change of the samples. Whereas the use of 0.05% sodium polyphosphate resulted in a pH of approximately 6, the addition of 0.05% sodium metasilicate resulted in a pH of approximately 11.5 (actual pH=11.44). Again, since pH can have an effect on zeta potential, the zeta potential of *E. coli* (ATCC 25922) at pH 11.5 had to be compared to the zeta potential of *E. coli* (ATCC 25922) with 0.05% sodium metasilicate. For *E. coli* (ATCC 25922), it is also important to remember that the zeta potential became less negative instead of more negative at pH 11.5. While pH adjustment to 11.5 resulted in a mean zeta potential of -13.6 mV (SD=2.34), the addition of 0.05% sodium metasilicate had a mean zeta potential value of -26.0 mV (SD=2.64) (P=0.000). This suggests that the addition of sodium metasilicate resulted in a change to the surface charge of *E. coli* (ATCC 25922) different from that which occurred by pH adjustment. Conversely, the

mean zeta potential of *E. coli* (ATCC 11775) was similar for pH 11.5 samples (-10.4 mV; SD=0.698) and samples that contained 0.05% sodium metasilicate (-10.8 mV; SD=1.45) (P=0.535). Again, there is a marked difference between the zeta potential response to similar water sample conditions for *E. coli* (ATCC 25922) and *E. coli* (ATCC 11775).

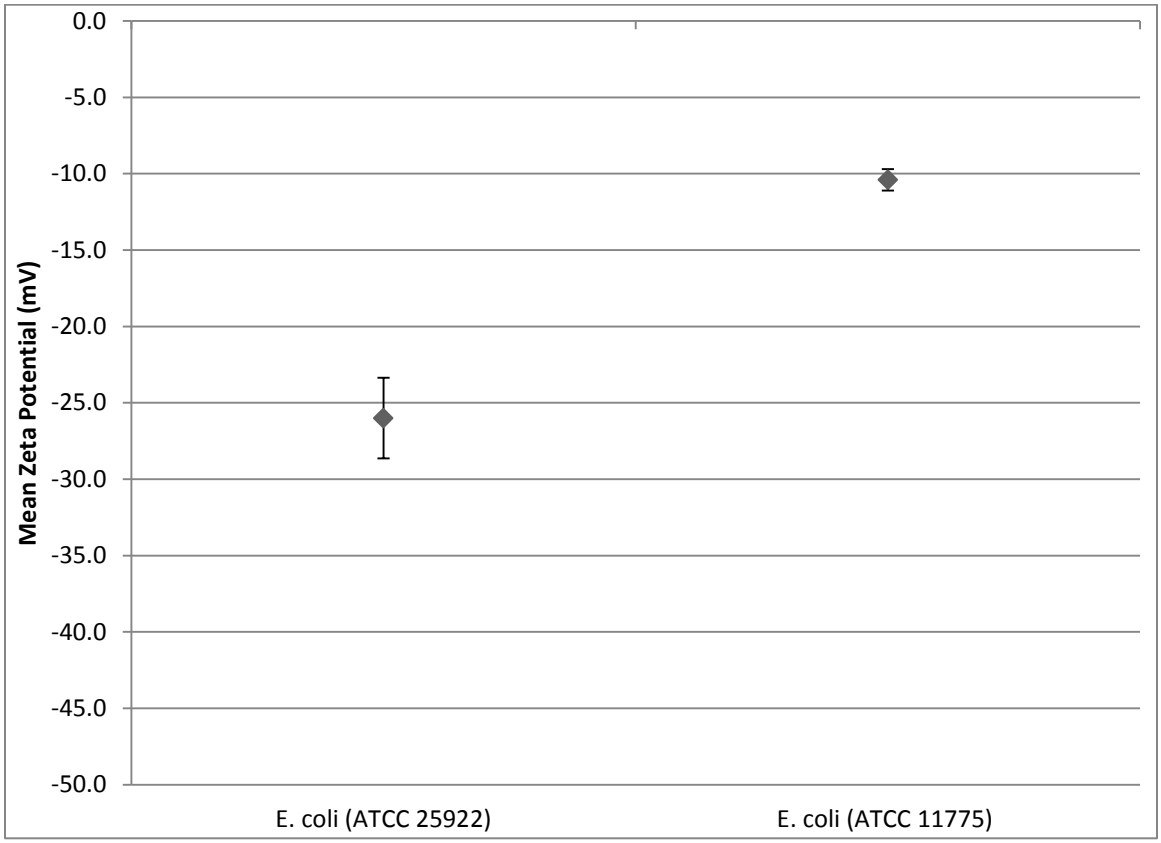

FIGURE 25: Comparison of zeta potential for two strains of *E. coli* in model water one with 0.05% sodium metasilicate

As shown in Figure 26, *E. coli* (ATCC 25922) samples (n=5) were also titrated with increasing concentrations of sodium chloride (0.00% to 0.10%). The addition of NaCl did not result in a pH change of the sample being tested, but an increase in the concentration from 0.00% NaCl to 0.10% NaCl resulted in a change in ionic strength of the sample from 1.76 mM to 3.93 mM. Typically, as ionic strength increases, zeta

potential becomes less negative. At the initial condition of no added sodium chloride, the mean zeta potential of *E. coli* (ATCC 25922) was -11.4 mV (SD=5.26), and the zeta potential did not significantly change at any titrated concentration. Additionally, grab samples of *E. coli* (ATCC 25922) in model water one were also analyzed with 0.00%, 0.02%, 0.05%, and 0.08% NaCl. For grab samples (n=3), the mean zeta potential values for *E. coli* (ATCC 25922) were more negative than those produced during the sodium chloride titrations, but values were within the standard deviation of readings. *E. coli* (ATCC 25922) in model water one had a mean zeta potential value of -10.0 mV (SD=2.07), while the mean zeta potential was -17.7 mV (SD=0.793) when a 0.02% concentration of NaCl was added, -16.3 mV (SD=2.29) for a 0.05% concentration of NaCl, and -18.7 mV (SD=1.39) for a 0.08% concentration of NaCl. While the zeta potential results were similar for all three concentrations of sodium chloride, the zeta potential was significantly more negative at each concentration (P=0.000 for 0.02% NaCl, P=0.000 for 0.05% NaCl, and P=0.000 for 0.08% NaCl) than at the initial condition. As a result of these differences, a second set of grab samples (n=3) was analyzed at each of the three sodium chloride concentrations. These results, which were similar to those achieved in the first set of grab samples, are shown in Figure 26.

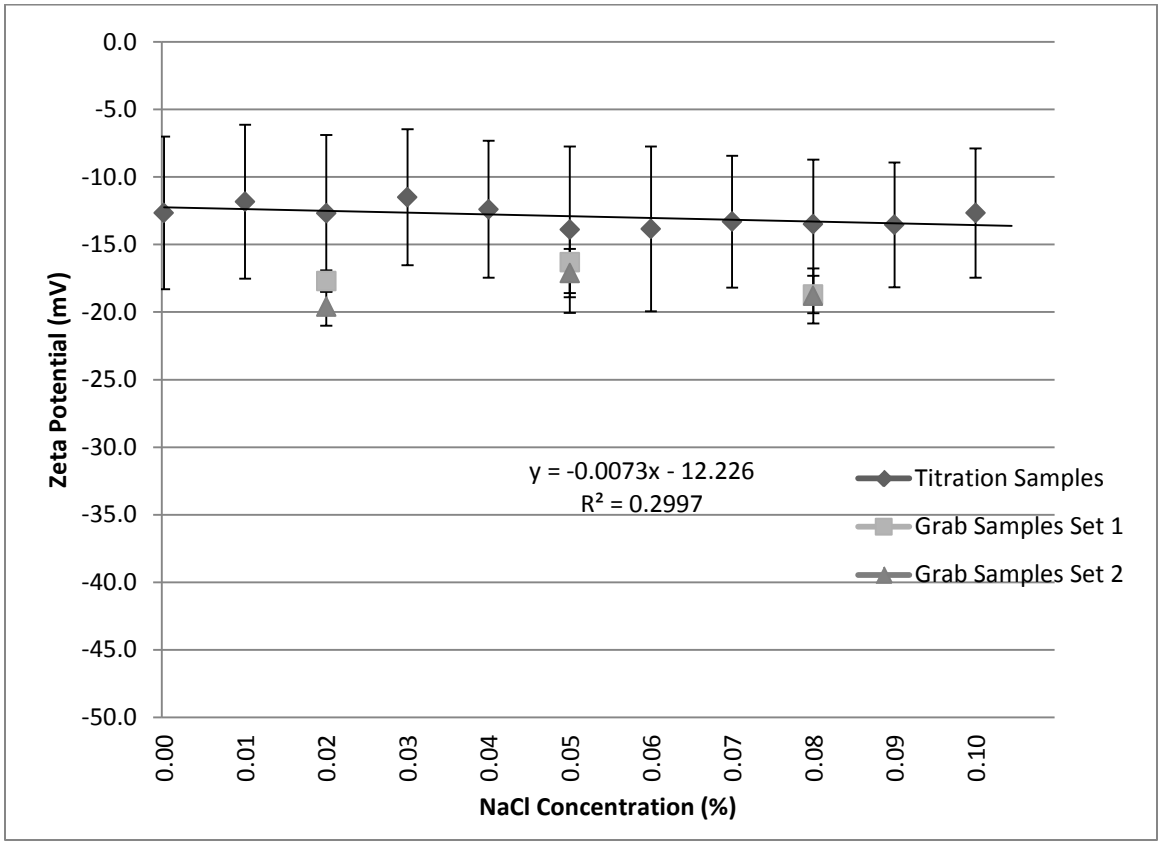

FIGURE 26: Mean zeta potential of *E. coli* (ATCC 25922) particles at increasing concentrations of sodium chloride

These results are in contrast to the expected response of zeta potential as ionic strength is increased. However, the concentration range of sodium chloride tested only spanned one order of magnitude. Additionally, this is not unprecedented in the literature, as Lytle and colleagues (1999) reported that electrophoretic mobility values for *E. coli* O157:H7 were more negative at an ionic strength of 9.15 mM than at an ionic strength of 0.915 mM. Additionally, as shown in Table 19, the mean zeta potential values of *E. coli* at 0.05% sodium chloride are significantly less negative than for either 0.05% sodium polyphosphate or 0.05% sodium metasilicate.

TABLE 19: Summary results for ionic strength effect for *E. coli*

	E. coli ATCC 25922[1]	P-value	*E. coli* ATCC 11775[1]	P-value
Mean Zeta Potential (0.05% NaCl)	-16.3 mV (SD=2.29)		-2.56 mV (SD=1.27)	
Mean Zeta Potential (0.05% NaPP)	-26.8 mV (SD=4.73)	0.000	-7.11 mV (SD=0.490)	0.000
Mean Zeta Potential (0.05% NaMeta)	-26.0 mV (SD=2.64)	0.000	-10.4 mV (SD=0.698)	0.000

[1]Initial condition ionic strength of 1.76 mM

As with the previously discussed additives, grab samples with a second strain of *E. coli* (ATCC 11775) (n=3) were analyzed to determine if zeta potential differences existed between the two *E. coli* strains at a 0.05% concentration of sodium chloride. As previously discussed, at the initial condition, the mean zeta potential for *E. coli* (ATCC 25922) was determined to be significantly more negative than the mean zeta potential (-3.54 mV; SD=0.679) for *E. coli* (ATCC 11775) (P=0.000). The addition of 0.05% sodium chloride produced a less negative zeta potential (-2.56; SD=1.27) for *E. coli* (ATCC 11775), but this zeta potential difference was not significant (P=0.064). The results are shown in Figure 27.

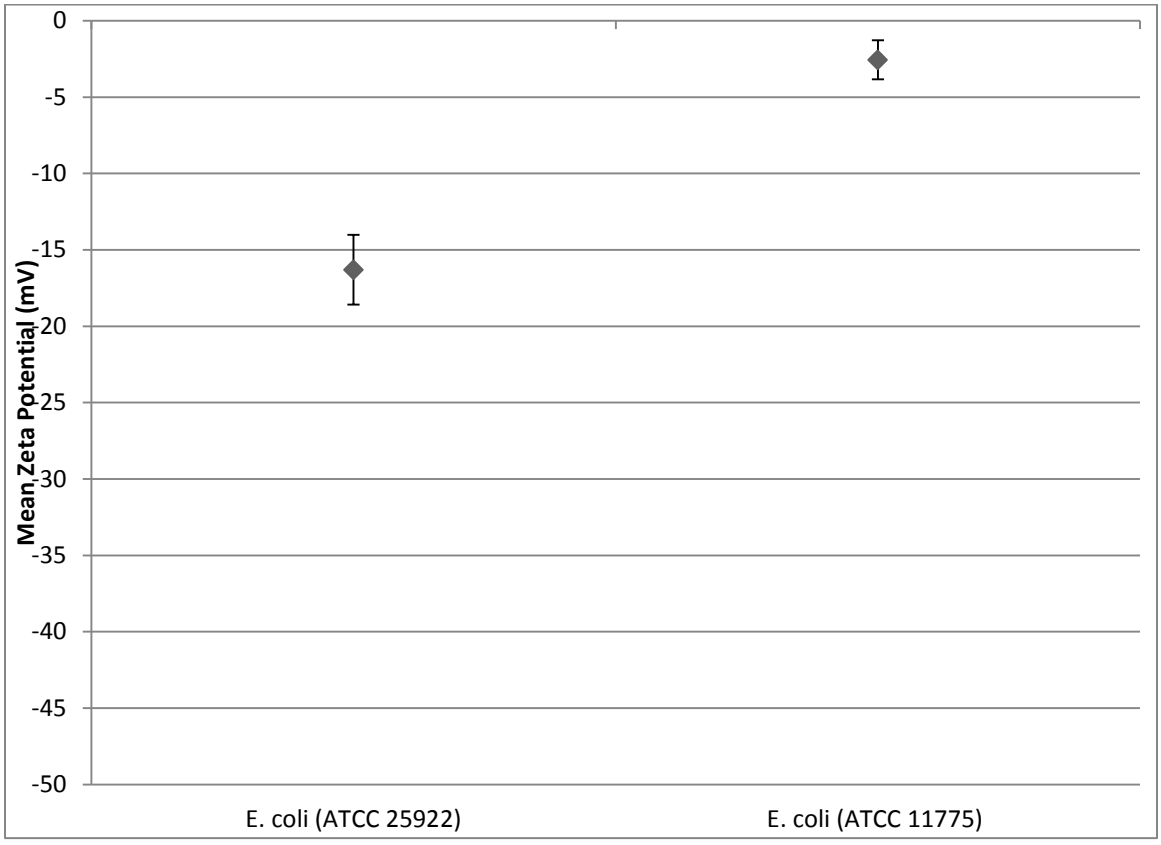

FIGURE 27: Comparison of zeta potential for two strains of *E. coli* in model water one with 0.05% sodium chloride

Next, one titration sample, with *E. coli* (ATCC 11775), was performed for each the constituents of the model water solution. Total organic carbon in solution (pH 6.20) was titrated to the model water sample in one mg/L increments from the initial concentration of 1 mg/L to 10 mg/L. Based upon the results of the titration sample, three grab samples of *E. coli* (ATCC 11775) in model water one were analyzed at a TOC concentration of 8 mg/L. At a TOC concentration of 8 mg/L, the mean zeta potential value of *E. coli* ATCC 11775) in model water one grab samples was determined as -7.51 mV (SD=1.19), while the titration sample had a zeta potential value of -8.96 (SD=0.420) (P=0.012). Additionally, as shown in Figure 28, the mean zeta potential at the initial

concentration of 1 mg/L TOC as C was -6.35 mV (SD=0.661). There was no change in sample pH as TOC was increased from 1 mg/L as C to 10 mg/L as C.

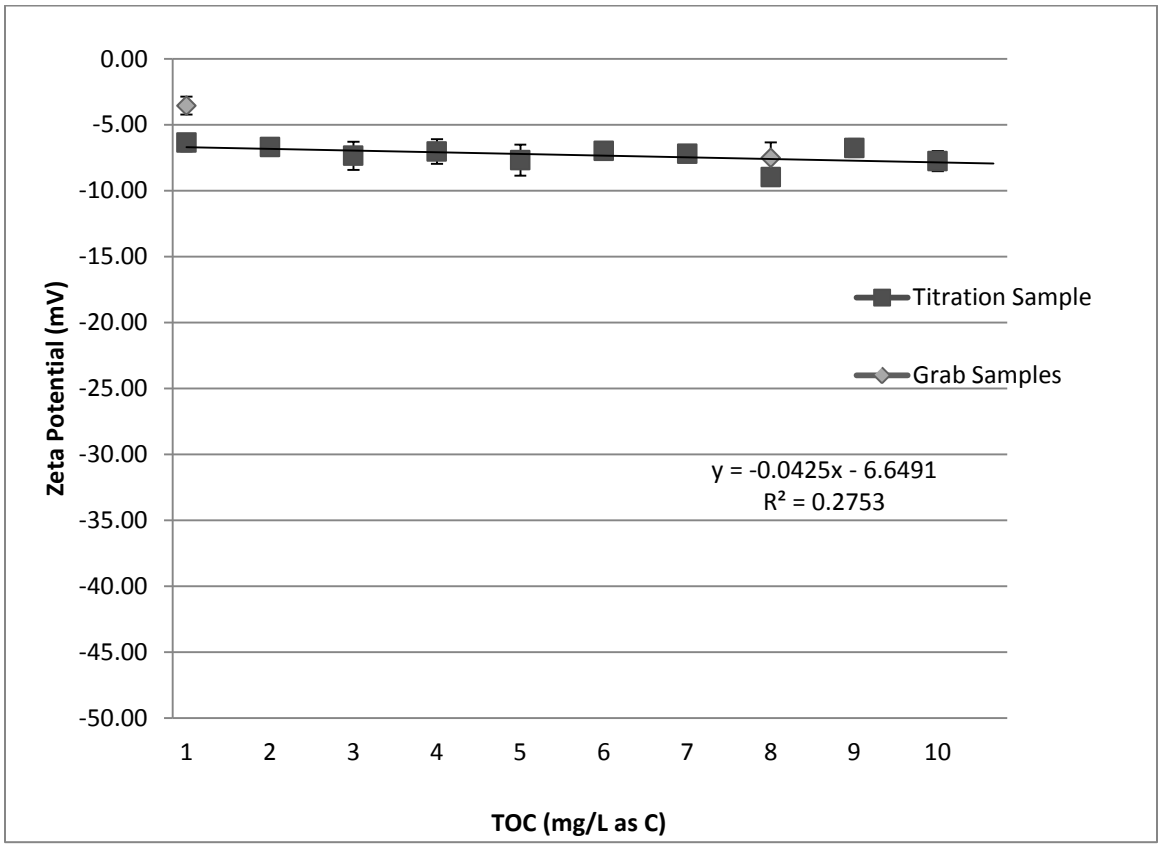

FIGURE 28: Zeta potential values for *E. coli* (ATCC 11775) particles in model water one at varied TOC concentrations

Similarly, one titration sample was analyzed to determine how increased concentrations of alkalinity, in the form of $NaHCO_3$, would affect the zeta potential values of *E. coli* (ATCC 11775). The stock $NaHCO_3$ solution that was used as a titrant had a pH of 8.93. After the titration had been completed, three grab samples of *E. coli* (ATCC 11775) in model water one were analyzed for zeta potential. The zeta potential at the initial condition was also similar for grab samples (-3.54 mV; SD=0.679) and for the

titration (-3.77 mV; SD=0.773) (P=0.680). As shown in Figure 29, the addition of more $NaHCO_3$ (up to 100 mg/L) had no significant effect on zeta potential values.

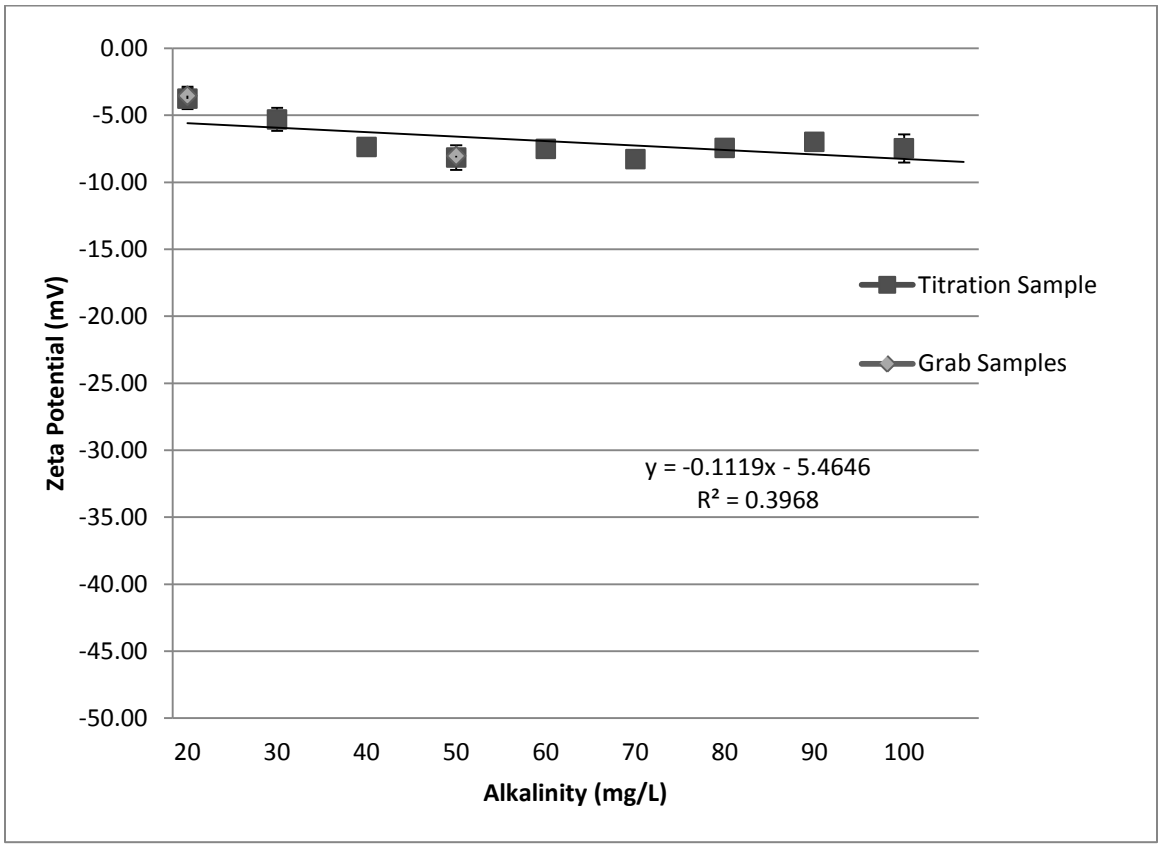

FIGURE 29: Zeta potential values for *E. coli* (ATCC 11775) particles in model water one at varied alkalinity concentrations

As was done with TOC and alkalinity, one titration sample with *E. coli* (ATCC 11775) in model water one was performed with hardness (as $CaCO_3$) concentration levels adjusted from 20 mg/L to 100 mg/L, and grab samples (n=3) were analyzed based upon titration results. At an initial hardness level of 20 mg/L, the mean zeta potential of grab samples was determined as -3.54 mV (SD=0.679), while the zeta potential value for the initial condition was determined as -6.80 mV (SD=0.641) for the titration sample (P=0.005). In general, as shown in Figure 30, as the concentration of hardness increased,

the zeta potential readings became less negative. During titration, the pH decreased from an initial pH of 3.81 to a final pH of 2.81 as the $CaCO_3$ solution (pH 1.40) was added. Grab samples with 100 mg/L concentration were also at a pH of 2.81. In addition, ionic strength changed from 1.60 mM to 5.59 mM during titration.

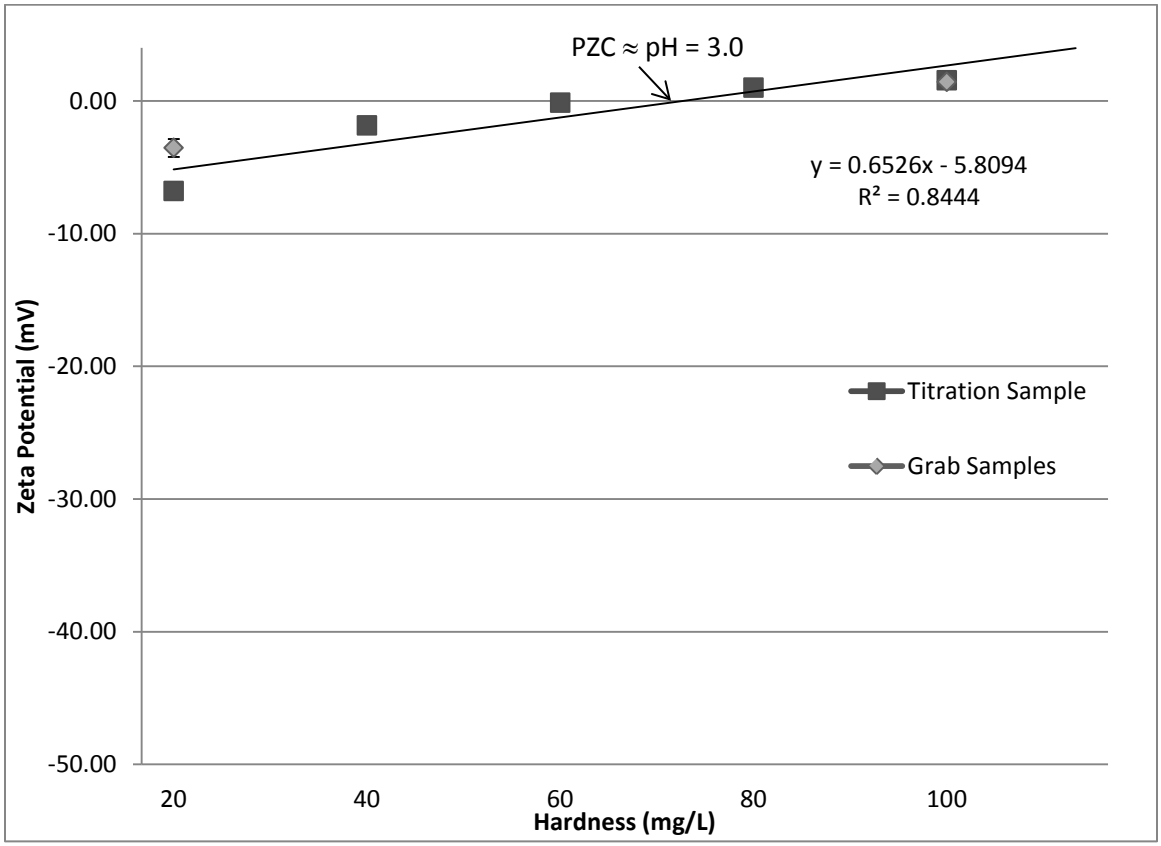

FIGURE 30: Zeta potential values for *E. coli* (ATCC 11775) particles in model water one at varied hardness concentrations

5.4.7 Model Water Variations

Lastly, both types of *E. coli* were analyzed, separately, as grab samples with two variations of the model water. One variation to the model water solution was the use of calcium chloride ($CaCl_2$) instead of calcium carbonate ($CaCO_3$) in HCl to add hardness to the model water solution. The use of $CaCl_2$ resulted in an increased pH (pH=7.05 to

7.85) of the model water solution from a pH of approximately 4 for $CaCO_3$ in HCl. For *E. coli* (ATCC 25922), the mean zeta potential for grab samples analyzed in suspension in this solution (n=3) was -19.9 mV (SD=2.11). While this zeta potential value was significantly different from the zeta potential value of *E. coli* (ATCC 25922) in model water one (P=0.000). When compared to model water one (adjusted to a pH of 7), the zeta potential values are similar (P=0.441). Similarly, when *E. coli* (ATCC 11775) was analyzed in this water, the mean zeta potential was more negative (-17.3 mV; SD=1.04) (P=0.000). Mean zeta potential results for each zeta potential *E. coli* strain are shown in Figure 31.

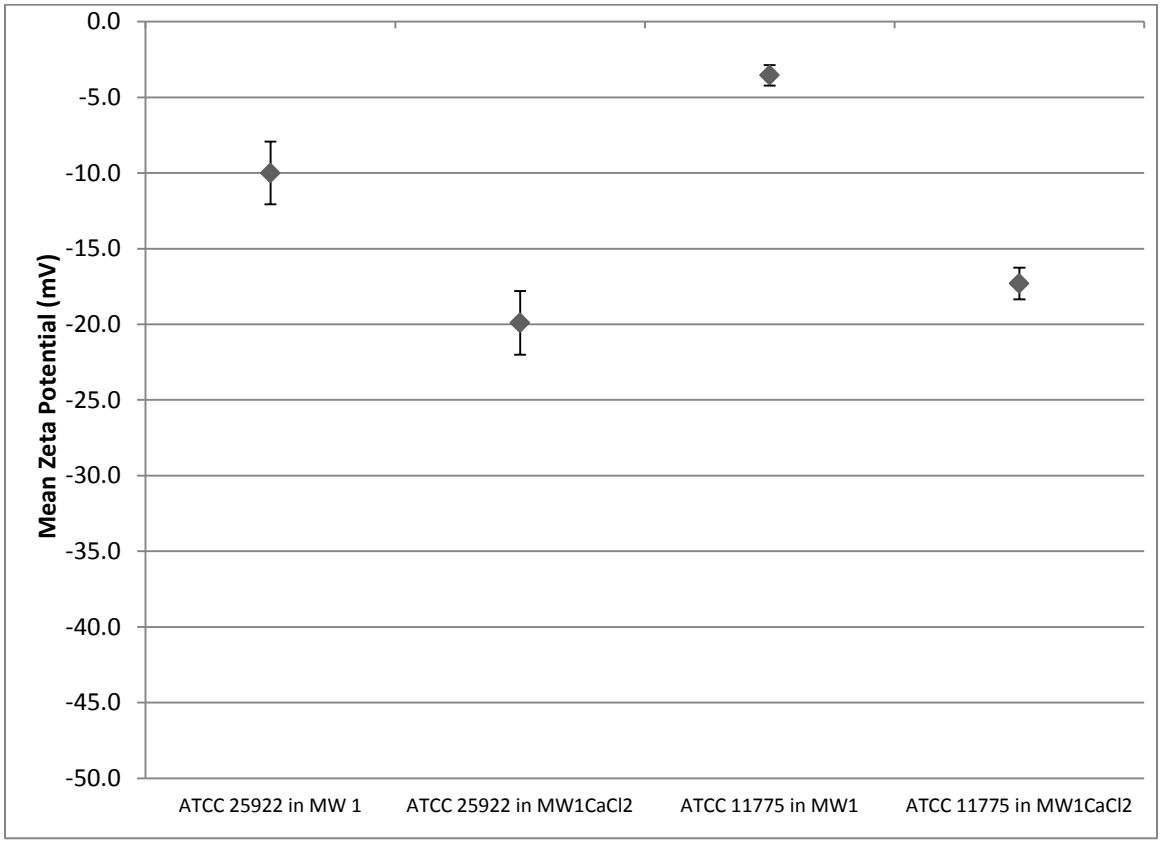

FIGURE 31: Zeta potential values for *E. coli* (ATCC 25922 and ATCC 11775) in model water one (MW1) and model water one made with $CaCl_2$ ($MW1CaCl_2$)

The second model water variation was a solution of model water one but with components (instant coffee and $CaCO_3$) added independently to deionized water and then filtered prior to being added to deionized water to make the model water solution. This was done in order to verify results with filtered components. For *E. coli* (ATCC 25922), the mean zeta potential of the grab samples (n=3) analyzed in suspension in this model water was -16.8 mV (SD=2.13). While the pH of this suspension was more basic (0.6 pH unit) than the model water suspension used previously, the mean zeta potential of these samples was significantly more negative than previous samples (P=0.000). When *E. coli* (ATCC 11775) was analyzed with the same model water variation, the pH was, again, more basic (0.4 pH unit) than the previous model water suspension. Similar to *E. coli* (ATCC 25922), the mean zeta potential (-9.19 mV; SD=0.601) of these grab samples (n=3) was significantly more negative than *E. coli* (ATCC 11775) in the initial model water suspension (P=0.000). However, *E. coli* (ATCC 11775) still produced less negative zeta potential readings than *E. coli* (ATCC 25922). Zeta potential data for each *E. coli* strain is shown in Figure 32.

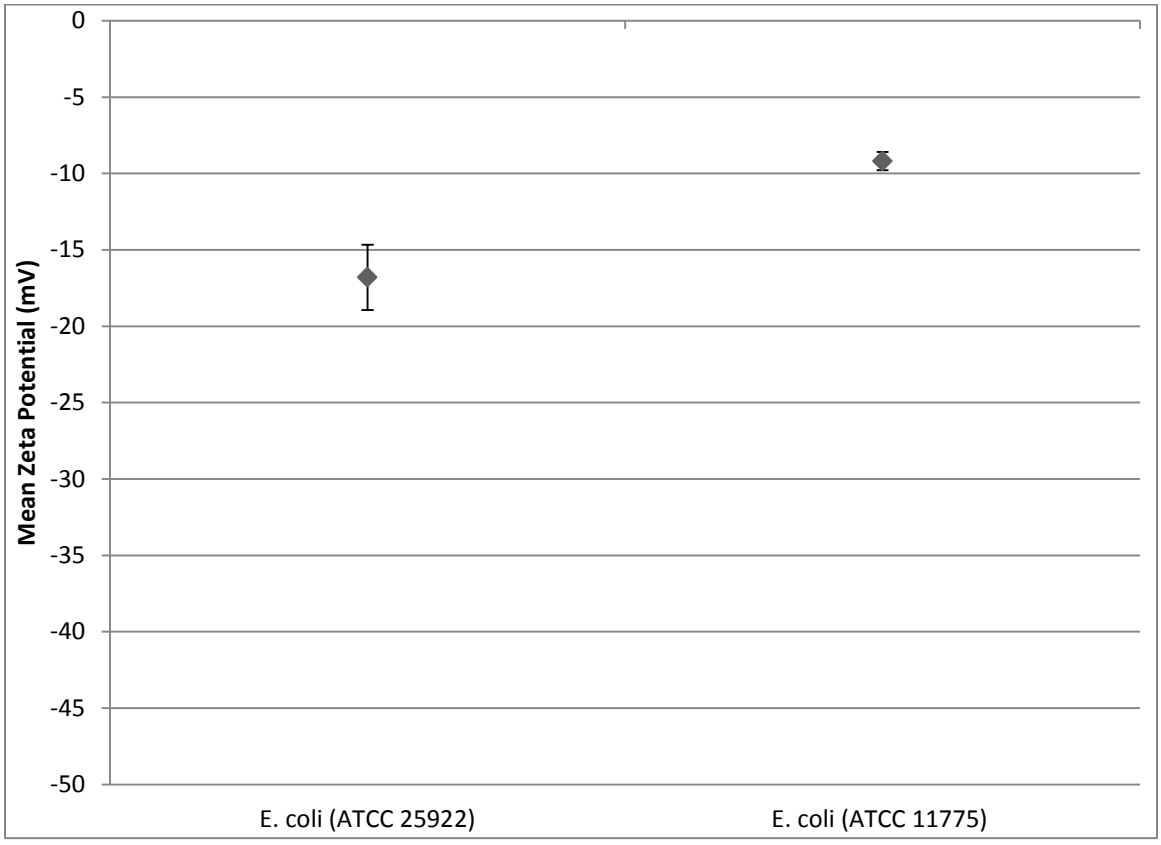

FIGURE 32: Zeta potential values for *E. coli* (ATCC 25922 and ATCC 11775) in model water one made with filtered components

To briefly recap this chapter, settling tests, alone, cannot provide the complete picture of the mechanisms of action for the two chemical dispersants used in this study. Although the use of a chemical dispersant with kaolin allowed for some significant improvements in the retention of the particles in suspension, the maximum percentage of particles that could be retained in suspension was 22%. Additionally, the use of sodium metasilicate provided better retention of kaolin particles at only two tested conditions, while the use of sodium polyphosphate produced significantly higher results more frequently. When silica, diatomaceous earth, and alumina were tested as the particles of interest in settling tests, there were no significant improvements to particle retention when either dispersant was used. This could, in part, be due to settling.

With regard to zeta potential analyses, sodium polyphosphate did make the zeta potential of kaolin significantly more negative with the most negative reading at a 0.05% sodium polyphosphate concentration. The use of sodium metasilicate was less straightforward as the zeta potential of kaolin was most negative at a sodium metasilicate concentration of 0.09%. Since sodium metasilicate increased sample pH to greater than 11, the influence of pH had to be considered, and kaolin samples analyzed at an adjusted pH of 11.5 had a significantly more negative mean zeta potential value than the mean zeta potential at any sodium metasilicate concentration tested. When each chemical dispersant was used with alumina as the particle in suspension, the results were again, mixed. The most negative zeta potential for alumina with sodium polyphosphate use occurred at a concentration of 0.04%, while the use of sodium metasilicate did not have a significant effect at any of the concentrations tested. The use of either sodium metasilicate or sodium polyphosphate did reduce (i.e., make more negative) the zeta potential of *E. coli* (ATCC 25922) but not *E. coli* (ATCC 11775). Therefore, the strain of *E. coli* can be a very important consideration since the zeta potential values of the two strains of *E. coli* were dissimilar and behaved differently under the same conditions. However, there are several factors that can affect the zeta potential of different strains of *E. coli*, including pH and ionic strength.

In addition to differences in response to sodium polyphosphate and sodium metasilicate, the two strains of *E. coli* used in this study responded differently to pH changes and ionic strength variations. As pH was increased from 6 to 11.5, the zeta potential of *E. coli* (ATCC 25922) became less negative. Alternatively, when *E. coli* (ATCC 11775) was tested at pH values of 6 and 11.5, the zeta potential was more

negative at the higher pH value. A difference between the two *E. coli* strains was also noted when ionic strength was varied. When ionic strength was increased, the zeta potential of *E. coli* (ATCC 25922) became more negative, but this did not occur when *E. coli* (ATCC 11775) was used. Figures 33 and 34 summarize the zeta potential results of *E. coli* (ATCC 25922) and *E. coli* (ATCC 11775) for pH, ionic strength, and chemical dispersants.

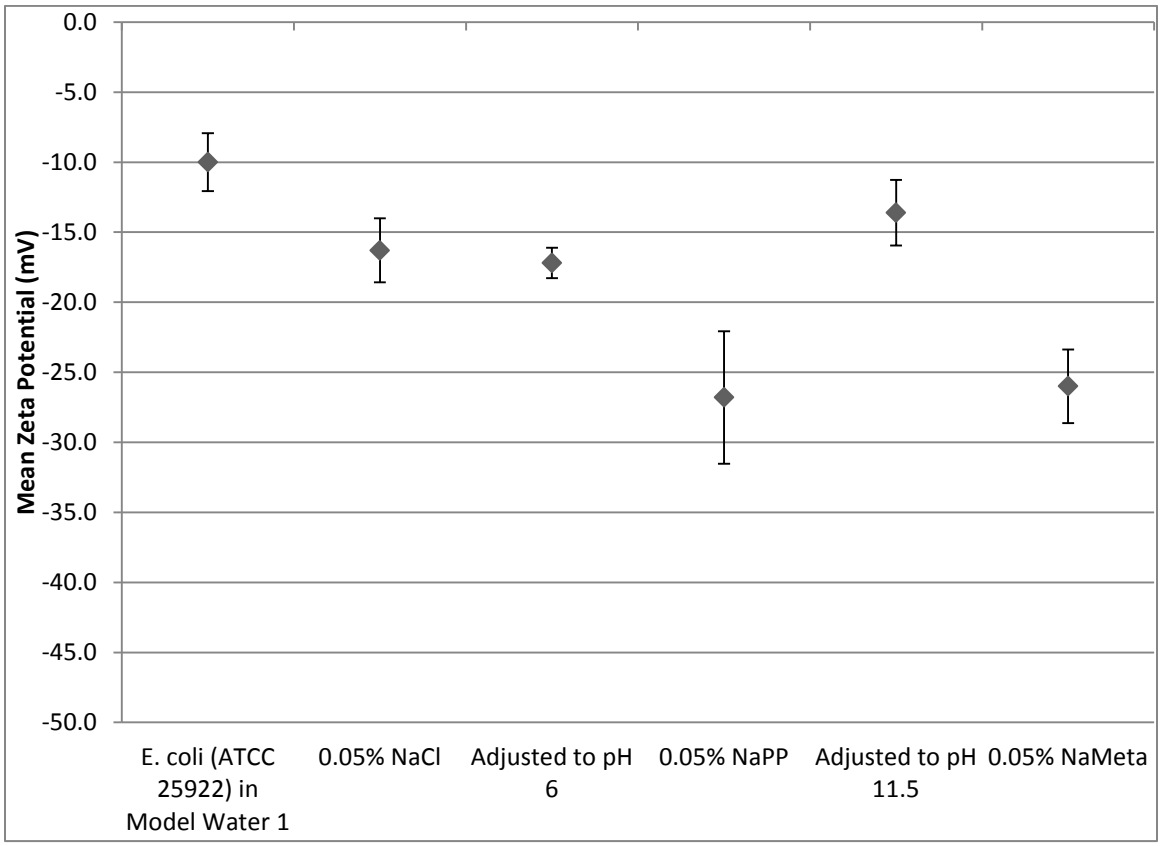

FIGURE 33: Summary of all additives in grab samples with *E. coli* (ATCC 25922)

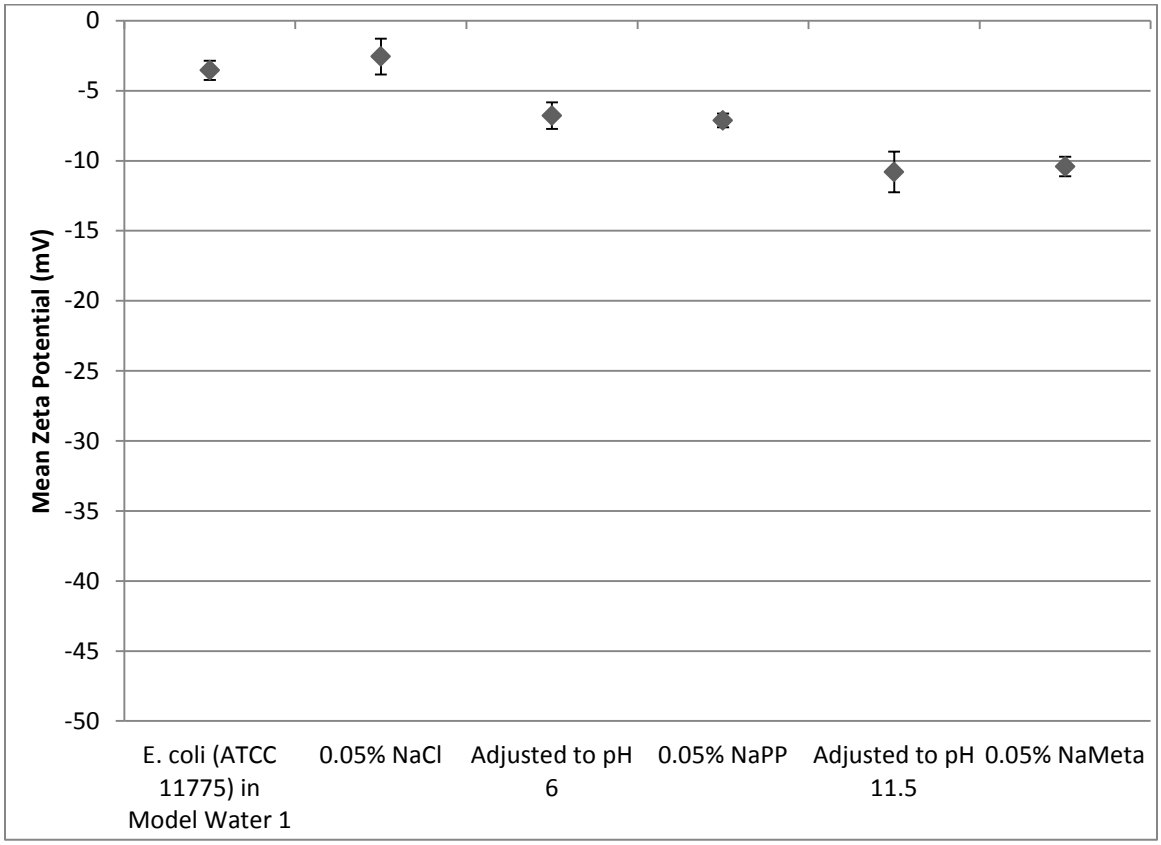

FIGURE 34: Summary of all additives in grab samples with *E. coli* (ATCC 11775)

5.5 Conclusions

The various physical properties of water samples can affect the surface charge of particles, including microbes, in suspension. While previous work with microbes and dispersants has shown some success, all microbes will not behave similarly in the presence of sodium polyphosphate or sodium metasilicate. The recognition that there can be significant differences in microbe responses to water sample conditions and sample additives will be important in the development of future microbial methods that include chemical dispersants.

When sodium polyphosphate was added to kaolin or *E. coli* (ATCC 25922) suspensions, this addition caused a change in the surface charge of the particles that was

not the result of pH increase. When sodium metasilicate was added to suspensions of kaolin or alumina, the change in surface charge was not a result of the sodium metasilicate addition but a result of the increased pH produced by sodium metasilicate. However, for *E. coli* (ATCC 25922), the mechanism of zeta potential reduction was a result of the sodium metasilicate addition and not just a function of pH increase. The most effective concentration of sodium polyphosphate was observed for kaolin and *E. coli* (ATCC 25922). For kaolin, the addition of sodium polyphosphate up to 0.05% resulted in significantly more negative zeta potential readings (P=0.000), but additional sodium polyphosphate (up to 0.10%) did not continue to produce more negative zeta potential values (P=0.621). A similar trend existed for *E. coli* (ATCC 25922). Up to a sodium polyphosphate concentration of 0.05%, the zeta potential became more negative (P=0.000), but from a concentration of 0.06%-0.10% sodium polyphosphate, zeta potential values did not get more negative (P=0.056). Comparable trends were not observed for sodium metasilicate.

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APPENDIX A: ULTRAFILTRATION PROCEDURE

Sample Preparation

1. Place the 10 liter sample in a cubitainer on a stir plate.
2. Add stir bar (magnetic octagonal 2”x3/8” or magnetic dumbbell-style 2”) and stir moderately.
3. Add UF sample amendment to sample.
4. Allow to stir for 5 minutes while the UF is blocked.
5. To block the UF, place pipets in blocking solution (1 liter of 0.1% NaPP) and pump UF blocking solution through the UF at 1700 mL/min (170 rpm) until only 100 mL of the blocking solution is left in the media bottle.
6. Remove inlet pipet but allow the blocking solution held-up in the UF system to flow into the media bottle through the outlet pipet.
7. Turn off the peristaltic pump.

Filtration

1. Place both the inlet and outlet pipets into the sample.
2. Filter sample at 170 - 220 rpm (1.7 - 2.2 L/min).
3. Record start time.
4. Measure the retentate flow rate by removing the outlet pipet from the sample container and placing in a graduated cylinder for 20 seconds.
5. Calculate flow rate. Flow rate should be between 1 L/min and 1.4 L/min.
6. Pour retentate back into sample container.
7. Measure the permeate flow rate by removing the waste tubing from the permeate carboy and placing in a graduated cylinder for 20 seconds.
8. Calculate flow rate. Flow rate should be between 0.6 L/min and 0.8 L/min. (Permeate flow rate should be around ½ of the retentate flow rate.)
9. Pour contents of graduated cylinder into sample container.
10. Screw clamp can be tightened or loosened to alter the two flow rates (increase pressure to increase permeate flow rate; decrease pressure to increase retentate flow rate). If screw clamp is adjusted, the two flow rates must be measured after any adjustment.
11. Record flow rates and system pressure after adjustments are made.
12. Allow sample to filter until there is approximately 150 mL of retentate volume left in the sample container.
13. Remove the inlet pipet from the sample container but leave the outlet pipet in place until all of the held-up sample volume is out of the UF system.
14. Turn off the peristaltic pump.
15. Pour the retentate into a 500 mL conical centrifuge tube and record the volume (approx. 250 mL).

Backflush

1. Remove all tubing from the UF.
2. Remove UF from assembly.
3. Cap the top and bottom ports on the UF.
4. Hold UF over a sink.
5. Leave top side port uncapped and uncap bottom side port.
6. Allow water to drain from UF.
7. Cap top port and top side port of UF. (Leave bottom and bottom side port uncapped.)
8. Place UF back in assembly.
9. Connect appropriate tubing to the UF inlet tubing and the bottom side port of the UF.
10. Place a 250 mL beaker under the bottom port of the UF to collect backwash.
11. Place inlet pipet into a measured 150 mL aliquot of the UF backflush solution.
12. Turn the pump on at 60 rpm until the backwash solution can be seen in the bottom of the UF.
13. Turn pump to 220 rpm.
14. Backwash the ultrafilter.
15. Turn off pump.
16. Allow dripping from the bottom port to slow or stop.
17. Uncap top side port (this will release pressure in the system).
18. Disconnect tubing from the bottom side port and reconnect the tubing to the top side port.
19. Cap the bottom side port.
20. Empty contents of the beaker into the 500 mL conical centrifuge tube containing the retentate.
21. Replace beaker under the bottom UF port.
22. Place the inlet pipet into a measured 50 mL aliquot of backwash solution.
23. Turn the pump on at 220 rpm.
24. Backwash the ultrafilter.
25. Turn off pump.
26. Allow dripping from the bottom port to stop.
27. Disconnect tubing from the top side port (this will release pressure in the system).
28. Empty contents of the beaker into the 500 mL conical centrifuge tube containing sample.
29. Record final sample volume in centrifuge tube. (approximately 500 mL)

Centrifugation

1. Vortex the 500 mL centrifuge tube for 30 seconds.
2. Divide the contents of the 500 mL centrifuge tube evenly into two 250 mL conical centrifuge tubes.

3. Rinse the 500 mL centrifuge tube with DI water and add rinsate to one of the 250 mL centrifuge tubes.
4. Repeat the rinse and add rinsate to the second 250 mL centrifuge tube.
5. Balance the 2 centrifuge tubes.
6. Place in the centrifuge.
7. Centrifuge at 1,200 x *g* for 30 minutes with the brake off.
8. Remove from centrifuge.
9. Aspirate each centrifuge tube to 35 mL above any packed pellet.
10. Resuspend each pellet with 30 seconds of trituration followed by a 30 second vortex.
11. Combine the contents the both centrifuge tubes.
12. Centrifuge at 1,200 x *g* for 15 minutes.
13. Remove from centrifuge.

APPENDIX B: JOURNAL PUBLICATION - IMPROVEMENTS IN *CRYPTOSPORIDIUM* RECOVERY AND VARIABILITY THROUGH MODIFICATIONS TO UNITED STATES ENVIRONMENTAL PROTECTION AGENCY METHOD 1623

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Improvements in *Cryptosporidium* recovery and variability through modifications to United States Environmental Protection Agency Method 1623

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Keywords
Cryptosporidium; Envirochek HV; *Giardia*; Method 1623; pleated capsule filter.

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Abstract

The use of United States Environmental Protection Agency (USEPA) Method 1623 for the detection of *Cryptosporidium* in water often results in low and variable rates of recovery. According to data disseminated in Method 1623, less than a quarter of tested surface water samples had *Cryptosporidium* recoveries of 60% or greater. This research explored various method modifications in order to produce a method with higher recovery and less variability. Once method modifications were implemented, recoveries of *C. parvum* in reagent water increased by greater than 20% to an average recovery of 76%. In surface water, a significantly higher ($P = 0.049$) mean recovery of 67% for *C. parvum* was achieved with the modified method. *Giardia* is simultaneously detected when using Method 1623. The method modifications significantly increased the recovery of *Giardia* in reagent water by 37% ($P = 0.0002$) while also allowing for a 17% higher recovery in surface water samples ($P = 0.21$).

Introduction

Cryptosporidium is a chlorine-resistant human parasite responsible for a drinking water disease outbreak of more than 400 000 individuals (MacKenzie *et al*. 1994). Monitoring of *Cryptosporidium* by drinking water utilities has increased in recent years in response to this outbreak threat, but detection methods for *Cryptosporidium* often suffer from low and variable recoveries (Connell *et al*. 2000; Simmons *et al*. 2001; Hu *et al*. 2004). There are currently two methods that are approved by the United States Environmental Protection Agency (USEPA) for the detection of *Cryptosporidium*, which are Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA (USEPA 1997) and Method 1623: *Giardia* and *Cryptosporidium* in Water by Filtration/IMS/FA (USEPA 1999). Both methods provided significant improvements over the USEPA Information Collection Rule method (Clancy *et al*. 2003). However, when either Method 1622 or 1623 is used, recoveries in source or finished water samples commonly average less than 45%, with relative standard deviations as high as 47% (Connell *et al*. 2000; Simmons *et al*. 2001; Hu *et al*. 2004).

As mentioned earlier, the analysis of water samples for *Cryptosporidium* can pose a challenge. For example, when surface water samples are examined, the sample matrices can be highly variable from one location to another and even within the same location based upon different environmental factors (DiGiorgio *et al*. 2002). In addition, as USEPA Method 1623 entails the filtration of a water sample to capture and concentrate oocysts, separation of the oocysts from debris, and microscopic identification, if interfering components are not effectively removed, then these can cause problems with organism recovery and/or slide examination. Moreover, the specific step or steps of the method leading to low recoveries have not been clearly identified (Hu *et al*. 2004). Therefore, these problematic areas might involve the physical equipment (e.g. filters or centrifuges), reagents (e.g. elution buffers or stains) used or the way in which the analyst performs the method (e.g. sample transfer or sample separation). Another factor that might play a role in low or variable recoveries is the difficulty of accurate microscopic identification of the parasite. Slide examination in Method 1623 requires some expertise, and confirmation of an oocyst is often difficult to achieve (LeChevallier *et al*. 1995; Ware *et al*. 2003). Even with confirmatory steps required, false positives and false negatives are still possible (Sturbaum 2003). Nevertheless, reliable quantitation of *Cryptosporidium* is important. For example, in order to accurately evaluate water treatment efficiency and effectiveness, and to assess other health-based targets, such

as log removal of pathogens, the importance of an accurate assessment of the number of oocysts in water samples is crucial.

USEPA Method 1623 is a performance-based method, meaning that the published method can be modified by a laboratory provided that the required quality control criteria are met (Clancy *et al.* 2003). Previous researchers have made attempts to improve the performance of Method 1623 (or 1622) by changing filters, immunomagnetic separation (IMS) procedures and stains in attempts to improve recovery and decrease variability of results. This research will be summarized later.

Filters have been the focus of multiple studies. According to Hu *et al.* (2004), a significant decline in *Cryptosporidium* recovery occurs during the filtration step. When researchers compared Envirochek and Envirochek HV capsule filters (Pall Corporation, Ann Arbor, MT, USA), the HV filter performed better in some conditions; while the standard Envirochek performed better in other conditions (DiGiorgio *et al.* 2002; Quintero-Betancourt 2003). In alternate studies, the performance of hollow-fibre ultrafilters has been tested. One such study compared the use of the standard Envirochek filter to a hollow-fibre ultrafilter (Hemoflow F80A, Fresenius Medical Care, Lexington, MA, USA) for processing both reagent and surface water samples with Method 1622 (Simmons *et al.* 2001).

Simmons *et al.* (2001) reported that there was no significant difference between the capsule filters and ultrafilters when processing reagent water; but when surface water was analysed, the ultrafilter performed significantly better. The authors of a second study that also used the same type of hollow-fibre ultrafilters reported recoveries of 83% with ultrafiltration vs. 46% for Envirochek HV filters for *C. parvum* from seeded tap water samples (Hill *et al.* 2009).

Researchers have also examined IMS dissociation procedures. Ware *et al.* (2003) used heat dissociation compared with acid dissociation followed by heat and reported that a heat-only dissociation increased recovery 30% in seeded reagent water samples while also allowing for 44% higher 4′,6-diamidino-2-plenylindole (DAPI) confirmation rate. In surface water samples, recovery improved by 41%, and the DAPI confirmation rate improved by 25% (Ware *et al.* 2003).

A study by Quintero-Betancourt (2003) compared EasyStain to Aqua-Glo and found that although each was reported to be equally effective in the detection of *Cryptosporidium*, EasyStain was preferred for the lower levels of background fluorescence and non-specific binding.

Therefore, based upon the findings of previous researchers and the experiences of the authors, a research plan was developed in order to examine whether augmentations to USEPA Method 1623 could be achieved through guided method modifications. The primary objective of these modifications was to create a more efficient method for the analysis of *Cryptosporidium* in environmental water samples. The current study explored various method modifications to improve *Cryptosporidium* recovery efficiencies and method performance. These method modifications included a comparison of fluorescent antibody (FA) staining kits, methanol fixation, filters, sample resuspension techniques and elution buffers.

Materials and methods

The December 2005 version of EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA (USEPA 2005) was used as the baseline for all sample analyses. The procedures outlined in the method were followed with the exceptions of the items intentionally modified for the purposes of this study. All slide examinations and organism determinations were made in a manner consistent with those described in Method 1623 (USEPA 2005).

Oocysts and water matrices

Oocyst stocks were obtained from three sources. Viable, flow-cytometry sorted stocks were obtained from the Wisconsin State Lab of Hygiene (WSLH). These spiking suspensions were heat-inactivated at 80°C for 30 min prior to sample seeding. Additionally, preparations from Waterborne, Inc. (New Orleans, LA, USA) were obtained in one vial containing 10^6 oocysts. These preparations were enumerated by haemocytometer counts and diluted to appropriate seeding concentrations for slide and staining comparisons. Lastly, EasySeed (BTF, Sydney, Australia) gamma irradiated spiking suspensions were used for filter comparisons.

For the staining and methanol fixation comparison studies, serial dilutions of the Waterborne spiking suspension (New Orleans, LA, USA) were prepared. Aliquots (50 µL) of the diluted spiking suspension were placed directly on slides as opposed to performing the entire method prior to staining. This allowed for a faster determination of which stain and fixation produced better recoveries while eliminating variability from other method procedures.

For samples processed though the entire 1623 procedure, the water was obtained from two locations. Deionized water was used for method blanks and reagent water samples. Raw or untreated water samples were collected from a raw water influent tap at the Franklin Water Treatment plant (Charlotte, NC, USA). The source water came from Mountain Island Lake, with an average total organic carbon (TOC) concentration of less than 2.0 mg/L and turbidities that ranged from 2.5 to 4.6 nephelometric turbidity units. Source water samples were used for matrix and matrix spike samples. Matrix samples were unseeded source water samples that were analysed to determine whether or not oocysts and/or cysts were naturally present in the untreated water.

Filtration

Samples were filtered, and the filters were eluted as specified in Method 1623. Two pleated capsule filters, the standard Envirochek and the Envirochek HV, were compared in this study. Three 10 L raw water samples, seeded with EasySeed, were filtered through each type of capsule filter.

Elution

The Laureth-12-based elution buffer specified in Method 1623 was used for elution of all samples except those used for elution buffer comparisons. For the elution buffer modifications, the standard elution buffer was compared with two different modified elution buffer solutions: the first with an addition of 0.1% Tween-80 and the second with an addition of 0.01% sodium polyphosphate. The elution buffer comparison between the standard elution buffer and the Tween-80 addition involved the analysis of five seeded reagent water samples eluted with each buffer type. For the comparison of the standard elution buffer to the elution buffer with sodium polyphosphate, three spiked raw water samples were analysed using each buffer type.

Sample resuspension

Following elution, samples were further concentrated by centrifugation consistent with Method 1623. After centrifugation and aspiration, the liquid in the centrifuge tube was then either vortexed for 30 s or the sample was triturated (by repeatedly pipetting a 5-mL volume into and out of a 10-mL serological pipette) for 30 s prior to transfer to a Leighton tube. Five spiked reagent water samples were vortexed prior to sample transfer, while five additional samples were triturated prior to sample transfer.

IMS

IMS for all samples followed the procedure in Method 1623 with the use of the Dynal Dynabeads-GC Combo kit (Invitrogen Dynal, Oslo, Norway). One slight modification included the volume of acid added for dissociation. Method 1623 specifies 50-μL acid volumes, but this study used 100 μL volumes so that one-half of the acid dissociation could be used for quantitative real-time polymerase chain reaction (qPCR) analysis (data not shown). This variation was previously used by Quintero-Betancourt (2003) with no adverse effects.

Staining

In order to do another optimization comparison, five samples dried on slides were fixed with methanol, while five samples were not. Methanol fixation involved placing one drop of absolute methanol on the dried sample and waiting approximately 5 min for the methanol to evaporate. Additionally, six slides were stained with EasyStain (BTF), and six slides were stained with Aqua-Glo (Waterborne). Procedures recommended by the manufacturer were followed. Slides used were either Dynal or Waterborne.

Statistics

Recovery efficiency for each sample was calculated by dividing the number of recovered organisms by the number of organisms seeded into the 10-L sample. The resulting fraction was then multiplied by 100 to obtain a percent recovery. Statistical comparisons were made using analysis of variance (ANOVA), with statistical significance set at 0.05 (Minitab 15, Minitab, Inc., State College, PA, USA).

Discussion of results

Comparison of staining procedures

The first set of experiments involved placing aliquots of an oocyst suspension on slides, fixing them with methanol, and staining the sample with EasyStain or Aqua-Glo. The mean recovery for *Cryptosporidium* using EasyStain was 94% [standard deviation (SD = 12%)], while slides stained with Aqua-Glo produced a mean recovery of 82% (SD = 24%). The difference was not statistically significant ($P = 0.410$). For *Giardia*, the mean recovery with EasyStain was 97% (SD = 11%) and 103% (SD = 11%) for Aqua-Glo. As with *Cryptosporidium*, the difference for *Giardia* was not statistically significant ($P = 0.494$).

Following elimination of methanol fixation, slides stained with EasyStain yielded a percent recovery of 102% based on haemocytometer counts. However, when Aqua-Glo was used without methanol fixation, the percent recovery fell dramatically to only 28%. This difference was determined to be statistically significant ($P = 0.000$). Figure 1 shows a summary of

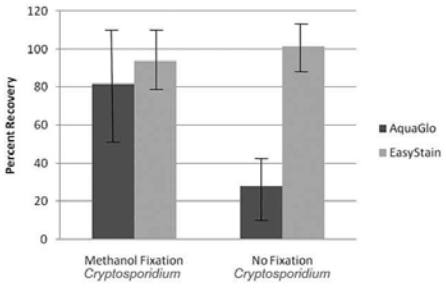

Fig. 1. Summary of results for positive control slide comparisons.

this information, with SD indicated by error bars. When slides were examined for *Giardia*, there was a 100% recovery with EasyStain, while Aqua-Glo recovery was 78% ($P = 0.081$).

EasyStain provided better percent recoveries whether methanol fixation was used or not. These findings are consistent with those of Quintero-Betancourt (2003) who reported that although the stains were equally effective, EasyStain provided improved enumeration and differentiation. The samples stained with EasyStain were easier to examine because there was less background fluorescence.

When EasyStain was used, methanol fixation did not significantly change recoveries, but the DAPI staining virtually disappeared without it. This was an important issue as DAPI staining patterns aid the analyst in making a positive identification. Without the DAPI staining, confirmatory identification was much more difficult. Methanol fixation was thus determined to be beneficial and used throughout the rest of this study.

Comparison of filters

For seeded surface water samples, the mean recovery of *Cryptosporidium* using the standard Envirochek filters was 29% (SD = 15%). The Envirochek HV filters produced a higher mean recovery of 40% (SD = 14%). However, this difference was not statistically significant ($P = 0.407$). The Envirochek HV also produced better results with regard to percent relative SD. Again, the results for *Giardia* were similar to those for *Cryptosporidium*. For *Giardia*, the standard Envirochek filter yielded a 51% recovery compared with a 64% recovery for the Envirochek HV filter ($P = 0.519$). The samples analysed with the standard Envirochek had a SD of 28% compared with 16% for the Envirochek HV. Table 1 includes a summary of the mean percent recoveries of *Cryptosporidium* and *Giardia*, and SDs for source water samples using each type of filter. Because better mean percent recoveries and a lower relative SD were achieved using the Envirochek HV filters, these filters were used for the remainder of the study.

Comparison of sample resuspension methods

The next modification involved the comparison of vortexing to trituration of the sample prior to transfer from the centrifuge tube to a Leighton tube. For this modification, seeded reagent water samples were used. For the vortexed samples, a mean percent recovery of 72% (SD = 15%) was achieved, while the mean percent recovery for samples subjected to trituration was 76% (SD = 15%). The difference in mean percent recovery between sample resuspension methods was not found to be significant ($P = 0.735$). When the recovery for *Giardia* was considered, vortexed samples had a mean recovery of 86% (SD = 11%), while samples subjected to trituration had a mean recovery of 78% (SD = 6%). As with *Cryptosporidium*, the difference in recovery of *Giardia* for each resuspension method was not statistically significant ($P = 0.199$). There was no significant difference between the methods, but samples that were resuspended using trituration did have better *Cryptosporidium* recovery (and the relative SD was not adversely affected). So, trituration replaced vortexing. While these two techniques showed similar performance for reagent water, it is possible that they could differ with a source water matrix. Table 1 includes a summary of these results.

Table 1 Summary of the mean percent recoveries of *Cryptosporidium* and *Giardia* for method modifications relating to filtration, sample resuspension and elution

Modification	*Cryptosporidium*, % (SD)	*Giardia*, % (SD)
Filtration		
Envirochek	29 (15%)	51 (28%)
Envirochek HV	40 (14%)	64 (16%)
Sample Resuspension		
Vortex	72 (15%)	86 (11%)
Triturate	76 (15%)	78 (6%)
Elution (1)		
Standard Method 1623[a]	76 (15%)	78 (6%)
Tween-80 Added[a]	71 (11%)	86 (6%)
Elution (2)		
Standard Method 1623[b]	67 (16%)	21 (15%)
Sodium Polyphosphate Added[b]	65 (11%)	25 (12%)

[a]Modification performed with reagent water samples.
[b]Modification performed with surface water samples.
SD, standard deviation.

Comparison of elution buffers

The fourth modification was alteration of the elution buffer. Two different elution buffer modifications were used with reagent water samples. For the first elution buffer modification (the addition of 0.10% Tween-80), the mean percent recovery for the five samples analysed using the standard elution buffer was determined to be 76% (SD = 15%), while the use of the modified elution buffer produced a mean percent recovery of 71% (SD = 11%). The difference in the mean percent recoveries between the two elution buffers was not statistically significant ($P = 0.566$). These results are further shown in Table 1. As for *Giardia* recovery, samples processed with the standard elution buffer had a mean recovery of 78% (SD = 6%), while samples processed with the modified elution buffer had a mean recovery of 86% (SD = 6%). The difference in *Giardia* recoveries was not statistically significant ($P = 0.082$). While these two elution buffers showed similar performance for reagent water, it is possible that they could differ with a source water matrix.

The second elution buffer modification (i.e. the addition of 0.01% sodium polyphosphate) was tested in raw surface

water samples and also achieved mixed results. Use of the standard elution buffer produced a mean percent recovery of 67% (SD = 16%), while use of the elution buffer with sodium polyphosphate produced a mean percent recovery of 65% (SD = 11%). While a small decrease in the mean percent recovery was found when sodium polyphosphate was added, this decrease was not significant ($P = 0.865$), although the variability was lower for the second elution buffer formulation. These results are summarized in Table 1.

When *Giardia* recovery was considered, the use of the standard elution buffer produced a mean recovery of 21% (SD = 15%), and the modified elution buffer allowed for a recovery of 25% (SD = 12%). As with *Cryptosporidium*, the difference in *Giardia* recovery was not statistically significant ($P = 0.313$).

To summarize the elution buffer results, the standard elution buffer described in Method 1623 produced a better mean recovery for *Cryptosporidium* than either modified elution buffer with Tween-80 or sodium polyphosphate added, but the variability was slightly greater in samples processed with the standard elution buffer. Because neither modification of the standard elution buffer improved recovery efficiency, the standard elution buffer was retained and used for future sample processing.

The final method modifications that were implemented or maintained for use included: EasyStain, methanol fixation, Envirochek HV filters and standard elution buffer. Use of the optimized method has produced a method with a mean percent recovery of 76% for *Cryptosporidium* and an SD of 15% in reagent water. In source water, the optimized method has produced a mean percent recovery of 67% for *Cryptosporidium*, with an SD of 16%.

Without the use of the method modifications, the average *Cryptosporidium* recovery that could be achieved in reagent water was 55%. However, when source water samples were analysed, the mean *Cryptosporidium* recovery was less than 30%. Therefore, the optimized method has allowed for a greater than 20% increase in mean recovery of *Cryptosporidium* from reagent water and a statistically significant increase ($P = 0.049$) in recovery from raw surface water. Results for the modified method versus the unmodified method are summarized in Figs 2 and 3.

Because Method 1623 allows for the simultaneous detection of both *Cryptosporidium* and *Giardia*, the desired goal of this study was to improve *Cryptosporidium* recovery while not negatively impacting the recovery of *Giardia*. To this end, the modified method has resulted in a significantly better mean recovery of *Giardia* in reagent water samples. The unmodified method produced a *Giardia* recovery of 41% in reagent water samples, while the modified method has produced a mean recovery of 78% ($P = 0.0002$). As for source water samples, the recovery of *Giardia* has improved from 20 to 37% ($P = 0.21$). Therefore, the study has produced a method that allows for better recoveries of both *Cryptosporidium* and *Giardia* in both reagent and source water samples.

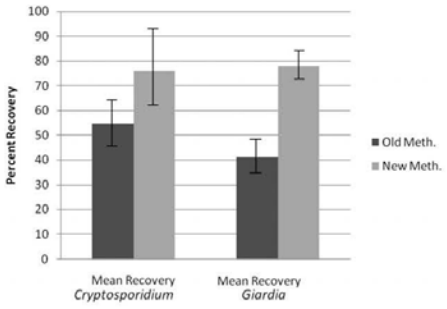

Fig. 2. Summary for the modified method for *Cryptosporidium* and *Giardia* in reagent water samples.

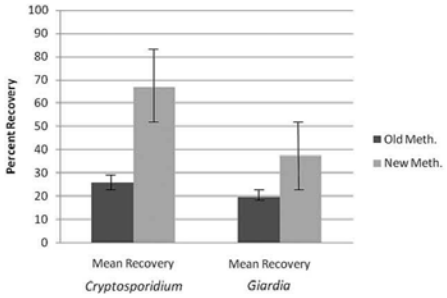

Fig. 3. Summary for the modified method for *Cryptosporidium* and *Giardia* in source water samples.

Conclusions

(1) Although not required by Method 1623, when methanol fixation is not performed, there could be a significant difference in recovery that is dependent upon the type of immunofluorescent assay stain used.

(2) With the exception of methanol fixation, no other single modification significantly improved method recovery, but the overall modifications offered significant recovery improvement of *Cryptosporidium* in surface water and *Giardia* in reagent water.

(3) Incorporated modifications that allowed for higher oocyst recoveries also enhanced recovery of *Giardia*.

(4) This method optimization research improved reagent

water recoveries of *Cryptosporidium* by 21% and significantly improved recovery of *Giardia* by 37%.
(5) Source water recoveries of *Cryptosporidium* were significantly improved (41%), and source water recoveries of *Giardia* improved by 17%.

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APPENDIX C: COMPARISON OF HOLLOW-FIBER ULTRAFILTERS WITH PLEATED CAPSULE FILTERS FOR SURFACE AND TAP WATER SAMPLES USING U.S. EPA METHOD 1623

Technical Note

Comparison of Hollow-Fiber Ultrafilters with Pleated Capsule Filters for Surface and Tap Water Samples Using U.S. EPA Method 1623

Gina H. Kimble[1]; James E. Amburgey[2]; and Vincent R. Hill, P.E.[3]

Abstract: The EPA method 1623 is designed specifically for the detection of *Cryptosporidium* and *Giardia*, but the method has some issues with low and variable recoveries. Ultrafiltration has been used effectively for microorganism recovery from water samples but is not approved by the EPA. To determine the efficacy of using ultrafiltration, 10-L tap water and surface water samples were seeded with *Cryptosporidium* and *Giardia* and concentrated with either a pleated capsule filter or a hollow-fiber ultrafilter. For *Cryptosporidium*, oocyst recovery in tap water was significantly higher for ultrafiltration (68%) versus the capsule filter (37%); ultrafiltration recovered 65% of oocysts in surface water versus 61% for the capsule filter. However, *Giardia* cyst recovery was mixed. In tap water, the capsule filter produced a significantly better recovery (85%) of *Giardia* compared with ultrafiltration (63%), but the surface water ultrafiltration recovery (81%) was significantly better than the capsule filter recovery (40%). Overall, ultrafiltration recoveries were equal to or better for *Cryptosporidium*, but recoveries of *Giardia* were varied depending on the filter used and the type of water analyzed. **DOI: 10.1061/(ASCE)EE.1943-7870.0000545.** © *2012 American Society of Civil Engineers.*

CE Database subject headings: Drinking water; Surface water; Microbes; Sampling; Filters; Laboratory tests; Methodology.

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Introduction

Cryptosporidium and *Giardia* are protozoa that can be present in surface water and can remain after conventional drinking water treatment processes, including disinfection with chlorine. Outbreaks of *Cryptosporidium* associated with drinking water have been documented in the United States since the early 1980s (Solo-Gabriele and Neumeister 1996). In 1993, an outbreak of *Cryptosporidium* in the municipal drinking water supply made more than 400,000 people ill and killed approximately 100 individuals in Milwaukee (MacKenzie et al. 1994). For healthy individuals, this illness can produce gastrointestinal symptoms, because the immune system works to fight off the infection. However, cryptosporidiosis can be fatal in immunocompromised individuals whose immune systems cannot fight the infection. Therefore, it is imperative that reliable methods be available for analysis of drinking water protect the public from waterborne *Cryptosporidium*.

When the Milwaukee outbreak occurred in 1993, *Cryptosporidium* testing requirements did not exist for public water suppliers. However, with the promulgation of the Long Term 2 Enhanced Surface Water Treatment Rule (LT2 rule), surface water monitoring for *Cryptosporidium* became a requirement for water suppliers with a population of 10,000 or greater (U.S. EPA 2006). Public water suppliers that were required to sample under the LT2 rule had to perform monthly monitoring for 24 months.

Neither *Cryptosporidium* nor *Giardia* can be detected with fecal indicator-organism tests that are common in the water industry, nor has any correlation been shown between the detection of fecal indicator organisms and either *Cryptosporidium* or *Giardia* (Sobsey 1989). To detect these organisms, a specific method of analysis must be used. Currently, the approved method for the detection and analysis of these two microorganisms is EPA method 1623 (U.S. EPA 1999). There can be problems with recovery and variability when method 1623 is used (DiGiorgio et al. 2002; Hu et al. 2004). Thus, researchers have tried different modifications to improve the performance of method 1623. One method alteration that has been tested involves the type of filter used. The most commonly used filter in method 1623 is a pleated capsule filter, the Envirochek HV (Pall Corporation, Ann Arbor, MI), but some researchers have tested the efficacy of hollow-fiber ultrafilters (Hill et al. 2005, 2009; Morales-Morales et al. 2003; Simmons et al. 2001).

One study that compared the standard Envirochek filter with a hollow-fiber ultrafilter (Fresenius Hemoflow F80A, Fresenius Medical Care, Lexington, MA) for processing both reagent and surface water samples with EPA method 1622 (*Cryptosporidium* only) reported no significant difference between the filters when processing reagent water, but the ultrafilter performed significantly better when surface water was analyzed (Simmons et al. 2001). A second study using the same type of hollow-fiber ultrafilter reported recoveries of greater than 80% for *C. parvum* from seeded tap water samples (Hill et al. 2005). A more recent study that compared

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ultrafiltration (UF) with method 1623 found that UF produced significantly better recoveries of *Cryptosporidium*, but not *Giardia*, in tap water samples (Hill et al. 2009).

The objective of this study was to determine the recovery of *Cryptosporidium* and *Giardia* from both surface water and tap water using the Envirochek HV (Pall Corporation, Ann Arbor, MI) and Fresenius Optiflux 200NR filters (Fresenius Medical Care, Lexington, MA). Fresenius Optiflux 200NR filters are high-flux, hollow-fiber, polysulfone dialysis filters with a surface area of 2.0 m^2, a fiber inner diameter of 200 μm, and a molecular weight cutoff of approximately 30 kDa; these filters were operated in the cross-flow mode for this study. Hollow-fiber ultrafilters have not gone through a Tier 2 validation study for approval by the EPA for use with method 1623. However, UF can be validated under the EPA performance-based measurement system through completion of a Tier 1 validation study as long as acceptance criteria are met.

Methods

Ten-liter tap water ($n = 5$ per filter type) and source water ($n = 5$ per filter type) samples were obtained from the Franklin Water Treatment Plant (Charlotte, NC), whose surface water comes from Mountain Island Lake. Turbidity for the tap water is generally in the range of 0.1–0.3 nephelometric turbidity units (NTUs), whereas the source water generally has turbidity values of less than 5 NTUs. Total organic carbon (TOC) averages approximately 1 mg/L in the tap water and is less than 2 mg/L in the source water. Flow-cytometry sorted oocyst/cyst suspensions obtained from the Wisconsin State Laboratory of Hygiene (Madison, WI) were seeded into the water samples. Different sets of spiking suspensions were used during the sample analyses, but each 15-mL suspension contained a specified number of cysts/oocysts in the range of 149–172, according to the associated specification sheets that were supplied with the suspensions.

Five tap water samples were processed with the pleated capsule filter, and five tap water samples were processed with the hollow-fiber ultrafilter. Similarly, five surface water samples were processed with each filter type. In addition, unseeded control samples of each water type were also processed, and neither *Cryptosporidium* nor *Giardia* was detected in any of the control samples.

Filtration of samples through the pleated capsule filters was performed with a diaphragm pump (Shurflo, Cypress, CA), and UF was performed with a peristaltic pump (Cole Parmer Instrument Company, Vernon Hills, IL). After filtration, samples were processed using method 1623 techniques, with the exception of the elution procedure. The pleated capsule filters were eluted as specified in method 1623, but the ultrafilters were backwashed according to the procedure used by Hill et al. (2005), with a solution that contained 0.2% Tween 80, 0.01% sodium polyphosphate, and 0.01% Antifoam A. Following concentration by centrifugation at 1,500 × g and aspiration of the supernatant, each sample was further processed using immunomagnetic separation (Dynabeads GC-Combo, Invitrogen Dynal, Oslo, Norway), and slides were stained (EasyStain, BTF, Sydney, Australia) according to the procedures in method 1623.

The two filtration methods are similar in the amount of time required for completion. The pleated capsule filtration required approximately 10 min, and the time required for the UF procedure was approximately 15–20 min. Because elution/backwash procedures were also different for the two types of filters, the time required for this step also varied slightly between the filter types. The elution procedure performed on the pleated capsule filter can be completed in 20–25 min, and the backwash procedure performed on the hollow-fiber filter can be completed in 5–10 min. Overall, each method can be completed in approximately 5–6h.

Recovery efficiency for each sample was calculated by dividing the number of recovered organisms by the number of organisms seeded into the 10-L sample. The resulting fraction was then multiplied by 100 to obtain a percent recovery. Statistical comparisons were made using one-way ANOVA with statistical significance set at 0.05 (Minitab 15, State College, PA).

Results and Discussion

For the tap water samples ($n = 5$ per filter type), as shown in Fig. 1, the mean recovery of *C. parvum* for the pleated capsule filters was 37% (SD = 17), whereas the use of the ultrafilters achieved a significantly higher ($p = 0.007$) mean recovery of 68% (SD = 10) for *C. parvum*. For *Giardia*, the pleated capsule filters produced a mean recovery of 85% (SD = 6), and UF achieved a mean recovery of 63% (SD = 8; $p = 0.001$).

Fig. 2 shows the mean recoveries for each filter type by organism in surface water samples. For the surface water samples ($n = 5$

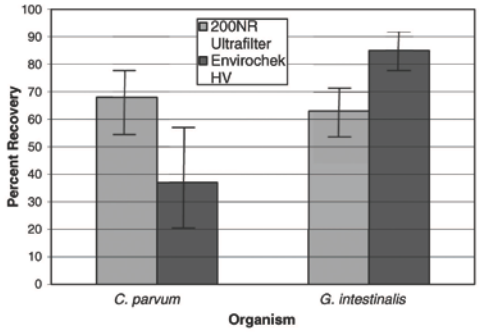

Fig. 1. Mean recovery of *Cryptosporidium parvum* and *Giardia intestinalis* in tap water samples for each filter type (error bars represent standard deviation)

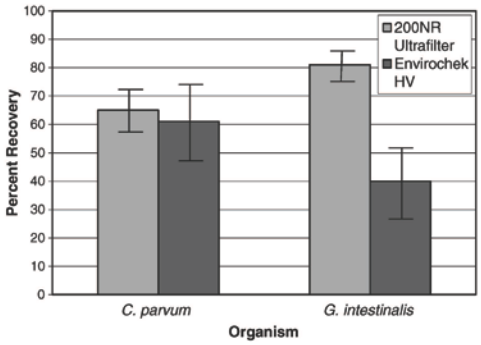

Fig. 2. Mean recovery of *Cryptosporidium parvum* and *Giardia intestinalis* in surface water samples for each filter type (error bars represent standard deviation)

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per filter type), the mean recovery of *C. parvum* was 61% (SD = 14) when using pleated capsule filters and 65% (SD = 7) when using UF. No statistically significant difference was found between the recoveries ($p = 0.63$). For *G. intestinalis*, recoveries in surface water for the pleated capsule filters averaged 40% (SD = 12). However, UF achieved a significantly higher mean recovery of 81% (SD = 5) for *Giardia* in surface water ($p = 0.00009$).

When compared with previous research, this study has produced similar and dissimilar results. As found in the current study, Hill et al. (2009) reported that UF produced significantly better recoveries of *Cryptosporidium* but not *Giardia* in tap water samples. Conversely, although the current study did not find a difference in recoveries of *Cryptosporidium* in surface water, Simmons et al. (2001) reported significantly better recoveries of *Cryptosporidium* with UF. However, these two studies used different models of ultrafilters and pleated capsule filters.

Conclusions

The results from this study demonstrate that UF can provide similar or better recoveries of *Cryptosporidium* and *Giardia* than recoveries from pleated capsule filters when applied to surface water. When applied to tap water samples, UF recoveries were significantly better than Envirochek HV filters for *Cryptosporidium*, but *Giardia* recoveries were better with the Envirochek HV (although overall method recoveries with UF were still greater than 60%). Solely on the basis of the results of this study with one single surface water, UF may be a viable option to improve *Cryptosporidium* and *Giardia* recoveries from both surface and tap water samples using EPA method 1623, but more samples of these and other types and sources of water need to be examined.

Acknowledgments

The use of trade names and names of commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or the U.S. Department of Health and Human Services. The findings and conclusions in this presentation are those of the authors and do not necessarily represent those of the Centers for Disease Control and Prevention.

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